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PHOSPHOLIPID RELATED ANTIGENS AND PROTECTIVE MECHANISMS: IMPLICATIONS FOR CARDIOVASCULAR DISEASES, HUMAN AUTOIMMUNITY AND INFLAMMATION

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Phospholipid related antigens and protective mechanisms: implications for cardiovascular diseases, human autoimmunity and inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mom and dad

If you are not ready for everything, you are not ready for anything.
Paul Auster

ABSTRACT

The pathogenesis of chronic inflammation, a common denominator in cardiovascular, metabolic and systemic autoimmune diseases, is influenced by a number of genetic, metabolic and immunological risk factors. Oxidation of cellular membranes and lipoproteins by reactive oxygen species generates a number of complex and distinct oxidized phospholipids (OxPL) thought to be involved in this pathogenesis. Since the discovery of OxLDL in atherosclerotic lesions, research on these oxidized phospholipids and protective factors/mechanisms has expanded in recent years. As a result, oxidized phospholipids and related antigens have been characterized extensively in connection not only with cardiovascular pathogenesis, but also with related diseases that also are associated with negative cardiovascular outcomes. As a consequence, these oxidized phospholipids are now known to play a key role not only in initiating inflammation, but also in mediating deleterious consequences.

In our first study, we examined the potential pro-inflammatory features of oxidized cardiolipins, phospholipids present in LDL, where they might be exposed at the surface following oxidation. Initially, we determined whether oxidized cardiolipin (OxCL) exerts pro-inflammatory effects on macrophages and neutrophils and, if so, whether Annexin-A5 can inhibit these effects. As expected, OxCL induced leukotriene production by these immune cells in a calcium-dependent manner through activation of the 5-LOX gene. Moreover, Annexin A5 bound to oxidized, but not native cardiolipin, thereby abrogating both the elevated leukotriene production and even intracellular mobilization of calcium. Annexin A5 also inhibited the expression of adhesion molecules on endothelial cells and might thus play an important role in preventing the binding of lymphocytes and monocytes to these cells and resulting inflammation around atherosclerotic plaques.

In our second investigation, we characterized the clinical significance of antibodies towards malondialdehyde, an important epitope exposed at the surface of both OxLDL and apoptotic cells, in 60-year-old patients with cardiovascular disease. An assay developed to measure circulating levels of anti-MDA IgMs revealed that these patients had lower levels than age- and gender-matched controls, especially the men (130.1 [107.8–155] versus 143 [120–165], p=0.001), RU/mL. The odds ratio and 95% CI below the 10^{th} [2.0; 1.19-3.36] and above the 66^{th} percentile [0.68; 0.48-0.98] indicated that these antibodies can predict risk in such patients. Amino acid sequencing showed less variation in these antibodies than in non-specific IgMs.

To extend these findings to systemic autoimmune diseases, we next assessed the prevalence of anti-MDA and anti-PC IgM in patients with RA, SLE, SjS, SSc, MCTD, and UCTD. The levels of anti-PC, but not ant-MDA IgM were strikingly lower in the case of MCTD. Moreover, levels of both IgMs were low among those with SLE or Sjogren's syndrome and high among patients with rheumatoid arthritis and primary phospholipid syndrome, with no difference in the case of UCTD. Furthermore, when anti-PC IgM was added exogenously, the number of immunosuppressive T cells (Tregs) increased, with no such effect with anti-MDA IgM. Finally, the amino acid sequences of these antibodies showed both certain similarities and differences.

In our last study, we assessed the prevalence of anti–PC IgG1 and IgG2 in patients with SLE. Low levels of IgG1 proved to be indicators of risk, whereas higher concentrations were protective. In addition, exposure of macrophages to monoclonal antibodies against phosphorylcholine (developed in-house) improved the efficiency of phagocytosis by these cells in a manner dependent on complement, which might explain why higher levels of these antibodies in patients with SLE appear to be protective.

Overall, the clinical and experimental evidence we provide here confirms the relevance of anti-PC and anti-MDA IgMs in connection with cardiovascular and autoimmune diseases. Both these natural protective antibodies and Annexin A5 could serve as prognostic markers and potentially be of value in treating inflammatory and autoimmune conditions.

LIST OF SCIENTIFIC PAPERS

I. Wan M, Hua X, Su J, **Thiagarajan D**, Frostegård AG, Haeggström JZ, Frostegård J. "Oxidized but not native Cardiolipin has pro-inflammatory effects which are inhibited by Annexin A5". *Atherosclerosis. 2014 Aug;235(2):592-8.*

II. **Thiagarajan D**, Frostegård AG, Singh S, Rahman M, Liu A, Vikström M, Leander K, Gigante B, Hellenius ML, Zhang B, Zubarev RA, de Faire U, Lundström SL, Frostegård J. "Human IgM Antibodies to Malondialdehye conjugated with Albumin are Negatively Associated with cardiovascular Diseases among 60-Year-Olds". *Journal of American Heart Association. 2016, Dec 20;5(12).*

III. **Thiagarajan D**, Oparina N, Lundström S, Zubarev R, Sun J, the PRECISESADS Clinical Consortium, Marta Alarcon-Riquelme and Frostegård J, "IgM antibodies against malondialdehyde and phosphorylcholine in different rheumatic diseases", *Manuscript to be submitted*.

IV. **Thiagarajan D**, Frostegård A, Steen J, Rahman M, Vikström M, Zubarev R, Lundström S and Frostegård J, "IgG1 antibodies against phosphorylcholine are associated with protection in SLE and atherosclerosis: potential underlying mechanisms". *Manuscript to be submitted*

CONTENTS

LIST OF ABBREVIATIONS

CVD	Cardiovascular Diseases
OxPL	Oxidized Phospholipids
PC	Phosphorylcholine
MDA	Malondialdehyde
OxCL	Oxidized Cardiolipin
PtC	Phosphatidylcholine
PS	Phosphatidylserine
4-HNE	4-hydroxy-2-nonenol
PUFA	Polyunsaturated Fatty acid
anti-PC	antibodies against Phosphorylcholine
anti-MDA	antibodies against malondialdehyde
ROS	Reactive Oxygen Species
PAMP	Pathogen Associated Molecular Pattern
DAMP	Danger Associated Molecular Pattern
PRR	Pattern Recognition Receptors
OSE	Oxidation specific epitopes
NAbs	Natural Antibodies
LDL	Low density Lipoprotein
HDL	High Density lipoprotein
OxLDL	Oxidized Low Density Lipoprotein
IL6	Interleukin 6
IL1β	Interleukin 1 beta
TNFα	Tumor Necrosis Factor alpha
SLE	Systemic Lupus Erythematosus
RA	Rheumatoid Arthritis
MCTD	Mixed Connective Tissue Disorder
UCTD	Undifferentiated Connective Tissue Disorder
SjS	Systemic Sclerosis
SSc	Sjogren's Syndrome

CD	Cluster of Differentiation
PAF	Platelet Activation Factor
IgM	Immunoglobulin M
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
ELISA	Enzyme Linked Immunosorbent Assay
BSA	Bovine Serum Albumin
HSA	Human Serum Albumin
IMT	Intima Media Thickness
NSAIDs	Nonsteroidal anti-inflammatory Drugs
DMRADs	Disease Modifying Agents for Rheumatic Diseases
5-LOX	5-Lipooxygenase
COX-2	Cyclooxygenase 2
VCAM-1	Vascular Cell Adhesion Molecule 1
ICAM-1	Intercellular Adhesion Molecule 1
HUVEC	Human vein Endothelial cell
LTB_4	Leukotriene B_4
FURA-2AM	FURA- 2 Acetoxymethyl ester
CDR3	Complementary Determining Region 3
POVPC	1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phoshocholine
PAF	Platelet Activating Factor
RU/mL	Relative Units per milliliter

1 INTRODUCTION

1.1 INFLAMMATION AND IMMUNITY

The concept of inflammation is defined as a response to external insult such as bacteria, virus, fungi or even toxins. Inflammation, has existed since ancient times when Greek associated heat and redness with diseases. Later, in the beginning of first century AD, Roman physician Celsus, identified ***Calor*** (heat), ***Rubor*** (redness), ***Dolor*** (pain) and ***Tumor*** (swelling) as cardinal signs of inflammation with ***Functio laesa*** (loss of function) being added later on by Galen[1]. Inflammation, is an innate response involving macrophages, dendritic cells, neutrophils and other immune that recognizes Pathogen- or Damage associate molecular pattern (PAMP/DAMP), via a series of specialized receptors called Pattern recognition receptors(PRRs)[2].

The family of PRRs includes both membrane bound Toll like receptors (TLRs), and C-type lectins and cytosolic NOD like receptors (NLRs)[3]. These recognizes not only conserved motifs in the pathogen such as flagellins, sugars and a number of cell wall components like lipopolysaccharide (LPS), peptidoglycan but also endogenous danger signals released from the injured or damaged cells, eg. dsDNA and uric acid crystals[4]. The recognition by the PRRs on macrophages and neutrophils stimulates the production of a range of cytokines such as TNF, IL1β, IL6 and certain chemokines to combat the initial insult. Neutrophils are the first cells to be recruited to site of inflammation, where they bind to the endothelial cells, secreting pro-inflammatory mediators like histamines and platelet activating factors (PAFs), that help in clearing the initial trigger. However, when the innate immunological response becomes inadequate and excessive, the adaptive system including T and B-lymphocytes is recruited for the clearance[5].

Although inflammation generally involves external antigen, this process is also essential for tissue repair and homeostasis[6]. Tissue damage often generates of lot of dead cells and cellular debris, as in case of necrotic cell death, that must be removed promptly and swiftly by innate immune cells resulting in ***sterile inflammation***[7]. However, when the offending agent is not removed, and the inflammation remains unresolved, ***chronic inflammatory diseases*** develop with a variety of detrimental effect on the host including excessive release of reactive oxygen species (ROS). Cellular production of ROS affects a number of biomolecules, orchestrating a variety of inflammatory response, which involves phospholipids. In addition to being an

essential signalling molecule, ROS mediates inflammation and are thus implicated in a number of degenerative diseases such as **atherosclerosis**.

1.2 CARDIOVASCULAR DISEASE AND ATHEROSCLEROSIS

Cardiovascular diseases, a leading cause of mortality, affecting almost 17.9 million individuals, as of 2015 and accounting for 31% total deaths among CVD patients[8]. More than 75% of these deaths occurred in the middle and low-income countries. Cardiovascular diseases include many different diseases associated with the heart and blood vessels: ***coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart diseases, congenital heart disease and deep vein thrombosis and pulmonary embolism*.**

Among these, coronary heart disease and cerebrovascular complications account for approximately 85% of mortality worldwide, with atherosclerosis as the major underlying cause. The word atherosclerosis means hardening of the arterial wall, derived from Greek word, (skelros = harden), through deposition of lipid containing immune cells with a porridge like consistency (athero means porridge-like) which begins as a simple fatty streak at a younger age and develops into advanced lesions at later stages of life. Atherosclerosis gives rise to these clinical consequences, through the formation of lesion, narrowing of arteries, plaque rupture and thrombosis, lead to acute coronary syndrome (ACS) where, myocardial infarction and stroke are the most common forms of cardiovascular disease.

A number of traditional and non-traditional risk factors are involved in triggering the initiation and progression of atherosclerosis. The Framingham study, one of the largest epidemiological study in the field of cardiovascular diseases marked the important breakthrough in terms of assessing the associated risk factors[9]. Smoking, hypertension, family history, diabetes mellitus, male gender and old age were classified as classical risk factors of CVD[10]. In fact, the concept of cholesterol as proposed risk factor came from this study. People with family history of CVD and increasing age were more susceptible to cardiovascular events. The above factors contribute to increased production of ROS, from endothelial and smooth muscle cells, which leads to clinical consequences[11]. The involvement of cholesterol in triggering atherosclerosis, was demonstrated by *Ignatowski* and *Anitschkow* in pioneering studies, where rabbits were fed animal protein and fat rich diet[12]. Later, this conclusion was strengthened when *Goldstein* and *Brown*, discovered the LDL receptors, that regulates cholesterol metabolism, earning them the Nobel prize in 1985[13-16].

Later, Ridker and colleagues, proposed some novel risk factors including C-reactive protein, lipoprotein-A, homocystein and fibrinogen all related to coronary artery diseases[17].

1.3 INFLAMMATION IN ATHEROSCLEROSIS

About 40 years ago, atherosclerosis was simply regarded as diseases resulting from lipid accumulation[18-20]. Its complexity lead to proposal of a number of theories attempting to explain the disease initiation and progression. Although, originally researchers based their models on the ***"Response to injury"*** theory, inflammation came later to regard as a key driver at all stages of the disease[21-24].

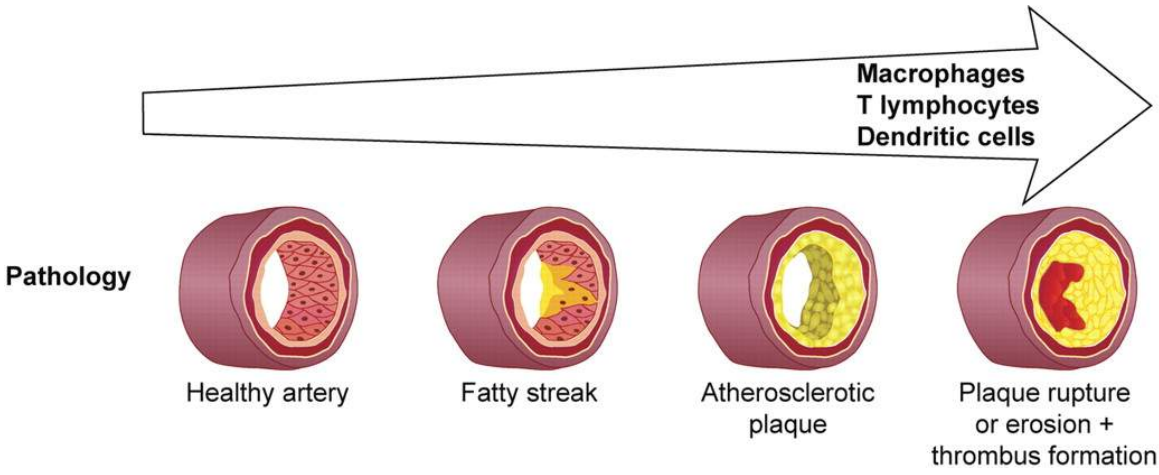


Figure 1: Distinct stages in the development of clinical atherosclerosis. Figure reprinted with permission[25].

Dysfunction of the endothelium is the hallmark of atherogenesis. Unlike smooth muscles cells or fibroblast, endothelial cells, grows in monolayers[26] and are connected by tight morphological junctions, performing a plethora of functions ranging from acting as a barrier and performing transport of molecules across the endothelium; vasodilation and contraction through secretion of nitric oxide (NO), provision of a surface to which lymphocytes do not adhere, and modification/oxidization of low density lipoprotein (LDL), which might cause endothelial dysfunction[26, 27].

Increased oxidative stress is proposed to be key factor in endothelial dysfunction[28]. The key factors responsible for endothelial activation includes: Oxidized low-density lipoprotein (OxLDL), mechanical injury, hypercholestremia, hypertension, smoking and genetic factors[29]. The cellular damage is inflicted when ROS reacts with nitric oxide (NO), thereby promoting injury on endothelial and smooth muscle cells. This damage does not occur all over the artery, the hotspots being arterial bifurcation, leading to expression of the surface expression proteins VCAM-1 and ICAM-1[30]. The adhesion molecule and chemo-attractants

such as MCP-1 released by endothelial cells facilitate the migration of lymphocytes and monocytes are also considered hallmark of atherosclerosis[31]. Upon migration, monocytes mature into macrophages which uptakes oxidized LDL, through their scavenger receptors CD36, SR-A1 and SR-B1. Excess uptake of OxLDL by macrophages eventually leads to formation of foam cell[32]. Over time, the chronic accumulation of foam cells, ultimately leads to fatty streak, fibrous cap, complex plaque, thrombosis and clinical events as shown in figure 1.

Smooth muscles cells (SMCs) are present in both the fatty streak and fibrous cap of atherosclerotic lesion, with macrophages predominating the former and smooth muscle cells in the latter. The extent of proliferation of smooth muscle in the fibrous cap determines the future clinical outcome since such proliferation leads to connective tissue that can accumulate lipids[33]. In addition, SMC express receptors for LDL and secrete derivatives of prostaglandin that act as growth factors[34-36]. SMCs also respond to a number of chemoattractant protein, helping them to the migrate to intimal layers where they develop into the fibrous cap. Moreover, SMCs secretes collagen and apoptosis of SMC results in less collagen and vulnerable plaque. The SMCs migrate from tunica media to intima, by degrading the extracellular matrix with the help of matrix metalloproteases (MMPs)[37]. Also, CD40L produced by platelets plays an important role in this phase of disease[38]. Eventually, the platelets elicit atherogenesis by adhering to a dysfunctional endothelial monolayer. They are known for their chemotactic and mitogenic properties secreting platelet derived growth factor (PDGF)[39] and epidermal growth factor (EGF)[40], that promote migration and proliferation of SMCs[40, 41]. Moreover, for these mitogens in fibroblast and monocytes are responsible for chemotactic activity of the monocytes[42].

A seminal publication by *Jonasson and colleagues* revealed abundant expression of HLA-DR in plaques, suggesting that involvement of inflammatory reactions[43]. Later through thorough investigation of plaque tissue, this same group, showed CD4+ as well as CD8+ T cells, few or no B-cell and lack of neutrophils, indicative of chronic inflammatory stage[44]. Subsequently, this finding and field of inflammation induced atherosclerosis was supported by the research of Peter Libby and others[21-24, 45]. However, this is not such a new concept, since immunological dominance was proposed to cause arterial injury by Rudolf Virchow and French in the 1860's [46, 47]. Indeed, Rudolf Virchow was the first to identify the inflammatory nature of the atherosclerosis describing diapedesis of lymphocytes on vessel wall [47].

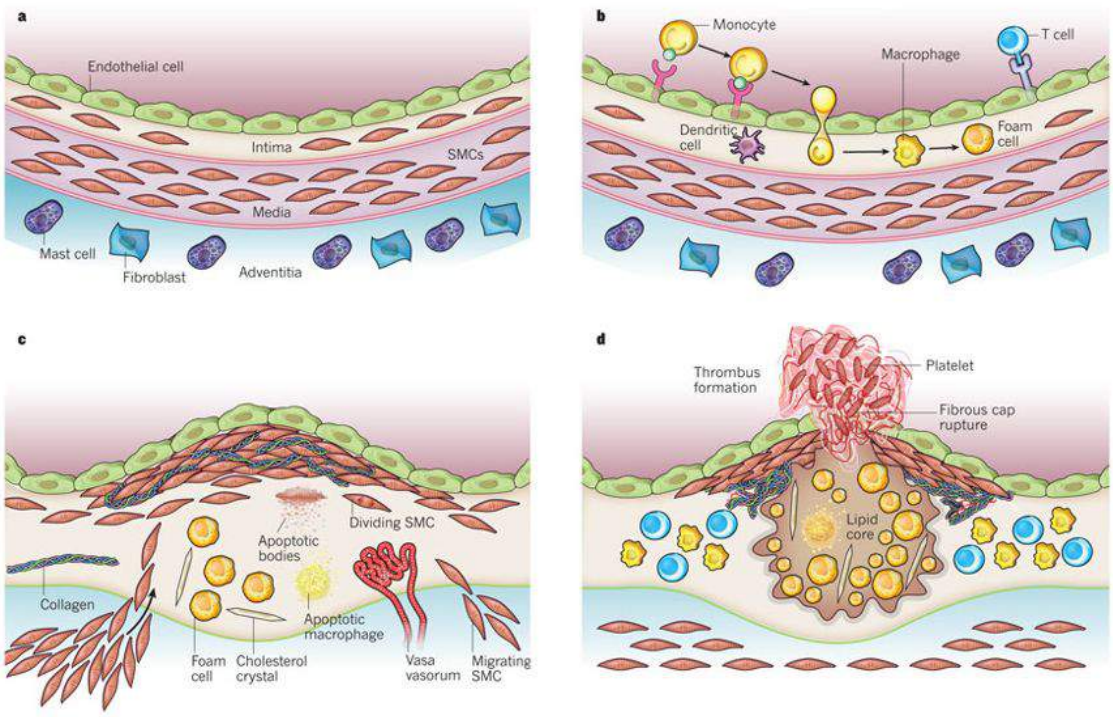


Figure 2: Inflammatory cascade leading to plaque rupture. Figure reprinted with permission[48]. a) Normal vessel structure showing tunica intima, media and adventitia, endothelial and SMC b) Adhesion and migration of monocytes and leucocytes into the vessel wall, c) Migration of SMC to intima leading to accumulation of apoptotic bodies, foam cells, cholesterol crystal and collagen and d) Thrombus formation and rupture of plaque leading to clinical consequences.

As mentioned above, excess uptake of OxLDL by macrophages eventually leads to formation of foam cell. Progressive accumulation of these cells leads to fatty streaks, atherosclerotic lesions and fibrous cap formation. At the same time, T cells, predominantly pro-inflammatory T cells (Th1) migrate into the subendothelial space a phenomenon also observed in connection with other autoimmune diseases such as RA[49]. Moreover, excessive production of the pro-atherogenic cytokines TNF-α, IL 6, IL 1β, and IL 17 were observed in atherosclerotic plaques. Formation of new blood vessels causing hemorrhage within plaques leads to thrombin formation, which in turn causes activation of endothelium and leucocytes. Now, in this stage we can see active involvement of endothelial cells and immune cells like macrophages and T-cells, SMCs thus suggesting a synergistic relation between inflammation and thrombosis [50].

The rupture of plaque is due to local inflammation in the plaque rather than the degree of stenosis itself [21]. The ruptured plaque usually consists of abundant macrophages and MMPs

which facilitate dysregulation of extracellular matrix causing plaque rupture [51]. This is due the physical disruption of the fibrous cap which causes the lipid containing thrombogenic core to get exposed to blood as shown in figure 2.

1.4 ROS AND OXIDATION OF LIPIDS

Utilization of oxygen is required for a number of physiological and metabolic process such as energy production, biosynthesis of cellular components associated with production of reactive oxygen species. Low levels of ROS are important for fighting infection but imbalanced production can lead to irreversible damage to DNA, lipids and protein and even cell death[52]. When this is process is unresolved, the process in-turn leads to release of free-radicals, which further promotes the oxidation. The biomolecules that are most affected by ROS includes lipids[52-54], specifically phospholipids that are abundant in the plasma membrane, cell organelles like mitochondria and also oxidized LDL. NADPH oxidase, Nox-2 and Nox-4 and mitochondria are powerful source of ROS in the endothelium[55]. Oxidation of Polyunsaturated Fatty acids(PUFA)[56-58] often referred as lipid peroxidation, produces a number of reactive and degradation products derived from the self. Often these degradation products, which are modified from the self, are known as oxidation specific epitopes(OSE) and are recognized by PRRs[59] are known to trigger inflammation. These OSEs play an important role in atherosclerosis, where, their exposure both on apoptotic cells and OxLDL plays a vital role in the pathogenesis[29].

1.4.1 Oxidized Low Density Lipoprotein

Convincing evidence from the group of Cleveland clinicians, showed oxidation was key process in converting LDL to OxLDL that render cytotoxic effect[60]. Later, *Steinbercher* and *colleagues*, in their interesting paper, proposed endothelial cell cells modifies LDL through peroxidation process[27]. OxLDL, as such is a complex biological compound, that contains about 700 different phospholipids, 600 molecules of free cholesterol, 1600 molecule of cholesterol ester and 185 different triglycerides and one molecule of apolipoprotein B-100 (apoB) as shown in figure 3. The extent of the phospholipid exposure at the surface of OxLDL, depends on the degree of oxidation[61]. Since oxidized LDL is the central molecule in the pathogenesis of atherosclerosis, a deeper understanding of the individual components, specially phospholipids are absolute necessary as they are prone to oxidation and recognized by immune cells.

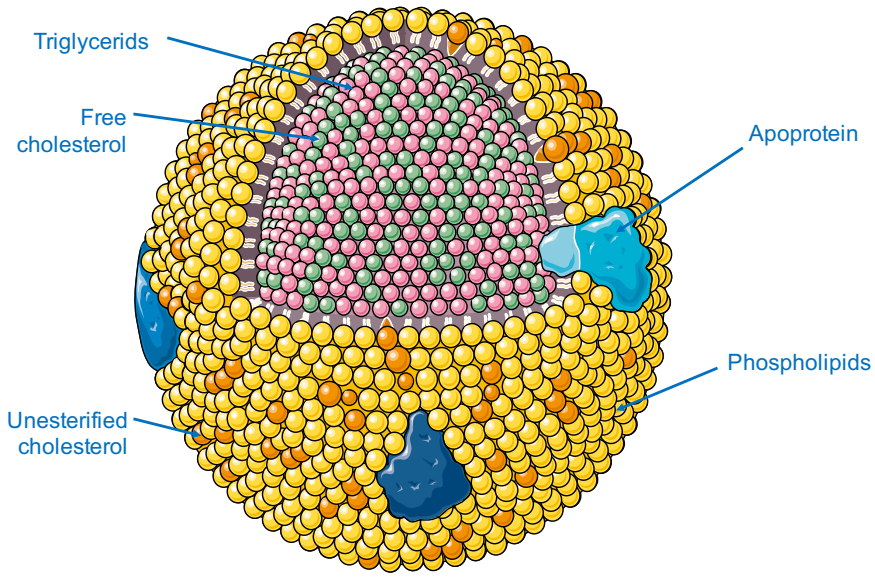


Figure 3: Structure of OxLDL exposing different components: phospholipids, triglycerides, apolipoprotein and cholesterol.

1.4.2 Oxidized phospholipids and oxidation specific epitopes

Through enzymatic and non-enzymatic mechanisms involving lipoxygenases, cyclooxygenases and free radicals, immensely facilitates oxidation of PUFAs in the biological membrane and the cytosol[57, 62]. Phosphatidylcholine(PtC), the most abundant phospholipid in both cell membrane and low-density lipoprotein results in production of a number of degradation which includes: phosphorylcholine-oxidized phospholipids (PC-OxPL), oxidized cardiolipin (OxCL), oxidized phosphatidylserine (Ox-PS), and highly reactive aldehyde called malondialdehyde (MDA) and 4-HNE, as shown in figure 4. All these oxidized phospholipids are endogenous triggers classified as Danger Associated Molecular Pattern (DAMP) recognized by both membrane bound and soluble Pattern recognition receptors of the innate immune system. This finding suggested that specific oxidized epitopes are dominant targets of innate immune cells with a focus on certain phospholipids in the context of atherosclerosis. Pertinent to this, OSEs also contribute to other inflammatory conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), diabetes and cancer[63]. These phospholipids especially the protective factors against them, such as natural antibodies and Annexin A5 are primary focus here in this thesis.

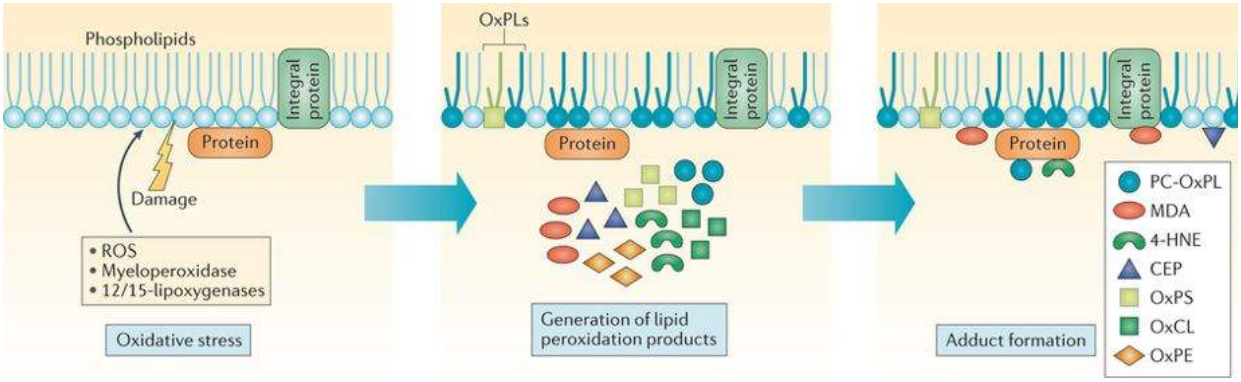


Figure 4: Schematic illustration of Phospholipid oxidation due to oxidative stress. Reprinted with permission[64].

1.4.2.1 Phosphorylcholine (PC)

The well characterized oxidation specific epitope (OSE) of phosphorylcholine has a unique structure that act as molecular mimic of both oxidized phospholipids and microbial antigens. It was first discovered in 1967 by Tomaz, is attached to polysaccharides in Gram-positive bacteria *Streptococcus pneumoniae*[65]. This epitope is also present in an array of other species of bacteria including, *Clostridium* spp.[66], *Lactococcus* spp.[67] and *Bacillus* spp[67]. of gram-positive bacteria. Oxidized phosphorylcholine is also abundant in filarial nematodes, and its's immunomodulatory effect has been extensively studied[68].

Oxidized phosphorylcholine is a dominant epitope implicated in the development of atherosclerosis and certain other chronic inflammatory diseases[69]. Though not exposed under normal physiological conditions, phosphorylcholine is an integral component of the most abundant phospholipid phosphatidylcholine (PtC), thus helping to maintain cell structure and integrity. In addition, this phospholipid is an integral part of low density lipoproteins (LDL) oxidation of which causes conformational changes exposing PC[70]. PtC contains one saturated and one unsaturated fatty acid. During inflammation, due to excessive ROS production and consequent oxidation at the Sn2 position, phosphorylcholine is exposed at the outer leaflet and becomes the immune dominant epitope in oxidized LDL and apoptotic cells which contain a great deal of PC, undergo this same modification to be identified by natural antibodies[71]. Phosphorylcholine is also an integral part of inflammatory mediator platelet activating factor (PAF) and other phospholipids such as sphingomyelin [72] .

1.4.2.2 Malondialdehyde (MDA)

MDA, a reactive aldehyde, is another important end-product of the lipid peroxidation and serve as the most commonly utilized marker of this process[73]. In-vivo, most MDA is

produced by oxidation of PUFA, containing two or more double bonds interrupted by methylene moiety. As proposed by Del Rio, as reviewed in[74], where MDA is the end product of peroxidation of either methyl linoleate, acrolein or prostaglandin. Physiologically, MDA epitope is predominantly found micro-vesicles, apoptotic cells, low density lipoprotein (LDL), high density lipoprotein (HDL), surfactants proteins and cartilage collagen[75]. Importantly, MDA forms adduct with histone on DNA, leading to mutations and eventually certain forms of cancer. Unlike PC, MDA is studied for its pathogenic properties that affects many cells types such as: macrophages, lymphocytes, endothelial and epithelial, causing apoptosis and production of pro-inflammatory cytokines[75]. Clinically, in addition to MDA has been linked to several types of cancers[76-80], Diabetes mellitus[81] and pre-eclempsia[82, 83] as well as in CVD.

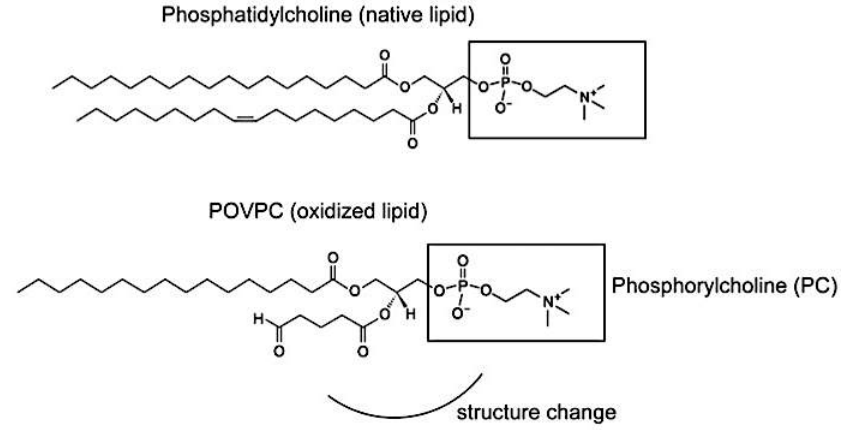
Oxidative modification of LDL is one of the best characterized in terms cholesterol accumulation by macrophage, where MDA plays a crucial role. Immuno-histochemical identification of atherosclerotic lesion of rabbits[84]. Extraction of LDL from human lesion confirmed presence of MDA epitopes[85]. This observation followed a series of studies, where immunization with in-vitro MDA modified LDL, lessened atherosclerotic lesion, indicating existence of protective response against them[86-88]. Alteration of LDL by MDA lead to cholesterol accumulation by human macrophages[89]. MDA forms adduct with amino group to form the N^{ϵ}-(2-propenal) lysine and generate lysine–lysine cross-links via 1-amino-3-iminopropene. Another potential toxic mechanism involves cross-inking of collagen, which in turn affects cardiovascular tissue[90]. A recent publication pointed out that in coronary tissues DNA methylation mediates MDA toxicity through the AKT-FGF2 pathway [91].

1.4.2.3 Oxidized Cardiolipin (OxCL)

The lipid cardiolipin (CL) has a unique structure containing two phosphatidyl groups bridged by a glycerol group. CL has two negative charges and four18-carbon fatty alkyl chains of which two are unsaturated fatty acids. This lipid is localized to membranes, where electron transport and phosphorylation common e.g., the inner mitochondria and bacterial cell membranes. Cardiolipin is an integral part of LDL, and oxidation the unsaturated fatty acids forms OxCL, which plays a role in Cardiovascular diseases. Important functions of cardiolipins include triggering apoptosis, serving as proton trap for oxidative phosphorylation, and regulating aggregated structures.

The structure of the above mentioned phospholipids are illustrated in the figure below.

a)



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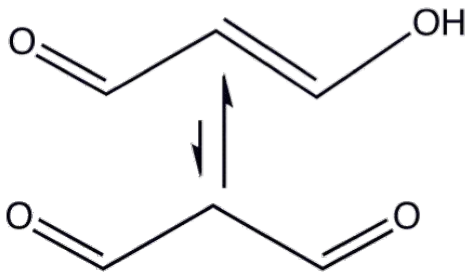

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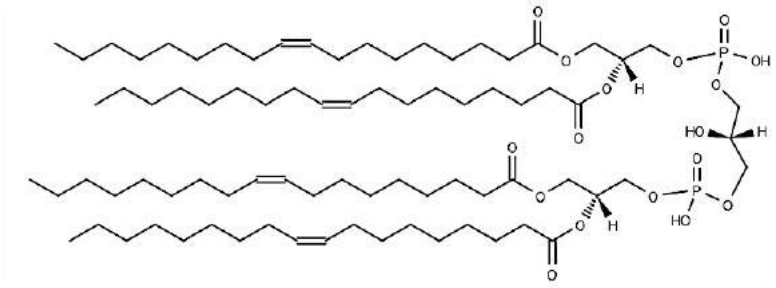

Figure 5: The structure of Oxidation specific epitopes: a) POVPC and PtC exposing Phosphorylcholine, b) Malondialdehyde and c) Cardiolipin.

1.5 PROTECTIVE FACTORS TO OXIDIZED PHOSPHOLIPIDS IN ATHEROSCLEROSIS

Oxidation specific epitopes play important roles in both physiological and pathological processes. As described earlier, OSE exposed at the cell surface present in biomolecules: and

when oxidized innate immune cells promptly remove them, thus maintaining normal homeostasis functions and tissue turnover. However, under pathological conditions, this homeostasis is disturbed due to the enormous burden associated with inflammation of the continuous oxidation reaction, and consequent inefficient clearance of apoptotic cells. This sensing and removal of OSE is facilitated by a broad range of pattern recognition receptors (PRR) both cellular and soluble expressed by the innate arm of the immune system.

Scavenger receptors and toll-like receptors constitute most of the cellular PRRs that recognizes oxidation specific epitopes. The scavenger receptors include: CD36, SR-A1 and SR-B1, LOX-1, CD68 predominantly found on macrophages [92, 93]. CD36, SR-A1 and SR-B1 are essential for the uptake of OxLDL by macrophages and silencing these receptors reduces this uptake by at least 70-80%[94]. Initially, these receptors as well as (SR-A1 and SR-B1) were characterized with respect to their ability to recognize PC-OxPL[95, 96], but they were later also found to bind to MDA-modified protein and 4-HNE[97, 98]. Similar to the scavenger receptors, TLRs also play a crucial role in sensing OSEs.

PC-OxPL, again has been one of the important targets of the TLRs, with E06 IgM inhibiting, IL6 secretion by the OxPAPC (which has PC epitopes exposed), but not LPS[99]. On the other hand, uptake of OxLDL, requires effective co-ordination with CD36, which requires CD36, for its recognition[100]. However, MDA and 4-HNE do not directly elicit production of any chemokines, which implies that other PRR might be involved in their recognition.

Often considered to be the marker of the acute phase protein in inflammation, CRP recognizes molecular patterns in both the pathogens and altered endogenous compounds, first identified during Streptococcus infection. Later, recognition was found to involve a common ligand, PC-OxPL found in them[101]. However, their ability to bind to MDA-LDL has not yet been examined. The role of complement proteins was studied initially in mice with *Ldlr deficient* animals, deficient in C3 complement leads to development of atherosclerosis[102]. Both C3 and terminal complement complexes(C5b–C9) can be detected in atherosclerotic lesions[103]. The figure demonstrating the recognition of oxidation specific epitopes and phospholipids by innate immune cells is shown below.

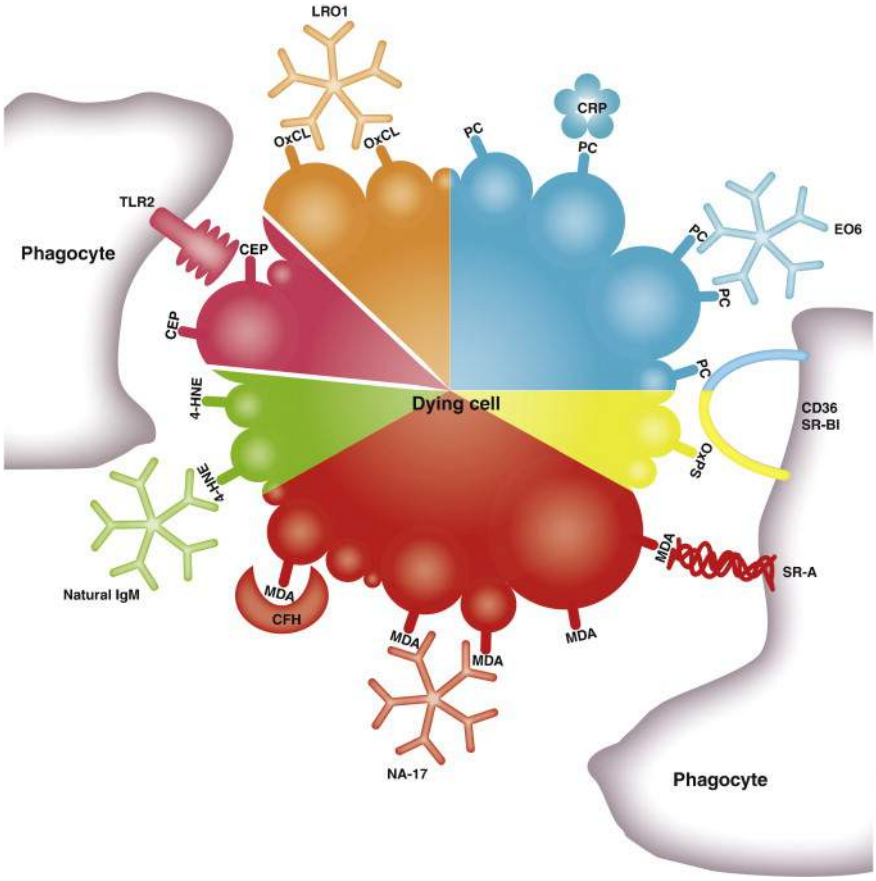

Figure 6: Soluble and membrane PRRs recognizes oxidation specific epitopes. Reprinted with permission[104].

1.5.1 Natural antibodies(Nab)

Although natural antibodies were probably first originated approximately 50 years ago in the late 19th century, Paul Ehrlich, proposed "horror autotoxicus"[105], i.e., autoantibodies that reacts with self-structures and are mostly involved in pathological process. Much later in 1991, Stratis Avrameas, proposed the term "Gnothi seauton" a term referring to repertoires of natural antibodies required for the self- structures are required for general homeostasis of the organism[106].

For many years these antibodies were regarded as insignificant but around mid 1920's evidence of their functions association with bacteria started to emerge[107] and 10 years later, *Kidd* and *Friendewald*, pointed out their functional specificity towards normal tissue constituents[108] opening a new field of these protective natural IgMs act in co-ordination with complement proteins to perform their innate functions[109].

Although, the IgM subclass has been most extensively studied, recent evidence indicates natural IgG antibody, mostly IgG3 subclass participate in certain innate functions in a manner

not completely understood. Purified natural IgG binds to sugar residue on gram-positive and gram-negative bacteria and specifically associate with ficolins to make uptake of bacterial more effective[110]. They also known to interact with CRP[111], TLRs[112, 113] and C1q[113]. Their potential protective function against chronic inflammatory conditions such as SLE or atherosclerosis is of considerable interest.

Phenotypically, circulating natural antibodies are IgMs, IgGs and IgAs and are poly-reactive in both humans[114] and in mice[115] recognizing antigens from endogenous as well as exogenous sources. Site-directed mutagenesis in mice revealed that CDR3 region is responsible for this poly-reactivity.[116, 117] By ELISA techniques these antibodies have been shown to bind both intracellular and circulating including thyroglobulin, cytoplasmic antigens in polynuclear neutrophils, intrinsic factor, Factor VIII (FVIII), anionic phospholipids and glomerular basement membrane[118, 119], which are also important targets in connection with autoimmune diseases. However, these antibodies differ from their pathological counterparts exhibiting fine specificity[120].

1.5.1.1 B1 cells - producers of protective natural antibodies

Plasma B cells produce Immunoglobulins(Igs) of five subtypes: IgA, IgD, IgE, IgG and IgM, characterized on the basis of the constant region. IgMs are the first subclass produced prior to class switching to effectively eliminate infection and maintain immune homeostasis. After contact with the antigen, IgMs class switch to one of the other four subtypes. IgGs are most prevalent class in the circulation and mucous. These antibodies function by activating complement, neutralizing antigen, priming immune cells and phagocytosing apoptotic cells. In addition of a unique subclass of antibodies called "**natural antibody**" is produced in both humans and in mice prior to infection.

While T-cells contribute to cellular immunity, B-cells produce antibodies to specific antigen, thus participating in humoral arm of the adaptive immunity. B cells are divided into two different subtypes, B1 and B2 cells. B2 -B cells, located in the follicular and marginal zone produce antibodies against foreign antigens, whereas B1 B-cells produce antibodies against self-antigens and can self-replenish. Although, B1 cells predominantly produce IgMs (about 80%), they also generate IgG and IgA.[121, 122].

Most of the functional studies related to B1 cells have been performed in mice. B1 cells were first identified among the $CD5^+$ population, which is associated with autoimmune disease [123]. B1 cells are divided into B1-a cells that express CD5 whereas B1-b cells on their surface and B1-a that do not. Although these cells share many similar properties, B1-a cells are more

often are self-replenishing in association with autoimmune conditions, while self-replenishing B1-b cells can also develop from progenitor cells from bone marrow [124-126]. B1-a cells appear in the mouse embryo as early as 8.3 days post fertilization [127] mostly in the peritoneal and pleural cavities[128]. However, the mechanism by which they generate autoantibodies of these antibodies class is still not well understood. The B1-cells are encoded in the germ-line and not mutated in their V_H and V_L regions [129].

Moreover, B1 cells can be subject to positive selection in the presence of self-antigens as shown by Hayakawa and co-workers utilizing transgenic mice and antigen recognition through B-cell receptors (BCRs) is essential for their development[130-132]. In this classical paper, they demonstrated the importance of BCR and their activation, by crossing B1 cells producing anti T-cell antibodies with the Thy-$1^{-/-}$ strain, in which B1-cells were unable to produce such antibodies[130, 132]. In addition, B1 cells interact weakly perhaps due to their inability to enter into the germinal centre to mature into high affinity autoreactive pathogenic cells. The B1-cells cells produce antibodies that bind not only oxidized phospholipids and certain self-antigens expressed on apoptotic cells but also lipids present in the cell wall of gram-positive bacteria such as *Pneumococcus,* thus protecting against atherosclerosis in several ways[133]. Antibodies produced by B1 cells are poly-specific and have low affinity for many different antigens.

Until recently, the existence of human B1 cells are a huge topic of controversy. Although quite challenging, Griffin and colleagues identified $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ in umbilical cord blood and peripheral blood cells of humans[134]. Furthermore, these number of these memory B1 cells decline with age perhaps explaining why the old people are more susceptible to cardiovascular problems and chronic infection. Although the B1-cells of mice produce antibodies in a T cell-independent manner encoded in the germ-line, protective autoantibody production in humans was recently shown to be hugely dependent on T-cells[135].

Approximately 50% of IgMs from human umbilical cord are autoreactive towards apoptotic cells and specifically reactive towards Cu-OxLDL and proteins modified by MDA as in mice[71]. Gene sequencing of the complementary determining region, CDR region of B1 cells isolated from healthy human showed somatic hyper-mutations, revealing that these antibodies are products of affinity maturation [136]. Although on the basis of microarrays of Merbl and colleagues claimed that (B1-derived) antibodies in new-borns bind almost 305 self-antigens [137]. ELISA measurements revealed, showed little or absent natural anti-phosphorylcholine antibodies in the serum of new-borns but presence of anti-malondialdehyde IgM[138, 139]. Antibodies against PC are well characterized and the relevance of antibodies against other

products of oxidation like MDA, CL and PS is starting to emerge. These antibodies produced by B1-b-cells differing in phenotype and mutation from their mice counterparts.

1.5.1.2 Function of natural antibodies

The function of natural antibodies depends on their signalling threshold with respect to the encountered antigen[140]**.** Natural antibodies were initially characterized in terms of their adaptive functions, but are now extensively studied for their innate properties. These T-independent antibodies in a genetically controlled manner[119, 141, 142] are produced by 5-15%[119] splenic B cells 20% of which are autoreactive[143].

1.5.1.2.1 Athero-protective natural antibodies

As indicated above low-density lipoproteins (LDL) play a central role in the pathogenesis of atherosclerosis. Oxidation of LDL results in the formation of neo-epitopes on both lipid and protein molecules[144] forming adducts with phosphorylcholine and malondialdehyde headgroups, that are detected by cells of both the innate and the adaptive immune system[145]. The ability of natural antibodies to bind oxidation specific epitopes (specifically lipids) has led to investigation of their prevalence in patients with cardiovascular disease and systemic inflammatory diseases associated with increased cardiovascular risk such as SLE.

Natural antibodies bind to one or more epitopes on OxLDL and prevent their uptake by macrophages, thus preventing/slowing the formation of foam cells[146, 147]. Immunization of mice with LDL modified with MDA or pneumococcus increases production of athero-protective anti-MDA IgM and anti-PC IgM, respectively in mice[148, 149]. These antibodies do not bind to native or non-oxidized phospholipids not even though those containing the same phosphorylcholine head group. These antibodies block uptake of OxLDL by macrophages, eventually reducing plaque formation[150].

Natural antibodies against PC-OxPS are among the most well characterized of their ability to recognize both pathogen and endogenous danger. Having been studied extensively in the 1970's and 80's they were initially identified as the TI5 idiotype providing protection function against streptococcal infection in mice[151]. The clones were given names TEPC-15 and M167 and found reacting to small hapten "phosphorylcholine"[152]. Most of the immunological and functional properties of these antibodies, such as genetic sequence, germ line encoding and class switching was validated during this same era. About 20 years later, IgM antibodies generated from naive ApoE$^{-/-}$ mice [150] were termed "E0" and found to be structurally and functionally related to the T15 idiotype that binds phosphorylcholine-

containing OxLDL[147, 150]. Nishinarita and Sawada, determined the concentration of anti-PC IgG and IgM in normal human serum to be 320 and 110 μg/mL respectively[153].

Several epidemiological studies performed by our group and others pointed out that high titres of antibodies against these lipid antigens are associated with protection against cardiovascular disease [154-162]. The levels of anti-PC antibodies among healthy individuals differed 100-fold and were correlated with CRP levels [163]. In fact, CRP and anti-PC IgM compete for same epitope, phosphorylcholine[101]. Furthermore, anti-PC IgM levels were higher among women than men [155] and found to decrease with age[164].

Among hypertensive patients those with high level of anti-PC exhibited low intima media thickness (IMT) progression, a hallmark of carotid atherosclerosis [155]. Interestingly, the anti-PC IgM level in serum of the Kitava population in New Guinea (with a low incidence of cardiovascular disease) are significantly higher than in age- and sex-matched Swedish controls[160]. Individual serological analysis of 60-year-old Swedish patients with CVD (both men and women) confirmed that a lower titre of IgM antibodies against phosphorylcholine and malondialdehyde are independent predictors of the risk for progression [69, 165]. In addition to the level antibodies against OxLDL in human sera is inversely proportional to the development of carotid atherosclerosis [166].

Protection by anti-PC IgMs is not only observed in the case of atherosclerosis but also with other chronic inflammatory diseases with cardiovascular risk such as SLE [156, 158]. Thus, low level of anti-PC antibodies in SLE patients is associated with increased the cardiovascular risk [157, 167] as well as elevated risk for stroke [162]. Earlier studies in mice showed that natural antibodies inhibit the development of inflammatory arthritis [168]. In line with this finding, anti-TNF therapy in patients with RA improved enhances serum anti-PC levels[169]. In addition the level of these antibodies predicts Alzheimer's disease[170]. All of these observations indicate that circulating anti-PC antibodies regulates several chronic inflammatory diseases.

Much of the protective effects against MDA-LDL was studied in animal models. Initial studies on mice, suggested these monoclonal antibodies against MDA recognize Oxidation specific epitopes in OxLDL[86-88, 102]. A study lead by Chou and colleagues, proposed MDA-modified LDL are the most dominant targets among other oxidation specific epitopes, in germ-free conditions that are recognized by innate immune cells, for example, natural antibody[71]. The same group, generated a monoclonal antibody against MDA-LDL, originated in spleen, recognizing both OxLDL, and apoptotic cells, specifically later apoptotic cells.

Even stimulation of B1 cells in-vitro generates antibody against MDA-LDL, in the same mice model, reviewed by Weismann[104].

The studies on human came much later, where mostly associations were done with MDA-LDL, but not with respect to anti-MDA IgMs. Although, Tsimikas and colleagues, in a longitudinal 15-year study, showed MDA-LDL was associated with lower risk in patients with CVD and stroke outcomes[171], rather extensive studies in the previous years were made with respect to OxLDL[155, 172, 173]. More studies focusing on IgMs against MDA-LDL are starting to emerge. In addition, the fab fragment of antibodies bound to atherosclerotic plaques and the antibodies also recognize microbial antigens of *Porphyromonas gingivalis*[174]. But in contrast, these antibodies of IgG1 subclass promote the pathogenesis of RA[175]. Unlike PC IgMs, IgMs MDA modified proteins is not well established with respect to different cardiovascular outcomes, deserves deeper understanding.

1.5.1.3 Other functions of natural antibodies associated with athero-protection

1.5.1.3.1 Protection against Infection

Since these antibodies have a broad specificity, they recognize a variety of bacterial and viral antigens including non-protein carbohydrate and lipid moieties. Anti-PC antibodies bind to PC in the cell wall of *Streptococcus pneumoniae* [176] and protect against pneumococcal infection both in mice and also in humans. Recently, low levels of anti-PC and anti-MDA antibodies among patients with HIV patients were found to be low [177]. In fact, vaccinating mice with pneumococcus found to reduce atherosclerotic lesion[149].

1.5.1.3.2 Promoting efficient phagocytosis of apoptotic cells

Everyday approximately hundred billion senescent cells die undergo apoptosis in order to maintain normal tissue homeostasis [178]. If not swiftly removed, these cells become necrotic, releasing cellular contents like dsDNA, HMGB-1 (High mobility group box-1) or HSP (heat shock protein) that can provoke an inflammatory immune response. One of the most important homeostatic functions of innate immune cells is to recognize the “eat-me” signals exposed on apoptotic cell surfaces and clear these cells efficiently[179, 180].

When a cell undergoes apoptosis, it expresses several neo-epitopes recognized by innate immune cells such as macrophages and dendritic cells[71, 181]. Moreover, natural antibodies aid in the recognizing of certain oxidation-specific epitopes (OSE) expressed specifically on the surface of apoptotic or stressed cells including phosphorylcholine (PC), malondialdehyde (MDA), phosphatidylserine (PS) and cardiolipins. (CL), and also recruit complement

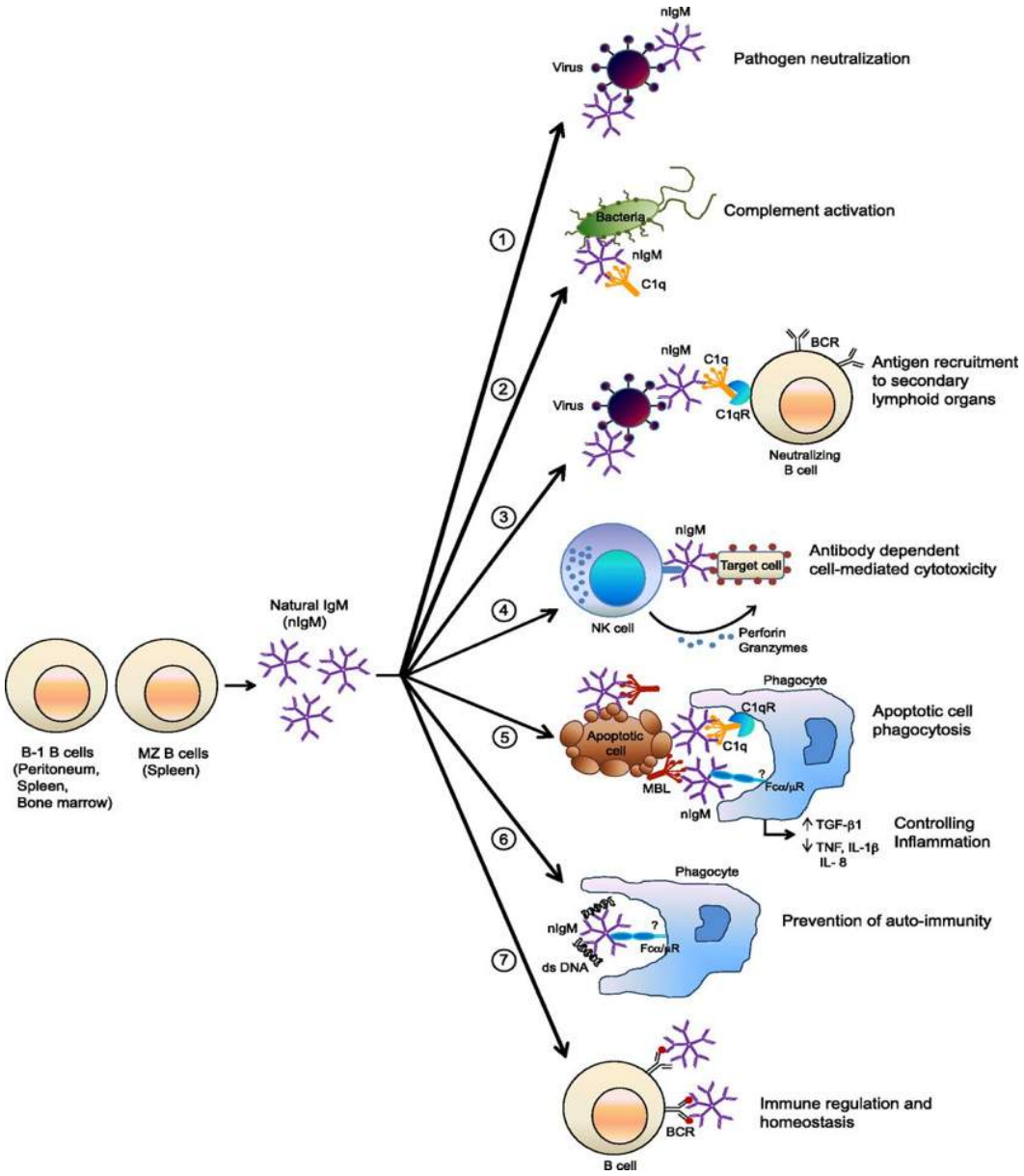

Figure 7: Functions of natural antibody in homeostasis, by aiding efficient phagocytosis, activating complement protein, restrict autoimmunity. Figure reprinted with permission[182].

components like C1q and MBL to initiate phagocytosis[183-185,186]. Mice with impaired production of IgM not only have problems clearing dead cells, but also suffer from increased pathogenic deposition of IgG in tissues [187, 188]. The pictorial representation of different functions performed by natural antibodies is demonstrated in the figure 7.

1.5.1.3.3 Influencing dendritic cells and regulatory T cells

The potential role of natural antibodies in improving dendritic cell functions has been characterized in mice models. Administration of IgM T15 improved the phagocytotic effeciency of these dendritic cells in C1q dependent manner. Moreover, these antibodies, prevents dendrtic cells uptake of various TLR ligands (TLR3, TLR4, TLR7 and TLR9) by reducing the expression of HLA-DR and CD40 expression[168]. In vitro addition of IgMs to regulatory T cells auguments their share in the T-cell population derived from human plaque both in pateints with SLE[189].

1.5.2 Annexin A5

Annexin-A5 (ANXA5), is a 35.7kDa protein, is a member of the Annexin super-family, that binds to phospholipids in a calcium-dependent manner[190]. Although, originally purified from the anti-coagulant fraction of the human umbilical cord artery and placenta[191], ANXA5 are also produced by endothelial cells and comprises 2% of intracellular protein[190]. Their conserved structure consists of 70 amino acid and a variable N-terminal region, which determines the function[138]. The genes encoding the ANXA5 are present in chromosome 4 at 4q26-g28[192]. Although mainly intracellular, they are also found circulating in plasma, urine and cerebrospinal fluid at a level of 1-28ng/mL[193-195]. These proteins are ubiquitous found in fungi and plants and play an essential role in cell signalling and endocytosis[190, 196]. ANXA5 has extracellular functions despite being part of intracellular cytosolic protein as reviewed in[197, 198].

1.5.2.1 Athero-protective properties of AnnexinA5

The anti-coagulant properties of ANXA5 are well known for their [191]. This protein binds with high affinity to phosphatidylserine on the surface of apoptotic cells in a calcium dependent manner. Phosphatidylserine, is normally found in the inner leaflet of the plasma membrane but becomes exposed when the cells are apoptotic or damaged. In fact, ANXA5 is commonly used by researchers to identify apoptotic cells and Reutelingsperger, showed ANXA5 can be used for detection of vulnerable plaques [199].

Annexin A5 acts as two dimensional “band-aid” structure on the surface of endotheial cell thereby helping in preventing anti-coagulation [200]. Moreover, it is anti-thrombotic by down-regulating the expression of tissue factor [201]. In addition, Annexin A5 inhibit Phopholipase A_2 (PLA_2), which plays an important role in synthesizing Platelet Activating factor (PAF) or phosphatidylcholine (PtC), thus facilitating chronic immune response that can lead to lesions. Evidence suggests that ANXA5 may have a novel function in disease associated with

cardiovascular symptoms such as SLE by shielding phospholipids and thus making them inaccessible to the coagulation cascade [198]. Moreover, ANXA5 binding to OxLDL could provide new clue in controlling chronic inflammation [202, 203].

1.6 SYSTEMIC AUTOIMMUNE DISEASES AND CO-MORBID CVD:

Each autoimmune disease has its own genetic and pathological abnormalities and consequent clinical manifestation. Often, patients with autoimmune diseases, share common symptoms with those other having diseases, especially cardiovascular conditions. Increased CVD co-morbidity is observed in Systemic Lupus Erythmatosus (SLE), Rheumatoid Arthritis(RA), Anti-phospholipid syndrome(APS) but such associations has been explored relatively less in patients with Systemic Sclerosis(SSc), Sjögren's syndrome(SjS), Mixed connective Tissue Disorder (MCTD), Undefined Connective Tissue Disorder (UCTD). Recent investigations in immunological pathway of atherosclerosis, indicates that it shares common pathway with a many of autoimmune diseases. Several of the common risk factors, both traditional and non-traditional which are shown in table 1. The immunopathogenesis of atherosclerosis has led to the understanding that activated immune competent cells plays crucial role in all the stages of disease. This understanding confirms the shared pathways between different autoimmune diseases and atherosclerosis[204, 205]. Of which, specifically patients with SLE and RA are extensively studied for the CVD co-morbidity due to their higher prevalence.

1.6.1 SLE and CVD

SLE is a complex systemic autoimmune disease, where various environmental, immunological and genetic factors play a vital role in the pathogenesis. One of the hallmark features of the SLE is impaired apoptotic clearance, and accumulation of autoantibodies against intracellular nuclear components like dsDNA, RO and La, potentially leading to immune complex formation, and causing considerable multi- organ damage like nephritis, malar rash, arthritis and vascular abnormalities[206]. This disease affects woman in their reproductive age. SLE patients are also characterized by elevated levels of lupus antibodies, and approximately 40% of the patients have circulating IgG cardiolipins[207]. Since this is a female centric disease, role of estrogen was also tested in the disease progression. Estrogen, helps in survival of autoimmune cells, by facilitating humoral response and B cell survival. As discussed above, inefficient clearance of apoptotic cell plays a pivotal role in disease initiation due to impaired function of phagocytes and complement functions[208, 209].

CVD co-morbidity among patients with SLE is well understood compared to any other autoimmune conditions. Until Urowitz *et al.*[210]. described bimodal pattern of mortality in

SLE patients and highlighted the CVD, the risk of CVD in SLE patients was under-appreciated. Accelerated atherosclerosis is the major co-morbid conditions associated with SLE, where the risk of stroke and myocardial infarctions are doubled in patients with SLE[211].

Systemic autoimmune diseases	Organs affected	Symptoms	CVD comorbidity (%)	Traditional Risk factor associated with CVD	Non-Traditional risk factor associated with CVD
Systemic Lupus Erythmatosus (SLE)	Immune damage to different organs (skin, kidneys, blood vessel, lungs)	glomerulonephritis, Malar rash, pulmonary emboli, atherosclerosis, myocarditis, joint stiffness	The risk for CVD is doubled in patients with SLE compared to controls	dyslipidemia, male gender, metabolic syndrome, obesity, smoking advanced age, family history of CVD,	polyautoimmunity, SLE per se, autoantibodies, immune cell per se, Inflammatory markers like CRP and ESR, SLE associated organ damage, Disease duration, vasculopathy
Rheumatoid Arthritis (RA)	Immune damage joints and synovium	swollen, tender joints, stiffness, polyarthritis, erosion of joint surface	30-50%	obesity, dyslipidemia, ' family history of CVD, hypertension	HLA-DRB1 SE, non -HLA, autoantibodies, high diseases activity, chronic pro-inflammatory state, glucocorticoids,
Primary antiphospholipid syndrome (PAPs)	Immune provocation of blood clot in arteries and veins causing complication of pregnancy	deep vein thrombosis, recurrent miscarriage, preterm birth, stroke	1.7-6% and can increase to 14%	smoking, type 2 Diabetes, obesity, metabolic syndrome,	phospholipid autoantibodies
Sjogren's Syndrome (SjS)	Effects onmoisture producing glands causing dryness of mouth, eyes and skin	Skin dryness, Dry mouth, Keratinoconjunctivitis sicca	5-7.5%	dyslipidemia, Type 2 Diabetes, advanced age	autoantibodies, poly-autoimmunity, glucocorticoids, long duration of the diseases, chronic pro-inflammatory status, glucocorticoids
Systemic Sclerosis (SSc)	Autoimmune disease of the connective tissue especially in the skin and small arteries and other visceral organs like they kidneys, heart, lungs and gastrointestinal tract	Thick skin, joint pain, pulmonary hypertension, hyperrenemia	20-30%	dyslipidemia, type 2 Diabetes, hypertension, hyperhomocystemia	autoantibodies, increased CRP
Mixed Connective Tissue Disorder (MCTD)	Often shares clinical feature with SLE, scleroderma, RA and myositis	Joint swelling, Raynaud phenomenon, sclerodactyly	Poorly defined	hypertension, hyperlipidemia,	CRP, LDL, autoantibody U1-RNP,
Undifferentiated connective Tissue Disorder (UCTD)	Undefined	dry eyes, dry mouth, hair loss, joint inflammation, joint pain, oral ulcers	Poorly defined		

Table 1: Characteristics of Systemic autoimmune diseases, traditional and non-traditional risk factors associated with CVD. Table generated based on review by Amaya-Amaya and colleagues[212].

Although overall mortality is reduced by approximately 3-20%[213], the prevalence of carotid plaques increases from 21% to almost 100% from young women to patients above 65

years[214]. CVD and SLE share some common risk factors as shown in table1 of which dyslipidemia and lipid peroxidation are important in-terms of CVD pathogenesis. Both the factors could equally contribute to oxidation of LDL eventual clinical consequence of atherosclerosis. Antibodies against OxLDL and phospholipids are relevant in this context. In consistent with these observation, natural antibody levels, against OxLDL, specifically against PC have been much under scrutiny. Various epidemiological observation done by us and others have showed, were concordant our observation from patients with CVD[139, 158, 167, 209] i.e., lesser circulating antibodies were associated with IMT progression and disease activity in SLE. Patients with SLE are showed decreased binding to the endothelium indicating Annexin A5 compete with antiphospholipid antibodies(aPL)[201], indicating Annexin A5 and aPL compete for same the target. Further insights into other isotypes of PC antibodies and mechanism of Annexin A5 are needed for better understanding of the disease with respect to phospholipid antigens and their protection against them.

1.6.2 RA and CVD

Extra articular manifestations are common among RA patients, virtually affecting multiple organs. As mentioned in table 1, patients with RA have increased risk for CVD[215] and the life expectancy of the patients with CVD risk is reduced by 3-10 years[216]. Atherosclerosis followed by ischemic heart disease are the major cause of death among the patients[217] preceded by vascular damage with dysfunction of endothelium[218, 219]. Disease modifying drugs such as MTX[220] and anti-TNF[221] and anti-malarial drugs[220] were shown to reduce CVD risk, by lowing CRP and IL6. Unlike SLE, the role of lipid peroxidation[222] is starting to emerge recently and prevalence of natural antibodies is not well understood[175, 223]. And we have previously showed anti-TNF and anti-CD20 treatment improves protective natural antibody titres in patients with RA[169]. Altogether, in order to have targeted therapies for patients, more studies are to be designed targeting lipid peroxidation and protective factors against.

Although CVD co-morbidity is quite prevalent in other autoimmune diseases like PAPs, SjS and SSc, role of small lipid moieties and pathogenesis associated with them is still poorly understood. Only large epidemiological studies could give more insight into this situation, to develop targeted therapies, for individual patients.

1.7 THERAPEUTICS IN CVD:

All these clinical and experimental understanding is aimed for developing targeted therapies to CVD patients and autoimmune patients with CVD co-morbidity. Various animal study and

large clinical study had led to better understanding of the therapeutic intervention. Traditionally stenting and coronary artery bypass surgery were long considered as classical intervention employed to treat cardiovascular disease[224]. Later drugs were designed to specifically reduce the deposition of cholesterol and thereby prevent plaque rupture with inhibiting HMG-CoA reductase, anti-hypertensives and thrombolytics. Inhibitors of HMG-CoA reductase marketed as statins are regarded as the successful since they reduce cholesterol synthesis by 20-45%. On the other hand, they have side effects affecting the liver function of 1-2% of those taking them[225, 226]. Experimental evidence showed statins could reduce activation of T-cells in a microRNA dependent manner[227]. The hypertensive drug amlodipine, showed reduction in cardiovascular events[228] but it's efficacy against IMT is limited, as demonstrated by large clinical trials.

On the other hand, anti-inflammatory therapies had been proposed to modulate and ameliorate inflammation either by targeting a molecule or by blocking the mechanism down-stream. Ongoing clinical trials are testing new or known therapeutics, which were used in other chronic diseases such as rheumatoid arthritis (RA) or SLE. These include monoclonal antibodies, glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying agents for rheumatoid disease (DMARDs). A retrospective study on the efficacy of methotrexate, a folic acid antagonist, proposed reduction in myocardial infarction but not associated with stroke[229]. Addition of methotrexate, downregulates adhesion molecules, lipoxygenase, cyclooxygenase and reducing production of cytokine[230].

Apart from this a number inhibitors of lipid mediators are used as potential therapeutic intervention for example, leukotrienes inhibitors,[231]while some other studies pointed out, inhibiting cyclooxygenase and phospholipase could improve cardiovascular outcomes[232]. Many animal studies have identified mi-RNAs targets associated with atherosclerosis, based on their tissue specific and time dependant patterns of expression[233]. For example, miR-30, miR-92 and miR-145 are potential targets in treating cardiac fibrosis, cardiac apoptosis and angiogenesis[234]. Another approach might be blocking CD40, which was highly effective in murine disease models of atherosclerosis but the clinical trial outcome is not yet known[235]. Other researchers have focused on dilmapimod, which inhibits p38MAP kinase, an intracellular signalling protein that mediates secretion of inflammatory cytokines.

Monoclonal antibodies, much appreciated for their specificity, are targeted against inflammatory cytokines such as TNF-α (etanercept, adalimumab and infliximab) and IL-1β (eg. Canakinumab) [232], though well known for their efficacy in treating RA and other autoimmune conditions such as SLE, their therapeutic potential is emerging in patients with

CVD. Clinical studies on TNF-α blockades were done in patients with RA with high CVD co-morbidity[236]. The study showed effectiveness of anti-TNF to reduce acute myocardial events. In response to high cholesterol levels, macrophages secrete IL-1β[237]. A phase II clinical trial was started with anti- IL-1β monoclonal antibody, in patients with diabetes showed reduction in CRP levels[238]. This lead to ongoing phase III trial, named CANTOS trial with 17,200 myocardial infarction patients[239], where the end point is reduction of stroke and cardiovascular death. Recently, inhibitors of PSCK9 were also effective in lowering cholesterol and reducing cardiovascular death in combination with statin treatment[240]. The antibody, also had similar effect in diabetic patients, where they reduced cardiovascular risk and incident diabetes[241]. About 10 monoclonal antibodies were approved by the FDA are listed below.

Type	Name	Target	Status
Human	Alirocumab	Blocks PCSK9	Phase III to IV
	Canakinumab	Neutralized IL1beta	Phase I to III
	Evinacumab	Blocks Angptl3, lower LDL	Phase I
	Evolocumab	Blocks PCSK9 and lowers LDL	Phase I to III
	Inclacumab	Blocks inflammatory cell extravasation	Phase II
Humanized	Eculizumab	C5 complement inhibitor	Phase IV
	Tadocizumab	Blocking interaction with fibrinogen and fibronectin	Phase II
	Tocilzumab	Block IL6 receptor	Phase II
	TS23	Inhibits alpha-2 anti-plasmin	Phase I

Table 2: Monoclonal antibodies against cardiovascular in clinical trials.

Though antibodies against phospholipids has been studied for athero-protective and homeostatic function, thus far only monoclonal "E06" was developed and studied exclusively in animal models. Development of human monoclonals will be a valuable tool as these antibodies will be helpful in assessing the functions in homeostasis and other pathogenic conditions, especially, atherosclerosis and autoimmune conditions.

Despite the profound knowledge on pathology, associated risk factors and targeted therapies, cardiovascular diseases, still remains leading cause of death in the world, and also among patients with CVD co-morbidity in autoimmune diseases. This emphasizes the necessity for

finding other novel risk factors, with the help of powerful epidemiological studies and subsequent experimental testing. The current knowledge on different risk factors and even the success of treatments was derived from such studies using robust statistical associations. Such testing might lead to improved therapeutic intervention and precise medicine specific for patients in CVD and autoimmune conditions leading to better quality of life and eventual reduction in death.

2 AIMS OF THE THESIS

The overall aim of the thesis is to understand the prevalence and function of protective factors against oxidation induced phospholipids, in inflammatory and autoimmune conditions and to elucidate their protective role using human cell culture system.

The specific aims include:

Study I: To investigate binding and anti-inflammatory property of Annexin A5 against oxidized cardiolipin.

Study II: To study prevalence of anti-MDA IgMs in 60-year Old CVD patients.

Study III: To study the prevalence of anti-PC and anti-MDA IgMs among different Systemic autoimmune patients(SADs).

Study IV: To study the role of anti-PC IgG1 in SLE pateints and functions of in-house generated anti-PC IgG1s in human cell culture system.

3 METHODOLOGICAL CONSIDERATION

In order to answer the specific research question, this section summarizes the human materials used to explore biomarkers related to cardiovascular diseases and methods employed to understand potential properties of Annexin A5 and monoclonal antibodies.

3.1 PATIENT COHORT

A cohort, in the medical field, is typically defined as a group of individuals with specific characteristics, recruited to determine either new incidence, cause or prognosis of a diseases[242]. In order to elucidate, the prevalence of natural antibodies and to evaluate relationship between the antibody circulating levels and outcomes patients with CVD and autoimmune diseases, we employed cohort based approach to answer our research question. We typically employed three different cohorts, as illustrated in the figure 8 below. The patient's serum samples were collected, and other demographic and disease related observation were recorded during initial recruitment are used for association analysis. Although we found the prevalence and association with the disease activity, we did not find any causal relationship using this type study.

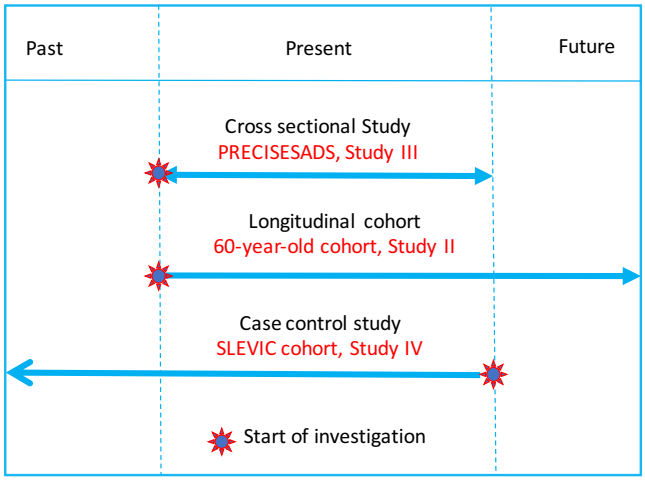

Figure 8: Different cohort used employed in the thesis.

3.1.1 60-Year-Old Cohort (Study II)

This is typical longitudinal cohort, where between July 1997 and June 1998, every third man and woman from Stockholm county who turned 60 was invited to participate in the cardiovascular health screening and 4232 (2039 men and 2193 women) agreed. approximately 78% response rate was recorded. The patients were followed up to identify cases of CVD, National Death Registry up until December 2003, and National In-Hospital Registry, until 2005 was examined resulting in 209 cases. Three random controls for every

case were matched for age and gender, the characteristics are presented below. The level of anti-MDA IgM in blood samples from all of these subjects were determined.

3.1.2 PRECISESADS Cohort (Study III)

PRECISESADS (Precision medicine strategies for Systemic autoimmune diseases) is a cross-sectional cohort, collected from December 2014 to October 2017, with participants from twelve different European countries. The 2656 participants were recruited that includes: 377 with rheumatoid arthritis (RA), 470 with systemic lupus erythematosus (SLE), 402 with systemic sclerosis (SSc), 385 with Sjögren's syndrome (SjS), 99 cases with mixed connective tissue disorder (MCTD), 106 with primary antiphospholipid antibody (PAP), 166 undifferentiated connective tissue disorder (UCTD) patients and 651 healthy controls (HC). Ethical permission was obtained from the countries, where patients and controls were recruited. The characteristics of the controls and patients with their established clinical score are presented in the table below.

3.1.3 The SLEVIC Cohort (Study IV)

The Systemic Lupus Erythmatosus Vascular Investigation cohort (SLEVIC) included patients who demonstrated clinical manifestation of CVD, and age and gender matched controls receiving care at Karolinska University Hospital, Stockholm, Sweden. All of these patients fulfilled revised American College of Rheumatology (ACR) for SLE. This study was approved by the Regional Ethical Review Board at Karolinska Institutet, Stockholm, Sweden and was performed in accordance with the Declaration of Helsinki. All subjects provided their informed consent before entering the study.

3.2 CLINICAL SCORING

3.2.1 CVD co-morbidity definition (Study III)

We estimated the cardiovascular (CV) score based on the presence of cardiovascular-related symptoms including: Arrythmia, Coronary artery disease, Hypertension, Pericarditis, Pulmonary arterial hypertension by right-heart catheterization, Pulmonary hypertension on Echo, Valve lesions, Arterial/Venous thrombosis, Gangrene of the fingers, History of Raynaud's phenomenon, History of recurrent miscarriage or pregnancy complications and Ischemic digital ulcers/Pitting scars. The present study did not differentiate the cardiovascular symptoms but categorized them as "2" if the cardiovascular symptoms are present, "1" if the cardiovascular scores were present in the past and "0", when cardiovascular symptoms are absent.

3.2.2 Carotid Ultrasound (Study IV)

The measurement of intima media thickness (IMT), is considered as a global score for atherosclerotic measurement. The right and left carotid arteries were examined with the B mode ultrasonography. The mean of the maximum intima thickness is determined in the far wall, as the distance between the echo from the leading edge of the lumen intima and lumen adventitia both in the common carotid and bifurcations. When the intima media thickening is greater than "1", it is defined as plaque. The vulnerable/echolucent plaque is graded between 1 and 4, when "1" is echolucent, "2" predominantly echolucent "3" predominantly echogenic, and "4" echogenic.

3.3 ENZYME LINKED IMMUNOSORBENT ASSAY

3.3.1 Biomarker measurement in cohorts (Study II, III and IV)

The circulating levels of these natural antibodies (IgMs and IgGs) among different patients and control groups, was measured using ELISA. For this purpose, we developed an ELISA procedure which was optimized for each individual experiment. PC-BSA or MDA-HSA (10μg/mL) was coated on 96 well NUNC immune plates and incubated overnight at 4°C. Thereafter, the plates were washed and blocked with 2% BSA for at least one hour after which the samples and standards were diluted with 0.2% BSA and added at 100μl for each well for two hours. The plates were washed and added with biotin/alkaline-phosphatase conjugated IgM/IgG secondary antibody at specified concentration for 2 hours. The samples were further incubated with Streptavidin (for biotin conjugated IgM, IgG1 or IgG2) or pnPP (for alkaline phosphatase conjugated IgM) 20 min and 75 min respectively. The reaction was stopped with 1N H_2SO_4/3M NaOH and the absorption were read at 450nm/405nm respectively. The values presented are relative units (RU/mL) depending on the OD values of the standards used in the cohort. This technique has a lot of advantages, that includes: high sensitivity, specificity and throughput and consuming lesser time, where we can use samples frozen for certain period of time. However, in this in-house technique, we did not find the concentration of the circulating levels in absolute values, rather quantitated them relative to the standard used, which makes it difficult to compare with other similar studies.

3.3.2 Monoclonal binding and competition assays (Study IV)

The microtiter plates were coated with different antigens in their oxidized or native states to understand the binding of in-house produced antibodies. The antigens were coated at specific concentration and antibodies were serially dilutes and added at different concentration from 20μg to 0.16μg per milliliter and studied for the binding effect. For the competition assay, the

antibodies were incubated with different concentration of the competitor and the competition was assessed in percentage by calculating the difference in the OD values in the presence or absence of competitor.

3.4 AFFINITY PURIFICATION OF NATURAL ANTIBODIES (STUDY II, III)

The anti-PC/MDA IgMs and their non-binding counterparts, were extracted from human IgM (Sigma Aldrich). Enrichment of anti-PC and anti-MDA IgM was achieved with Hi-trap NHS column (GE healthcare, Sweden). Briefly, MDA-human serum albumin and PC-bovine serum albumin (1mg/mL) was coupled to this column after extensive equilibration with solution of high and low pH, was then loaded with human IgM and incubated for several hours. Unbound IgMs were eluted as flow-through (FT). After continuous washing with buffer, anti-PC and anti-MDA IgMs were eluted using glycine-HCl elution buffer. The obtained antibodies and their flow-through counterpart were desalted using PD-10 desalting column (GE Healthcare, Sweden). Finally, the enriched antibody solution was passed through a 0.22um filter and stored at -20°C until future use.

3.5 MONOCLONAL PRODUCTION AND GENE SEQUENCING (STUDY IV)

Monoclonals antibodies are directed against one specific epitope are now widely used to treat many inflammatory and autoimmune diseases. Monoclonal antibodies (mAbs) against phosphorylcholine were isolated from single PC-reactive B-cells from healthy human donors. The corresponding genetic sequences were determined and c-DNAs (complementary DNA) synthesized were cloned into expression vectors coding for human Igγ (Ig gamma heavy chain), Igλ (lambda light chain) or Igκ (kappa light chain). The antibodies were produced by co-transfecting into exponentially growing human embryonic kidney (HEK) cells. After seven days of cultures, the proteins(antibodies) were purified in Protein G chromatography column. The antibody protein purity and the expression of heavy and light chains were confirmed by SDS-PAGE. Advantages of this method includes its specificity to one particular antigen and easy production. Also, these are fully human antibodies, which can be employed directly to the human cells. However, one limitation here is we were not able to assess the systemic effect of these antibody in in-vitro cell culture models, for example immune complex formation.

The antibody affinity to PC-hapten was measured by Surface plasmon resonance on Biacore X-100(GE healthcare, Uppsala, Sweden). For every B cells on the 96 well plates, matching IgH and Igκ/Igλ were obtained were sequences at the genes encoding them determined employing the Eurofins MGW operon. The sequence was analysed for somatic hyper

mutation and CDR3 variability using IMGT tools and the sequence were compared using multiple sequence alignment tool (MUSCLE) to understand the similarity and differences between the generated antibodies.

3.6 DE-NOVO SEQUENCING USING MASS SPECTROMETRY (STUDY II, III, AND IV)

The extracted IgMs were subjected to proteomics analysis to characterize the peptide sequences of anti-PC IgM, anti-MDA IgM and monoclonal antibodies. The extracted IgMs were reduced using dithiotreitol and subjected to alkylation at 37°C. The mass spectrometry was based on principle of liquid chromatography. The samples were injected into the nano-liquid chromatography system. The mass spectra were obtained in the range of *m/z* 300-70 with a resolution of 12,0000. The raw data were processed using spotlight approach which is combination of de-novo sequence and existing sequence. The abundance of peptides were normalized.

3.7 CELL CULTURE

3.7.1 Primary cell culture

Buffy coats were purchased from Karolinska University Hospital. cultivation of ***neutrophils*** (Study I), buffy coats were subjected to dextran sedimentation followed by gradient centrifugation on lymphoprep (Axis-Shield PoC AS, Oslo, Norway). The isolated neutrophils are used for calcium mobilization and LTB4 production study. In case of ***Macrophages*** (Study I and IV), isolated PBMCs were made positive selection for CD14 positive beads (Milteyni Biotech). The CD14+ cells were differentiated into M2 macrophages with MCSF (50ng/mL) stimulation for 4 days. The macrophages were subjected to study the improvement in the phagocytosis efficiency in the presence or absence of IgG1 anti-PC antibodies. ***Regulatory T cells*** (Study III), were generated by pre-coating with anti-CD3 antibody (10 μg/mL) (eBioscience), together with soluble anti-CD28 antibody (1 μg/mL) (eBioscience), IL-2 (10 ng/mL), TGF-β1 (10 ng/mL) (Immuno Tools), in culture medium for six days and Tregs were stimulated with phorbol myristate acetate (50 ng/mL) and Ionomycin (1 μg/mL) for 5 hours on the harvest day.

3.7.2 Cell Lines

3.7.2.1 HUVECS (Study I)

Human Umbilical Vein endothelial cells (HUVECs) were purchased from Life technologies. and were subjected to stimulation with Oxidized Cardiolipin(OxCL) to study the pro-

inflammatory effects exerted by OxCL, in activating the endothelial cells, by studying increase in surface expression markers VCAM and ICAM.

3.7.2.2 THP1 cells (Study IV)

THP1 cells were purchased from ATCC. The cells were differentiated into macrophage like using PMA (Phorbol 12-myristate 13-acetate) for 72 hours and stimulated with different concentration of LPS to study the effect of anti-inflammatory property of IgG1 anti-PC.

3.7.2.3 Jurkat cells (Study IV)

Jurkat cells were also purchased from ATTC. Jurkat cells were subjected to apoptosis, to study antibody binding, complement deposition and efficiency of phagocytosis uptake by the macrophages.

3.8 PHAGOCYTOSIS ASSAY (STUDY IV)

Buffy coats were purchased from Karolinska University Hospital. PBMCs were isolated followed by positive selection for CD14 (Milteyni Biotech). The CD14+ cells were differentiated into M2 macrophages with MCSF(50ng/mL) stimulation for 4 days. For the phagocytosis assay, apoptotic jurkat cells were incubated with antibodies, with or without serum for 1 hour. Prior to apoptosis induction, Jurkat T-cells were subjected to TAMRA staining (microscopic examination) or CFSE (FACS analysis) for 20 minutes at serum free conditions before. Antibodies tagged apoptotic cells were incubated with macrophages for 1 hour at 10% IgG deficient serum. The cells were washed and fixed with paraformaldehyde, prior to microscopic examination. The cells counterstained with DAPI. Images were recorded using fluorescent microscope. Phagocytosis efficiency was determined by number of macrophages that uptake apoptotic jurkat to the total number of macrophages using flourscent microscopy.

3.9 GENE EXPRESSION ANALYSIS (STUDY I)

To study gene expression of COX-2 and 5-LOX, RNA was extracted using QIAGEN mini kit. The extracted RNA was converted to cDNA using reverse transcriptase kit. The gene expression of COX-2 and 5-LOX was studied using designed primers. The obtained values were normalized against β-actin and presented as relative measures.

3.10 FLOW CYTOMETRY (STUDY I AND IV):

Flow cytometry is a robust tool where it finds its application in medical field, especially in single cells to study expression of surface markers, intracellular markers, complement

deposition, to mention some. Cultivated human macrophages, HUVECS, Jurkat, regulatory T cells were subjected to flow cytometric analysis to study the expression of surface markers and phagocytosis efficiency of macrophages.

3.11 STATISTICAL ANALYSIS (ALL STUDIES):

A pairwise t-test was employed for all clinical studies. A binary logistic regression was used for clinical associations such plaque development and SLE disease index (Study II and IV). For assessing the difference in antibody level among different autoimmune patients, using 2-tailed t-test along with non-parametric epps-singleton test. Spearman Rank correlation was employed for the correlation between antibody level and cardiovascular score (CV) (Study III). Student t-test was employed for all the experimental analysis (Study I and IV). For the peptide characterization, we employed both univariate and multivariate approach for principle component analysis and orthogonal projection to latent structures discriminate analysis using Simca 14.0, Umetrics (Study II and III). Statistical test where $p<0.05$ is considered significant.

4 RESULTS AND DISCUSSION

4.1 Oxidized but not native Cardiolipins is pro-inflammatory

The double bond of linoleic acid moiety in cardiolipin is prone to oxidation[243], which occurs during apoptosis or oxidative stress, releasing cytochrome-c through the action of cardiolipin specific peroxidase[244, 245]. Although oxidized cardiolipins were found in both atherosclerotic lesion and apoptotic cells[246], their immunogenic properties were not clarified in this context. First, we wanted to determine whether this phospholipid in its oxidized form promotes the production of inflammatory mediators, such as leukotrienes, due to their potential inflammatory role in atherosclerosis, stroke and myocardial infarction[231]. Leukotrienes mediates inflammation through autocrine and paracrine signalling, particularly by secreting chemokines to attract neutrophils to site of injury and facilitates cytokine production by immune competent cells[247]. Accordingly, this study was designed to elucidate some important aspects of inflammation induced by oxidized cardiolipin.

Since atherosclerotic lesion are abundantly filled with monocytes/macrophages and recent evidence suggested neutrophils deposition, we wanted to know if addition of OxCL to immune competent cells like macrophages and neutrophils stimulates the production of LTB_4. Indeed, increased leukotriene secretion by both cell types in a dose dependent manner, induced by OxCL was dependent on calcium. In fact, neutrophils were more potent than macrophages (Fig 9). The intracellular calcium mobilization as augmented by OxCL, was measured fluorimetrically with FURA-2A. This response by macrophages is particularly interesting because of their significance in the pathogenesis of atherosclerosis[248-250]. Calcium mobilization plays an integral role in many physiological processes, especially in connection with neurotransmission, contraction of muscle fibres, but excessive mobilization might have significant role in apoptosis and necrosis (Reviewed by *Pinton*)[251] which could possibly have detrimental role in atherosclerosis. Calcium flux is also known to affect mitochondrial membrane[251].

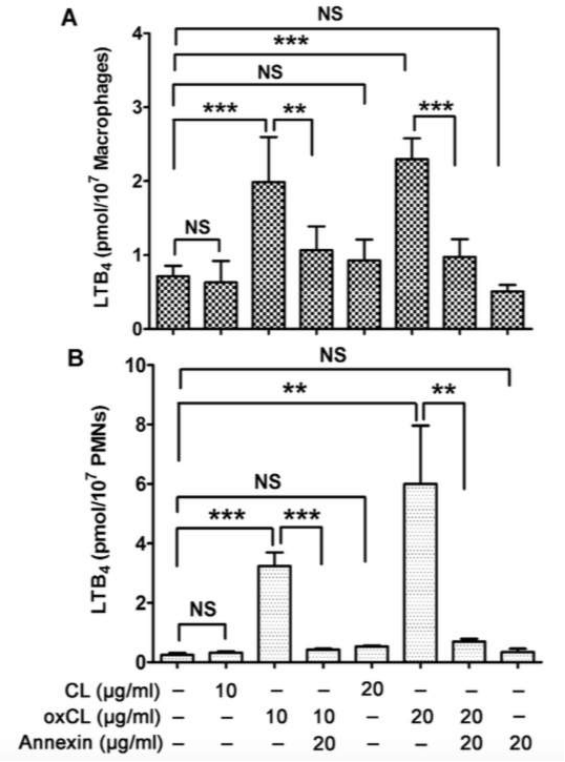

Figure 9: Leukotriene production by human monocyte derived macrophages and neutrophils upon oxidized cardiolipin stimulation and subsequent abrogation by Annexin A5.

Addition of OxCL to human macrophages or neutrophils, increased 5-LOX expression but did not enhance the expression of COX-2, which is consistent with the production of leukotrienes by 5-LOX. It is well known that arachidonic acid is the end product of linoleic acid degradation, which could be due to oxidation or enzymatic reaction. 5-LOX, also known as arachidonate 5-lipoxygenase, a lipoxygenase, converts essential fatty acid such as arachidonic acid into leukotrienes[252], a potent inflammatory mediator, in a calcium dependent manner[253] and is known to play a significant role in plaque rupture. 5-LOX was also found to be positively associated with atherosclerosis severity[250].

4.2 Annexin A5 binds to OxCL

Annexin A5, being a cytosolic protein, known for its binding to phospholipids. To determine whether the inflammatory burden caused by oxidized cardiolipin could be abrogated by Annexin-A5 we first looked for potential binding of Annexin A5 by classical ELISA assay. We demonstrated that Annexin A5 binds more strongly to OxCL than to native (CL) or reduced cardiolipin (R-CL) as shown in figure 10. The minimal oxidation when CL is exposed to air that occur could explain this binding to the cardiolipin not oxidizing intentionally.

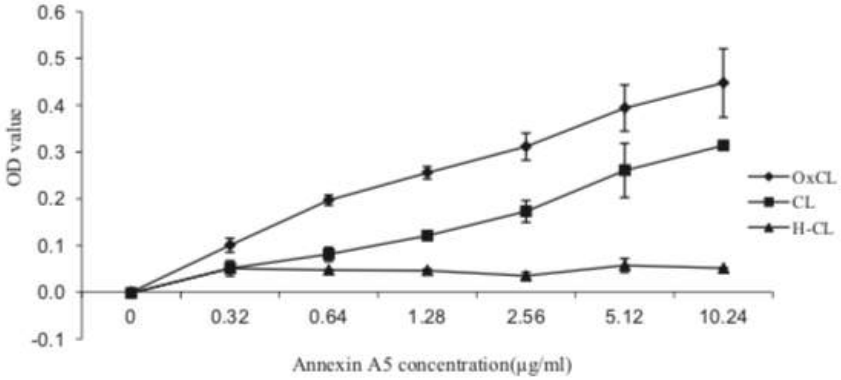

Figure 10: Annexin A5 demonstrates higher binding efficiency to oxidized cardiolipin than to native or reduced cardiolipin.

Next, we wanted to elucidate if Annexin A5 has any anti-inflammatory role. We found that Annexin A5 abrogated and in fact totally eliminated the increase in leukotriene production by macrophage and neutrophils caused by OxCL and calcium mobilization. OxCL, also enhances VCAM-1 and ICAM-1 at the cell surface, a hallmark of atherogenesis that allows monocyte and lymphocytes to bind to the endothelial cells and this validates the OxCL could also be potential candidate in vascular inflammation. Once again, this effect was abrogated by Annexin-A5 addition, primarily by preventing OxCL from binding to endothelial cells.

There are two possible mechanisms, for oxidation of cardiolipin: one, through oxidation of LDL itself, as cardiolipins are part of LDL, and another, by exposure of mitochondrial membrane in apoptotic and necrotic cells accumulated in the lesion. In both the cases, oxidative mechanism converts cardiolipin to oxidative cardiolipin which could lead to this downstream inflammatory function as we demonstrate in this study, thereby contributing to pathogenesis. It should also be noted that binding of Annexin A5 and CRP to OxLDL was demonstrated earlier, although they had different binding sites[202]. CRP is known to recognize PC moiety both in OxLDL and apoptotic cells[101], so Annexin A5 might bind to OxLDL plausibly recognizing OxCL.

Though we addressed an important question in this study, we did not demonstrate the clues concerning the exact site at which Annexin A5 binds to OxCL. In fact, we did not demonstrate the competition of Annexin A5 to other inflammatory phospholipids such as phosphatidylserine or OxCL was not done here or their binding to the atherosclerotic plaque was demonstrated. Although we proposed LTB_4 as one inflammatory mediators secreted by immune competent cells, we did not address other possible inflammatory mediators such as platelet activating factor or lyso-phosphatidylcholine which are also relevant in the pathogenesis.

4.3 Anti-MDA IgM and the risk for CVD

Previous findings indicate a negative correlation between serum levels of anti-PC IgMs and the risk for CVD among 60-year-old patients[172]. Here, we investigated the same potential correlation for anti-MDA IgM in the same set of patient and compared these to matched control. The prevalence of smoking, BMI, glucose and hypertension, the classical risk factors, were higher among the cases. Moreover, patients with CVD have lower amount of circulating IgMs against malondialdehyde (MDA-HSA) than matched controls.

In the cohort, we identified with patients with cases with one of the following outcomes: 77 with myocardial infarction (MI), 85 patients with angina pectoris and 49 with ischemic stroke. When considering MI and stroke, the odds of these patients developing CVD were twice as high for controls in the lowest tertile, below 10^{th} percentile, [OR(CI), 2.16(1.31–3.55)], although the odds were much improved after adjusting for smoking, hypertension, BMI and Type 2 diabetes. The associations were similar, when the MI and angina were treated as outcome.

Further, when stratified for gender, men showed higher risk, compared to women. For the men, the odds ratio was as high as 2.62 and 2.73 for MI/stroke and MI/angina respectively, in the lowest tertile, (below 10^{th} percentile) and the risk increased, when adjusted for confounders. Although, antibodies were associated with a protective effect above 66^{th} percentile, the odds reduced to 0.54 and even more so above the 90^{th} percentile (0.34). With better effect on men these finding indicate that IgM against malondialdehyde protect against CVD in 60-year old patients. Although this biomarker was strongly associated with men, no such association was observed for woman. One reason for this bias could be lesser female subjects in the entire study.

4.4 Polyclonal anti-MDA IgMs differs from other IgMs

The non-MDA binding IgMs (flow- through, FT) contained a larger number of peptides than MDA IgMs indicating that the latter are more conserved. Anti-MDA IgMs are produced by B1-cells, which produce natural antibodies and these antibodies are presumed to exhibit little or no somatic hyper-mutation. To our knowledge, the peptide sequences of these IgMs have not been documented before. Of the 2429 peptides identified, 1061 were identified through de novo sequencing and 738 showed distinct sequence homology. Only 20% of the peptides present in the FT were also present in the anti-MDA IgM.

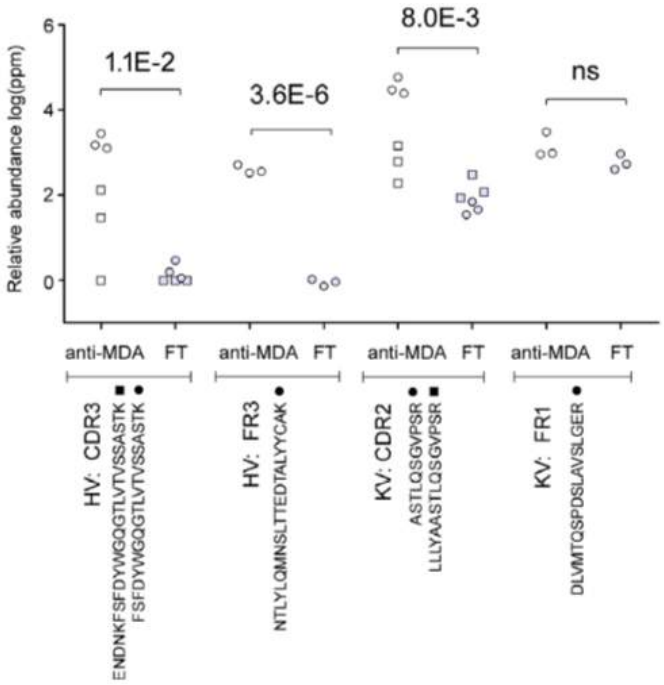

Figure 11: Difference between anti-MDA IgM and control FT in the variable region of heavy and light chain.

More specifically, there was no difference with respect to the heavy chain, but peptides were present in the kappa chain of anti-MDA IgM whereas relatively higher abundance of lambda chains was observed in the non-anti-MDA IgM. Thus, it was apparent that these antibodies were more homogenous compared to the FT, confirming these antibodies must not have undergone mutation over time as they recognise conserved sequence in the antigen.

Next, we wanted to understand how these antibodies, recognize the antigen, we examined the complementary determining region (CDR3 and CDR2) and also Framework (FR) region, as shown in figure 11. Compared to the FT controls the sequence ENDNKFSFDYWGQGTLVTVSSASTK and FSFDYWGQGTLVTVSSASTK were enriched in CDR3 and the sequence ASTLQSGVPSR and LLLYAASTLQSGVPSR were enriched in CDR2 region MDA IgM. It should be noted that these IgMs are polyclonal and are germ-line encoded.

The CDR3 region of the antibody can be regarded as fingerprint of an antibody. Given its poly-specificity nature, and previous finding suggesting their T –cell dependency[135, 136], we were curious to investigate the variability in the CDR3 region of the heavy and light chain. Our data suggested that the antibodies were homogenous compared to the controls. This is indicative from the previous studies on mice models, where, monoclonals, E014[147] and NA17[147] against malondialdehyde modified LDL, generated from germ-free mice did not show variability in the antigen binding region and were much comparable to T15 idiotype.

On the whole, through this study we characterized human natural occurring antibodies against malondialdehyde modified HSA.

4.5 Antibodies against PC and MDA are different among different rheumatic diseases

Although the elevated risk among patients with low-level of anti-PC and/or anti-MDA IgMs (*study II*) have been studied extensively in connection with cardiovascular disease patients the putative roles of these antibodies in other systemic inflammatory autoimmune diseases had not been documented. A present novel finding, among patients with different diseases of this sort, serum levels of anti-PC IgM were reduced in those with MCTD vs controls, RU/mL *(61.5 ± 38.7 vs 73.9 ± 34.3*), with no difference in the anti-MDA level *(62.9 ± 33.3 vs 69.3 ± 34.2*) as shown in figure 12.

In agreement with our previous observations, a clear reduction in IgMs against PC was observed patients with SLE compared to the controls *(68.3 ± 36.5 vs 73.9 ± 34.3).* Although lower levels were also common among those with other diseases like systemic sclerosis (SSc), these values did not differ significantly from those of controls. In contrast, elevated levels of both antibodies were detected in patients with rheumatoid arthritis (RA) and primary phospholipid syndrome (PAPs). Also reported previously[175], but only the level of anti-MDA IgG was associated with Disease activity score(DAS28).

Given the ubiquitous nature of the phospholipids, their relevance seemed to gain importance not just in lipid driven diseases like atherosclerosis but also, many other inflammatory condition, where oxidative bursts are one among the causative. Autoimmune diseases are characterized by elevated levels of pathogenic IgG autoantibodies directed toward altered self-antigens. Here we have elucidated the circulating levels of protective autoantibodies, also directed towards altered self-antigens in patients with different autoimmune diseases. The most interesting case among these group of patients was MCTD, due to their rare occurrence and degree of complexity and sharing clinical manifestation with other diseases. In fact, patients with MCTD were also presented with severe cardiovascular co-morbidity, which could partly explain why specifically anti-PC but not anti-MDA is not illustrated in this disease. Patients with MCTD present with features such as arthritis, swelling of the hands and fingers, thickening of the skin, Raynaud's syndrome and pleuritis to mention a few. Some population based studies have pointed out the prevalence rate as 3.8-6.5 per million per year[254, 255], in certain population with strong association to human leukocyte antigen[256]. Association of anti-phospholipid antibodies are studied in relevance to pathogenesis[257], although only antibodies against U1 small nuclear ribonucleoprotein (U1 snRNP) has been

strongly associated the diseases, not to be confused with levels in patients with SLE, where the levels are much higher in latter compared to patients with MCTD[258]. Due to it's rarity and complexity, the need for other novel risk markers was absolute necessary. Prevalence of low anti-PC IgM in this cohort is quite novel. Investigation concerning mechanisms linking anti-PC IgM and disease pathogenesis has to be studied.

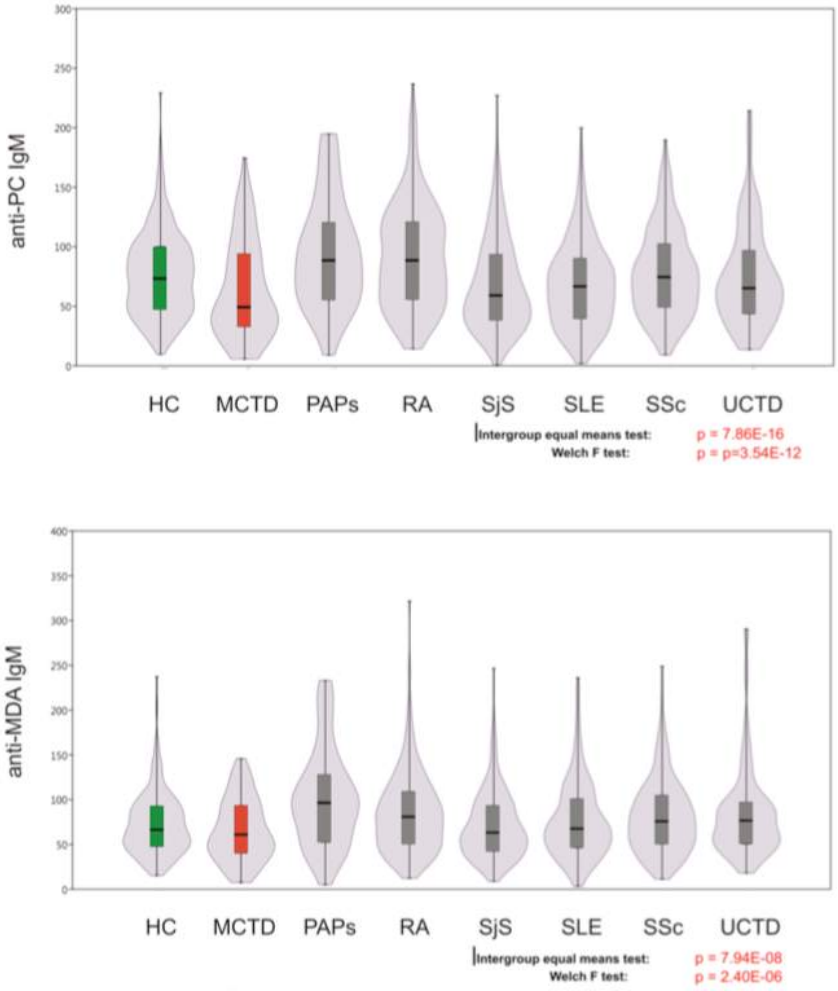

Figure 12: IgM antibodies against PC and MDA exhibited different profile in different rheumatic diseases.

Though PC and MDA are known to be present as dominant epitope in apoptotic cells and OxLDL, the circulation levels of antibodies against them are quite different, in fact level of MDA IgM does not differ much from the controls, except for PAPs and RA. Taken together, we can see both these antibodies were uniformly distributed, in fact in higher levels in serum of the patients PAPs and RA, which is consistent with previous findings by *Grönwall* and *colleagues*[175]. Although both IgM and IgG antibodies against PC and MDA were elevated in RA patients, only MDA IgGs were associated with the disease activity score. Therefore, this elevated level in the RA does not always account for pathogenicity, suggesting some alternate functions associated with this prevalence. Similarly, patients with PAPs have higher

circulating levels of these antibodies. In general, antibodies against phospholipids are elevated in this set-up. The most studied ant-phospholipid antibodies are anti-cardiolipins in a β_2-glycoprotein dependent or independent manner.

Although the cross-sectional design of the study could be one limitation to understand the causation effect of these antibodies, it was interesting to note how these antibodies are presented differently in different autoimmune diseases. Correlation analysis with other pathogenic autoantibodies and genetic factors, example. HLA DR, specifically comparing B1 cell distribution among patient and control (probably achieved by constructing nest-case-control) deserves further study.

4.6 Antibody levels and CVD comorbidity

The protective role of antibodies against phosphorylcholine and malondialdehyde is well understood in connection with cardiovascular outcomes, as is the fact that patients with autoimmune diseases often exhibit a variety of co-morbid conditions, especially cardiovascular symptoms. In our cohort, patients with different cardiovascular and other comorbid conditions were identified and stratified on the basis of CVD co-morbidity. It is to be noted the patients in different group exhibited different cardiovascular scores. Patients with systemic sclerosis had more cardiovascular co-morbidity, followed by MCTD, as illustrated in figure 13. Although patients with PAPs and RA had significantly lower cardiovascular score compared to others.

In the patients with MCTD and co-morbidity, there was a negative correlation between CVD scores and the levels of both antibodies, (anti-PC IgM: rho=-0.37, p=0.002 and anti-MDA IgM: rho=-0.32, p=0.01). We in our lab have demonstrated negative correlation of antibodies with CVD[155, 162, 172], finding in patients with MCTD are novel. Previously, increased levels of HSP60, were also linked to cardiovascular co-morbidity[259]. Similar to our previous findings, SLE patients also showed negative correlations, although that with anti-PC IgM was statistically significance. The results for patients with UCTD were similar. Interestingly, patients with rheumatoid arthritis also exhibited the same tendency, although their IgM levels were higher than those of controls, (anti-PC IgM: rho=-0.16, *p=0.007* and anti-MDA IgM: rho=-0.12, *p=0.04)*. Among patients with Sjögren's syndrome the statistical significance was borderline, whereas patients with systemic sclerosis or primary phospholipid syndrome demonstrated no correlation. Although patients with systemic sclerosis showed the highest cardiovascular scores, the levels of antibodies did not differ significant. Unlike our other

studies, cardiovascular score included in this study, consists of broad spectrum of outcomes. Therefore, this limits our conclusion, to any particular co-morbid condition.

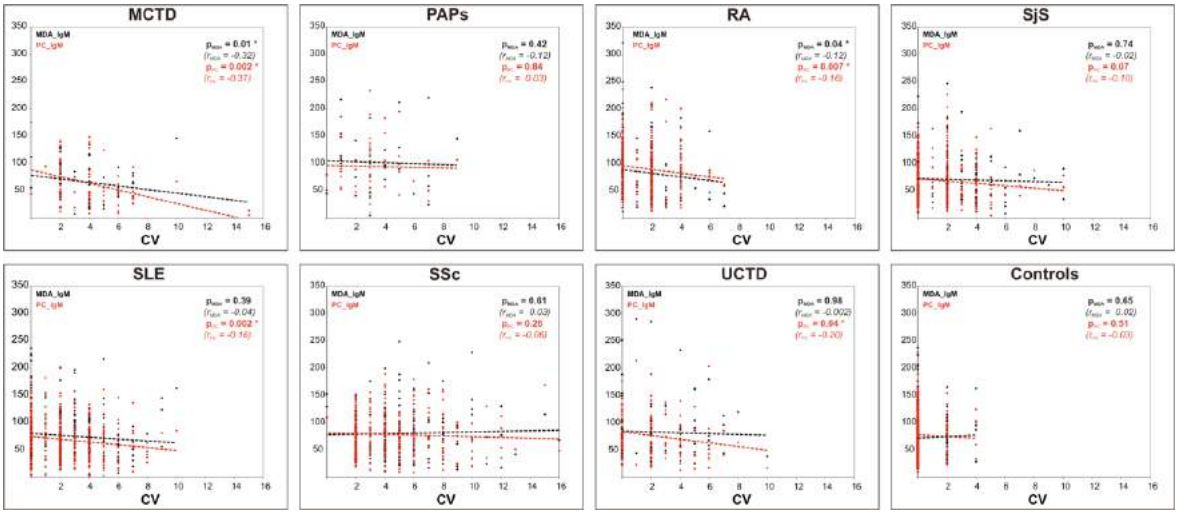

Figure 13: Correlation analysis of anti-PC and anti-MDA IgMs with different cardiovascular co-morbidity in systemic autoimmune diseases.

4.7 Antibodies influence regulatory T-cells in different fashions

Dysfunction of regulatory T cells are not only important in regulating homeostasis and self-tolerance but are believed to be vital cause in many autoimmune diseases such as RA, SLE. Here we found that in-vitro stimulation of anti-PC but not anti-MDA IgM increases the number of T-reg cell frequency. *Charolette and colleagues*, in their review described potential challenges and controversies of T regs associated with autoimmune diseases[260]. In fact, anti-PC IgMs enhanced this number even among regulatory T cell from SLE patients and atherosclerotic plaque[175]. Known for their suppressive functions a steady decline in regulatory T-cells occurs in patients with active autoimmune diseases such as SLE[261, 262] and rheumatoid arthritis[263] and even in animal models of atherosclerosis. Recently, T-regs were found to improve macrophage efferocytosis function in an experimental mice model[264]. Another study in mice showed that adoptive transfer of T-regs reduced macrophage and T-cell accumulation in atherosclerotic lesion[265]. An analysis of T-reg population in humans with coronary artery diseases and acute coronary syndrome revealed fewer in circulation[266, 267]. Depressed level of these cells are also common among MCTD[268], sjogren's syndrome[269] and systemic sclerosis[270].

The results show one of the important immunomodulatory function imposed by anti-PC IgM on regulatory T cells. In terms of atherosclerosis, increasing T reg population could probably

decrease foam cell formation and increase in anti-inflammatory macrophage population. In case of patients with RA, anti-TNF treatment enhance the T-reg population[271] and we have shown anti-TNF treatment improves serum levels of anti-PC IgM level in patients with RA[169].

4.8 The peptide sequence of antibodies to phosphorylcholine and malondialdehyde are different

Natural antibodies were characterized in the 1970's and 1980's, but mostly in mice[272]. To look for difference in the antibody peptide sequence of anti-PC and anti-MDA antibodies using de-novo sequencing followed by univariate and multivariate analysis. To our knowledge, this has not been done before. In general, both of these antibodies had a lower number of lambda regions than their flow-through counterparts. The CDR3 antigen recognition sequence in the heavy variable ENDNKFSFDYWGQGTLVTVSSASTK and FSFDYWGQTLVTVSSASTK, were present in both, whereas the sequence EESFWGQGTLVTVSSASTK was unique in anti-PC IgM, as demonstrated in table 3. Similarly, there were four peptide sequence that were specific for anti-PC IgM but not anti-MDA IgM nor their flow-through counterparts. Interestingly, in the light chain, the sequence of the CDR2 region of the variable kappa chain was similar to PC and MDA IgM.

The multivariate OPLS- DA analysis showed distinct separation of flow-through from anti-MDA and anti-PC IgMs along the x-axis and separating anti-PC and anti-MDA IgM along y-axis. Therefore, through this analysis we identified eight peptide segments that are common between antibodies and the flow-through, eight peptide segments specific for anti-PC IgMs and three peptide segments specific for anti-MDA IgMs. This shows the both commonality between anti-PC and anti-MDA IgM, which could probably explain its redundant function with the uptake of apoptotic cells and other similar homeostatic function. Whereas, the unique sequence explains the functional differences related to identifying peptide sequences specific for them, which in-turn determines their fate.

Type	Region	Sequence	anti-PC	anti-PC-FT	anti-MDA	anti-MDA-FT	p-values -anti PC vs anti-PC-FT	p-values -anti PC vs anti-MDA	p-values -anti PC vs anti-MDA-FT
HV	CDR3	**EESF**WGQGTLVTVSSASTK	2.4±0.1	0.3±0.3	-	-	1.4E-05	-	-
		ENDNKFSFDYWGQGTLVTVSSASTK	3.1±0.2	0.5±0.5	1.2±1.1	-	1.1E-04	8.8E-02	-
		FSFDYWGQGTLVTVSSASTK	3.9±0.1	1.6±0.4	3.2±0.2	0.2±0.2	1.7E-05	2.1E-03	7.9E-07
	CDR2	GLEWLG**YVYYSGVTR**	1.5±1.1	0.1±0.3	-	-	7.6E-02	-	-
		GLEWVA**VVSSDGR**	2.3±0.3	0.1±0.1	-	-	3.5E-06	-	-
		GLEWVS**SLNSDSK**	1.6±0.5	-	-	-	-	-	-
		GLEWVS**SMSASDGGTY**YADSVK	1.8±1.3	0.4±0.3	-	0.6±0.6	7.7E-02	-	1.9E-01
	FR3	NTLYLQMNSLTTEDTALYYCAK	3.3±0.2	1.1±0.2	2.6±0.1	0.0±0.0	1.2E-05	4.7E-03	9.3E-05
KV	CDR2	**ASTLQS**GVPSR	4.5±0.2	2.0±0.2	4.5±0.2	1.7±0.2	5.3E-06	8.0E-01	1.2E-05
		LLLY**AASTLQS**GVPSR	2.7±0.2	2.4±0.1	2.7±0.4	2.2±0.3	4.6E-02	9.0E-01	4.0E-02
	FR1	ELTQSPATLSLSPGER	1.1±0.8	-	-	-	-	-	-
LV	CDR1	VTL**SCSGSSANLGK**	2.2±0.3	-	-	-	-	-	-
		NYVSWYQQLPGTAPK	2.0±0.6	1.3±0.1	-	1.3±0.1	8.9E-02	-	9.2E-02
	CDR2	LLLY**GNNERPS**GLPDR	2.0±0.8	0.1±0.3	-	0.3±0.5	4.5E-03	-	7.6E-03

Table 3: Antibody peptide characterization of anti-PC and anti-MDA IgM.

4.9 Serum levels of anti-PC IgG1 are negatively associated with clinical outcome patients with SLE

Serum levels of both anti-PC IgG1 and IgG2 were higher among patients with SLE than controls (IgG1: 68±4.4 vs 55.8±2.7, p=0.02) and (IgG2: 101.5±16.8 vs 114.28±12.2, p=0.53). Our patients exhibited vascular inflammation and more CVD than the control. In patients with plaque, the levels of these antibodies were even lower in SLE patients, although only significantly so in the case of anti-PC IgG1 (55.2±3.4 vs 78.2±7.4p=0.006) alone reached significance. This difference was even more pronounced when comparing vulnerable plaques, especially in left carotid.

Next, we wanted to determine whether these antibody levels were correlated with clinical outcome with respect to SLE, specifically with SLEDAI, SLAM and SLICC activity and vascular complications eg: plaque and CVD complications. The level of anti-PC IgG1s were divided into quartiles, and the odds ratio and confidence intervals for different clinical consequences calculated. Overall, this level was negatively associated with the clinical complications. The negative correlation of antibodies for determining plaque vulnerability was already shown using correlation analysis[167]. Similarly, in hypertensive subjects, Igg1 was shown to be associated with IMT porgession[164]. Here, we showed he IgG1 anti-PC but not IgG2 were negatively correlated with plaque vulnerability, just like anti-PC IgM. Of course, this did not reach significance, which could be probably due to higher variation among the levels of patients. Similarly, we observed anti-PC IgG1s correlated with increased

atherosclerosis measures and cardiovascular events, below 25th percentile [OR, CI; 2.71, (1.03-7-09)], and even stronger at 10th percentile, [OR, CI; 2.71, (1.03-17.4)]. Similarly, the anti-PC IgMs were negatively correlated with organ damage index SLICC and diseases activity score SLEDAI, but not SLAM. Since these antibodies change with time, we did not adjust the outcome with confounders. Of the three, SLEDIA was correlated the most, below 10th percentile, OR, CI [5.1, (1.3-20)]. And these antibodies were protective by at least 20-30% with these scores.

Impaired clearance of apoptotic cell, is the characteristics of patients with SLE and leads to the release of potential danger signals, including nuclear antigens, against which pathogenic IgG autoantibodies are produced. Furthermore, cardiovascular diseases are one of the major co-morbid conditions among patients with a 50% higher prevalence than in controls. Atherosclerotic plaque contains a necrotic core with foam cells loaded with lipids and other cellular debris. This study gave an insight into the direct correlation of the antibodies with CVD related outcomes and SLE related outcomes indicating, not just IgM but also IgGs especially IgG1 could play potential role in SLE prognosis.

4.10 Dynamism among clonally selected antibodies

Since under such circumstances efficient clearance of apoptotic cells is absolutely necessary, we generated fully human IgG1s against phosphorylcholine to understand their function in connection with this process. Phosphorylcholine, is a dominant epitope of both apoptotic cells and oxidized LDL. Three monoclonal antibodies, A01, D05 and E01 each with different binding affinities were chosen; the corresponding gene sequences were analysed with IgBlast tool, to determine whether the antibodies were germ-line encoded or had undergone any somatic hyper-mutation and the amino acid sequences was compared using Multiple Sequence Alignment Tool (MUSCLE). The was a major difference in the amino acid sequence of the complementary determining region (CDR3), where the monoclonal antibodies A01 and D05 were similar, but E01 lacked peptides in the concerned region. Binding of these antibodies to phosphorylcholine and other lipids in both the native and oxidized states was examined by ELISA. As expected all in a similar manner bound to phosphorylcholine linked to BSA (PC-BSA). However, D05 demonstrated highest binding capacity to oxidized LDL, followed by A01 and E01. Moreover, D05 bound strongly to POVPC, whereas E01 and A01 did not bind at all.

On the other hand, none of these antibodies bound to native protein, LDL, PtC or BSA, demonstrates their specificity for oxidized phospholipids. In the case of phosphorylcholine, a

component of the *Streptococcus pneumoniae*. E01 exhibited the highest binding affinity, followed by D05 and then A01. Unlike in eukaryote systems, where it is bound to protein or lipid, PC in Streptococcus is bound to a carbohydrate. One plausible reason for this differential recognition is the difference in the amino acid sequence of the CDR3 region, in the heavy chain. We, in our previous study showed the difference in recognition of phosphorylcholine by IgG1 and IgG2 subtypes[164], where IgG1 were shown to be important in recognizing both phosphorylcholine and p-nitrophenyl-phosphorylcholine, NPPC (a PC hapten), whereas IgG2 only recognized NPPC, binding to the phenyl ring, indicating their crucial difference in recognizing PC haptens. Therefore, this identification lead to the division of PC specific antibody into two groups: group1 consisting of anti-PC IgMs, Igg1s and IgA, performing similar function and group II consists of IgG2 helps in identification of carbohydrate associated PC antigens. This was validated, using PC-IgG2, were elevated in patients with periodontitis suffered from increased risk for CVD. Overall, this interesting difference might provide a valuable tool to studying not only anti-PC antibodies that bind endogenous antigens but also fatal streptococcus and developed as vaccine for future.

4.11 Anti-PC IgG1 improves the efficiency of phagocytosis by macrophages

Since PC exposed at the surface of the apoptotic cells, we next incubated the antibodies with apoptotic cells. The antibodies A01 and D05, but not E01 bound to apoptotic and necrotic but not viable cells. Isotype controls did not bind to the apoptotic cells, as was confirmed by FACS. In the presence of D05, the efficiency of the uptake of apoptotic jurkat cells by macrophages increased from 19% to 31%, also rising to the 23% in the presence of A01 but E01 or isotype controls. As expected, since none of the antibodies bound to live cells, they did not facilitate uptake of such cells. Even in serum free conditions, the D05 mAb exerted a similar significant effect indicating lack of serum dependency.

In the presence of serum deposition of complement protein C1q on apoptotic cells increased from 0.072% to 4.72% and antibody did not alter this deposition, indicating that uptake of apoptotic cell may involve Fc-gamma receptor. In contrast, complement protein C3b did not deposit either in the absence or presence of antibody. Again, the difference in binding specificity, could reflect whether PC is bound to protein or carbohydrate with difference in sequence from E01 antibody determining the antibody binding to the lipid. The elegant role of anti-PC IgMs in apoptotic cell uptake were clearly demonstrated in a number of mice and human studies[135, 147, 168, 183].

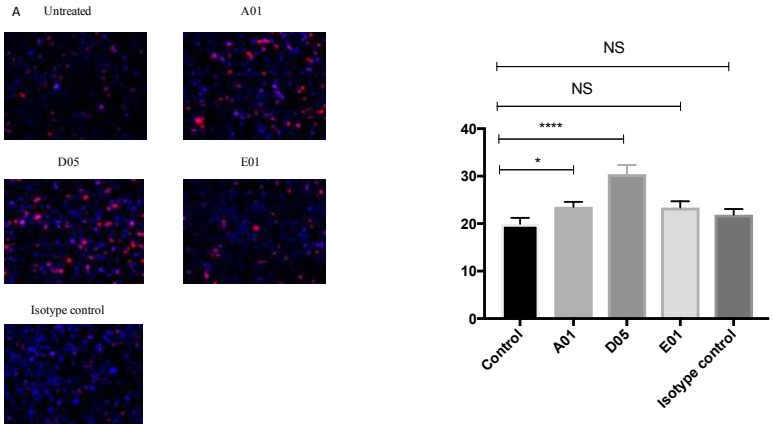

Figure 14: Monoclonal D05 (anti-PC IgG1) improves phagocytosis efficiency of macrophages.

As already described anti-PC IgMs and IgG1s are termed as group I antibodies, that could help in important homeostatic function such as removing apoptotic cells, binding to oxidized LDL, and subsequent inhibiting the uptake by macrophages[172]. The proposed role of anti-PC clone D05 in improving apoptotic cell uptake, thereby reducing further inflammation is shown in figure 15.

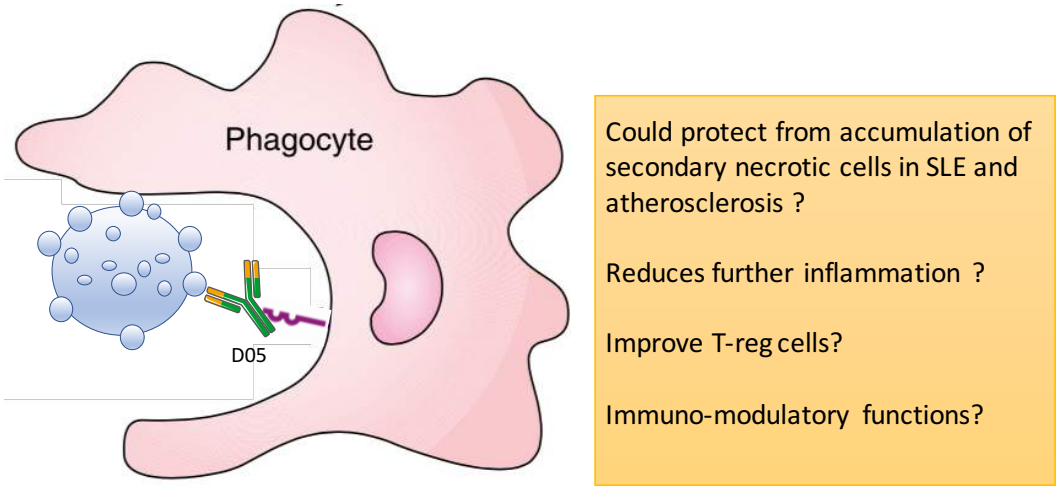

Figure 15: Proposed role of monoclonal D05 increasing apoptotic uptake by phagocytes

5 GENERAL DISCUSSION

The protective properties of natural antibodies especially, anti-PC IgM is well documented in the field of cardiovascular research[69]. The function of antibodies/factors against other relevant phospholipids are emerging. Biochemical characterization of the OxLDL lead to discovery of new phospholipid epitopes that are immunologically relevant. In fact, these oxidized phospholipids are the major targets of the innate immune system and has been a major topic of interest in the field of atherosclerosis, due to their protective property[71]. Persistent inflammation and increased production of ROS, shifts the paradigm towards autoimmune state. At the same time, the phospholipids are ubiquitous, and peroxidation can affect any cell type, thus could potentially lead to increased exposure of phospholipids, marking the beginning of inflammation. Unlike other pathogenic immune cells, B-cells or their products (antibodies), have not been sharply linked to pathogenic outcomes in CVD. Despite many traditional risk, the necessity for the search of other novel risk factors has never declined.

Oxidized phospholipids: a link between autoimmune disease and CVD?

Years ago, our group proposed the plausible role of oxidized phospholipids in patients with disease such as SLE, RA and anti-phospholipid syndrome (APS)[273]. Aforementioned, about 30-50% of autoantibodies against phospholipids are found circulating in patients with SLE and about one third develop APS with characteristic enhanced level of autoantibodies against cardiolipins (aCL), and anti- β2 glycoprotein (β2GPI) antibodies and lupus anti-coagulants. A number of studies pointed out accelerated atherosclerosis in both patients with PAPs as well as SLE, with increase in IMT as a classical sign. Some other studies showed interaction of OxLDL with β2GPI to form immune complex (IC), suggesting a plausible reason for vascular pathogenesis, that are inflicted in the case of SLE, APS and SSc[274]. In this thesis, we have discussed about prevalence of two antibodies that are epitopes of OxLDL. Although we focused on immunologically relevant epitopes of OxLDL, understanding the prevalence of these antibodies could shed light in phospholipid related immune activation and their possible role in rare chronic conditions. RA, though not a vascular related disease, dyslipidemia is associated with early stages of the disease. OxLDL seems to create pro-oxidative state in patients with RA, leading to further clinical consequences. In one of our studies, we showed, anti-PC IgM increased anti-TNF therapy, indicating dampening the inflammation alters lipid profile and this study also gives clue regarding reverting the protective natural antibodies with the help of anti-inflammatory therapy.

In study III we showed levels of natural antibodies and the associated CVD co-morbidity varied among patients with different rheumatic diseases. With the existing knowledge on OxLDL and cardiolipins in some autoimmune conditions, we wanted to extend our knowledge on PC and MDA to other conditions and specifically rare diseases, included within this broader group. We were able to confirm our finding with previous known data with respect to SLE, and find novel association with MCTD. Further investigation into other isotypes of antibodies and association with other classical autoantibodies could give insight in to possible clue to underlying disease mechanisms.

Natural Antibodies and Annexin, a possible therapy in CVD and autoimmune condition?

Given the clinical significance of these antibodies against OxLDL, especially small hapten like PC was a major interest in the animal models of atherosclerosis[275]. The authors showed active immunization with PC-KLH reduced plaque size and increased antibody titres (IgG and IgM)[275]. Years ago, immunizing of ApoE KO mice with OxLDL, in the neonatal stage, has showed reduction in atherosclerosis through deletion of reactive T cells[276]. Due to molecular mimicry between pneumococcus capsule and OxLDL, researchers even showed reduction in atherosclerotic lesion upon pneumococcus vaccination[149]. Experimental evidence using E06 (classical mouse T15 antibody) have shown to increase apoptotic cell uptake in mice models as well as reduce inflammatory arthritis[149]. A number of in-vitro experiment from our lab have pinpointed the reduction uptake of OxLDL[149] and increase regulatory T cell population[189]. Recently immunization with MDA-LDL also showed to reduce plaque size in atherosclerotic prone mice[277]. However, it is still hard to translate the finding to humans. Recently we developed fully human monoclonal antibodies (Study IV), against phosphorylcholine resulting in improved phagocytosis efficiency and dampened LPS induced inflammation. Both the property could be relevant in SLE and atherosclerosis where prompt removal of apoptotic cell is crucial. Immunization studies could possibly explain the systemic effect of the antibody in humans.

Similar to natural antibodies, Annexin A5 functions has been much studied in diseases such as SLE and anti-phospholipid syndrome. Annexin A5 has lower binding capacity to the endothelium in the patients with SLE[201]. A passive immunization could also be potential therapy in such patients, where Annexin A5 could potentially initiate antithrombotic events[201]. In the animal models Annexin A5 prevents vascular injury by accumulating and preventing the generation of thrombin. It is also proposed that Annexin A5 forms band-aid on the endothelial cells thus preventing them from rupture. Recently potential anti-inflammatory

role was proposed, where Annexin A5 ameliorate leukotriene production[203], which is of immense interest as they can dampen subsequent clinical adversity.

Considering potential benefits of the natural antibodies and Annexin A5, active or passive immunization would prove beneficial to patients with CVD and autoimmune diseases especially SLE or MCTD with CVD co-morbidity.

6 CONCLUDING REMARKS

In the present thesis, I document associations between circulating levels of IgM antibodies against three products of oxidization i.e., phosphorylcholine, malondialdehyde, and oxidized cardiolipin and protection against cardiovascular and systemic autoimmune diseases in humans. This protective role of natural anti-PC IgMs[155, 156, 162, 164, 278, 279], as well as Annexin A5[197, 198, 201] against CVD has also been observed previously. Here, we demonstrate a novel anti-inflammatory role played by Annexin A5 against oxidized cardiolipins and report on the prevalence of natural IgM antibodies in patients with cardiovascular and rare autoimmune diseases and employing pre-clinical studies to develop them into therapeutics for future.

In our first investigation, we found that oxidized cardiolipin exerts a pro-inflammatory effect on human macrophages and neutrophils in a calcium-dependent manner. In addition, we showed that Annexin-A5 binds to OxCL, but not native or reduced cardiolipin. OxCL stimulated the secretion of leukotrienes by macrophages and neutrophils in a manner dependent on 5-LOX and Annexin A5 abrogated this effect. Overall, Annexin A5 neutralized the pro-inflammatory effects of OxCL by binding directly to this epitope.

In our second set of experiments, we found associations between low levels of circulating IgMs against malondialdehyde in patients and increased cardiovascular risk, whereas higher levels appeared to be protective. In agreement with our previous observations[155], the levels of natural anti-MDA IgM were lower in patients than in age- and gender-matched controls, especially in the case of the men. Proteomics analysis of the polyclonal anti-MDA IgM revealed less sequence variability in the CDR3 region, which presumably contributes to their specificity.

In our third study, we extended these findings to systemic autoimmune diseases, demonstrating differences in circulating levels of anti-PC and anti-MDA IgM among patients with various autoimmune diseases. Furthermore, low levels of anti-PC, but not anti-MDA IgM were associated with diseases such as SLE and, in particular, with the rare and poorly defined autoimmune disease MCTD. When the patients were stratified on the basis of their cardiovascular scores, this negative association in the case of MCTD was even much stronger. Interestingly, higher levels of these antibodies were common among patients with RA. Peptide analysis of these antibodies showed certain similarities and differences that might contribute to their differences in recognition and thus involvement in various diseases.

Finally, we demonstrated that circulating levels of anti-PC IgG1, but not IgG2 were negatively associated with plaque vulnerability, history of CVD, and disease activity in patients with SLE. Our monoclonal antibodies targeted against phosphorylcholine improved the efficiency of phagocytosis by macrophages in a fashion dependent on Fc, also reducing inflammation. This indicates one possible mechanism by which anti-PC IgG1 could protect patients with SLE, since impaired clearance of apoptotic cells is a hallmark of this pathogenesis.

Our future efforts will be designed to understand potential associations between these and other pathogenic autoantibodies, as well as the mechanisms by which they regulate or modulate homeostasis and disease. Moreover, we will examine the effects of our monoclonal anti-PC antibodies in animal models of systemic autoimmune diseases. In addition, human monoclonal antibodies against other products of oxidation, including malondialdehyde and oxidized cardiolipins, will be developed and employed to elucidate the roles of these products in connection with oxidative pathogenesis. These belong to the repertoire of natural antibodies, the development of which from birth to adulthood in humans is still poorly understood. Mechanistic and omics-based approaches will be employed to monitor this development, which is important in understanding their involvement in pathological conditions. We hope that one day these protective factors can both provide valuable diagnostic markers and lead to the development of vaccines that can improve treatment of or perhaps even help prevent the development of inflammatory, cardiovascular and autoimmune diseases.

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8 REFERENCES

1. Gibbins I. the five cardinal signs of inflammation. *Med J Aust*. 2018;208:295.
2. Mahla RS, Reddy MC, Prasad DV and Kumar H. Sweeten PAMPs: Role of Sugar Complexed PAMPs in Innate Immunity and Vaccine Biology. *Front Immunol*. 2013;4:248.
3. Barton GM. A calculated response: control of inflammation by the innate immune system. *J Clin Invest*. 2008;118:413-20.
4. Takeuchi O and Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010;140:805-20.
5. Clark R and Kupper T. Old meets new: the interaction between innate and adaptive immunity. *J Invest Dermatol*. 2005;125:629-37.
6. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008;454:428-35.
7. Kono H, Onda A and Yanagida T. Molecular determinants of sterile inflammation. *Curr Opin Immunol*. 2014;26:147-56.
8. Thomas H, Diamond J, Vieco A, Chaudhuri S, Shinnar E, Cromer S, Perel P, Mensah GA, Narula J, Johnson CO, Roth GA and Moran AE. Global Atlas of Cardiovascular Disease 2000-2016: The Path to Prevention and Control. *Glob Heart*. 2018;13:143-163.
9. Mahmood SS, Levy D, Vasan RS and Wang TJ. The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet*. 2014;383:999-1008.
10. Dawber TR, Moore FE and Mann GV. Coronary heart disease in the Framingham study. *Am J Public Health Nations Health*. 1957;47:4-24.
11. Tousoulis D, Briasoulis A, Papageorgiou N, Tsioufis C, Tsiamis E, Toutouzas K and Stefanadis C. Oxidative stress and endothelial function: therapeutic interventions. *Recent Pat Cardiovasc Drug Discov*. 2011;6:103-14.
12. Buja LM. Nikolai N. Anitschkow and the lipid hypothesis of atherosclerosis. *Cardiovasc Pathol*. 2014;23:183-4.
13. Brown MS and Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science*. 1986;232:34-47.
14. Brown MS, Kovanen PT and Goldstein JL. Evolution of the LDL receptor concept-from cultured cells to intact animals. *Annals of the New York Academy of Sciences*. 1980;348:48-68.
15. Brown MS, Goldstein JL, Krieger M, Ho YK and Anderson RG. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J Cell Biol*. 1979;82:597-613.
16. Brown MS and Goldstein JL. Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proc Natl Acad Sci U S A*. 1974;71:788-92.
17. Ridker PM, Stampfer MJ and Rifai N. Novel risk factors for systemic atherosclerosis: a comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. *JAMA*. 2001;285:2481-5.
18. Ross R. The pathogenesis of atherosclerosis--an update. *N Engl J Med*. 1986;314:488-500.
19. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801-9.

20. Ross R. The pathogenesis of atherosclerosis. *Mech Ageing Dev*. 1979;9:435-40.
21. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation*. 2001;104:365-72.
22. Libby P. Inflammation and cardiovascular disease mechanisms. *The American journal of clinical nutrition*. 2006;83:456S-460S.
23. Libby P. Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2012;32:2045-51.
24. Libby P, Lichtman AH and Hansson GK. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity*. 2013;38:1092-104.
25. Klingenberg R and Hansson GK. Treating inflammation in atherosclerotic cardiovascular disease: emerging therapies. *Eur Heart J*. 2009;30:2838-44.
26. Gimbrone MA, Jr. Culture of vascular endothelium. *Prog Hemost Thromb*. 1976;3:1-28.
27. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL and Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A*. 1984;81:3883-7.
28. Kattoor AJ, Pothineni NVK, Palagiri D and Mehta JL. Oxidative Stress in Atherosclerosis. *Curr Atheroscler Rep*. 2017;19:42.
29. Steinberg D and Witztum JL. Oxidized low-density lipoprotein and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2010;30:2311-6.
30. Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr. and Boerwinkle E. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation*. 1997;96:4219-25.
31. Cybulsky MI and Gimbrone MA, Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science*. 1991;251:788-91.
32. Angelovich TA, Hearps AC and Jaworowski A. Inflammation-induced foam cell formation in chronic inflammatory disease. *Immunol Cell Biol*. 2015;93:683-93.
33. Burke JM and Ross R. Synthesis of connective tissue macromolecules by smooth muscle. *Int Rev Connect Tissue Res*. 1979;8:119-57.
34. Witte LD and Cornicelli JA. Platelet-derived growth factor stimulates low density lipoprotein receptor activity in cultured human fibroblasts. *Proc Natl Acad Sci U S A*. 1980;77:5962-6.
35. Chait A, Ross R, Albers JJ and Bierman EL. Platelet-derived growth factor stimulates activity of low density lipoprotein receptors. *Proc Natl Acad Sci U S A*. 1980;77:4084-8.
36. Bowen-Pope DF, Rosenfeld ME, Seifert RA and Ross R. The platelet-derived growth factor receptor. *Int J Neurosci*. 1985;26:141-53.
37. Johnson JL. Metalloproteinases in atherosclerosis. *Eur J Pharmacol*. 2017;816:93-106.
38. Mach F, Schonbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS and Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A*. 1997;94:1931-6.
39. Oka Y and Orth DN. Human plasma epidermal growth factor/beta-urogastrone is associated with blood platelets. *J Clin Invest*. 1983;72:249-59.

40. Ross R, Glomset J, Kariya B and Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci U S A*. 1974;71:1207-10.
41. Grotendorst GR, Chang T, Seppa HE, Kleinman HK and Martin GR. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. *J Cell Physiol*. 1982;113:261-6.
42. Deuel TF, Senior RM, Chang D, Griffin GL, Heinrikson RL and Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc Natl Acad Sci U S A*. 1981;78:4584-7.
43. Jonasson L, Holm J, Skalli O, Gabbiani G and Hansson GK. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. *J Clin Invest*. 1985;76:125-31.
44. Jonasson L, Holm J, Skalli O, Bondjers G and Hansson GK. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis*. 1986;6:131-8.
45. Libby P, Okamoto Y, Rocha VZ and Folco E. Inflammation in atherosclerosis: transition from theory to practice. *Circulation journal : official journal of the Japanese Circulation Society*. 2010;74:213-20.
46. French JE. Atherosclerosis in relation to the structure and function of the arterial intima, with special reference to th endothelium. *Int Rev Exp Pathol*. 1966;5:253-353.
47. Virchow RLK and Chance F. *Cellular pathology : as based upon physiological and pathological histology. Twenty lectures delivered in the Pathological institute of Berlin during the months of February, March and April, 1858.* 7th American ed. New York: R.M. De Witt; 1860.
48. Libby P, Ridker PM and Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317-25.
49. Steyers CM, 3rd and Miller FJ, Jr. Endothelial dysfunction in chronic inflammatory diseases. *Int J Mol Sci*. 2014;15:11324-49.
50. Croce K and Libby P. Intertwining of thrombosis and inflammation in atherosclerosis. *Current opinion in hematology*. 2007;14:55-61.
51. Galis ZS, Sukhova GK, Lark MW and Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *The Journal of clinical investigation*. 1994;94:2493-503.
52. Schieber M and Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol*. 2014;24:R453-62.
53. Catala A. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chem Phys Lipids*. 2009;157:1-11.
54. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am J Med*. 1991;91:14S-22S.
55. Kim M, Han CH and Lee MY. NADPH oxidase and the cardiovascular toxicity associated with smoking. *Toxicol Res*. 2014;30:149-57.
56. Esterbauer H, Schaur RJ and Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*. 1991;11:81-128.
57. Bochkov VN, Oskolkova OV, Birukov KG, Levonen AL, Binder CJ and Stockl J. Generation and biological activities of oxidized phospholipids. *Antioxid Redox Signal*. 2010;12:1009-59.

58. Salomon RG. Structural identification and cardiovascular activities of oxidized phospholipids. *Circ Res*. 2012;111:930-46.
59. Miller YI, Choi SH, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, Boullier A, Gonen A, Diehl CJ, Que X, Montano E, Shaw PX, Tsimikas S, Binder CJ and Witztum JL. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res*. 2011;108:235-48.
60. Morel DW, Hessler JR and Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J Lipid Res*. 1983;24:1070-6.
61. Miller YI and Shyy JY. Context-Dependent Role of Oxidized Lipids and Lipoproteins in Inflammation. *Trends Endocrinol Metab*. 2017;28:143-152.
62. Lee S, Birukov KG, Romanoski CE, Springstead JR, Lusis AJ and Berliner JA. Role of phospholipid oxidation products in atherosclerosis. *Circ Res*. 2012;111:778-99.
63. Gutteridge JM and Halliwell B. Comments on review of Free Radicals in Biology and Medicine, second edition, by Barry Halliwell and John M. C. Gutteridge. *Free Radic Biol Med*. 1992;12:93-5.
64. Binder CJ, Papac-Milicevic N and Witztum JL. Innate sensing of oxidation-specific epitopes in health and disease. *Nat Rev Immunol*. 2016;16:485-97.
65. Tomasz A. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus. *Science*. 1967;157:694-7.
66. Sanchez-Beato AR, Ronda C and Garcia JL. Tracking the evolution of the bacterial choline-binding domain: molecular characterization of the Clostridium acetobutylicum NCIB 8052 cspA gene. *J Bacteriol*. 1995;177:1098-103.
67. Gillespie SH, Ainscough S, Dickens A and Lewin J. Phosphorylcholine-containing antigens in bacteria from the mouth and respiratory tract. *J Med Microbiol*. 1996;44:35-40.
68. Al-Riyami L and Harnett W. Immunomodulatory properties of ES-62, a phosphorylcholine-containing glycoprotein secreted by Acanthocheilonema viteae. *Endocr Metab Immune Disord Drug Targets*. 2012;12:45-52.
69. de Faire U and Frostegard J. Natural antibodies against phosphorylcholine in cardiovascular disease. *Annals of the New York Academy of Sciences*. 2009;1173:292-300.
70. Boullier A, Gillotte KL, Horkko S, Green SR, Friedman P, Dennis EA, Witztum JL, Steinberg D and Quehenberger O. The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. *J Biol Chem*. 2000;275:9163-9.
71. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Backhed F, Miller YI, Horkko S, Corr M, Witztum JL and Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *The Journal of clinical investigation*. 2009;119:1335-49.
72. Karp G. *Cell and molecular biology : concepts and experiments.* 6th ed. Hoboken, NJ: John Wiley; 2010.
73. Uchida K. Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol Med*. 2000;28:1685-96.
74. Del Rio D, Stewart AJ and Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis*. 2005;15:316-28.
75. Busch CJ and Binder CJ. Malondialdehyde epitopes as mediators of sterile inflammation. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2017;1862:398-406.

76. Akbulut H, Akbulut KG, Icli F and Buyukcelik A. Daily variations of plasma malondialdehyde levels in patients with early breast cancer. *Cancer Detect Prev*. 2003;27:122-6.
77. Gonenc A, Ozkan Y, Torun M and Simsek B. Plasma malondialdehyde (MDA) levels in breast and lung cancer patients. *J Clin Pharm Ther*. 2001;26:141-4.
78. Manju V, Kalaivani Sailaja J and Nalini N. Circulating lipid peroxidation and antioxidant status in cervical cancer patients: a case-control study. *Clin Biochem*. 2002;35:621-5.
79. Bakan E, Taysi S, Polat MF, Dalga S, Umudum Z, Bakan N and Gumus M. Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer. *Jpn J Clin Oncol*. 2002;32:162-6.
80. Bakan N, Taysi S, Yilmaz O, Bakan E, Kuskay S, Uzun N and Gundogdu M. Glutathione peroxidase, glutathione reductase, Cu-Zn superoxide dismutase activities, glutathione, nitric oxide, and malondialdehyde concentrations in serum of patients with chronic lymphocytic leukemia. *Clin Chim Acta*. 2003;338:143-9.
81. Dierckx N, Horvath G, van Gils C, Vertommen J, van de Vliet J, De Leeuw I and Manuel-y-Keenoy B. Oxidative stress status in patients with diabetes mellitus: relationship to diet. *Eur J Clin Nutr*. 2003;57:999-1008.
82. Karabulut AB, Kafkasli A, Burak F and Gozukara EM. Maternal and fetal plasma adenosine deaminase, xanthine oxidase and malondialdehyde levels in pre-eclampsia. *Cell Biochem Funct*. 2005;23:279-83.
83. Yoneyama Y, Sawa R, Suzuki S, Doi D, Yoneyama K, Otsubo Y and Araki T. Relationship between plasma malondialdehyde levels and adenosine deaminase activities in preeclampsia. *Clin Chim Acta*. 2002;322:169-73.
84. Haberland ME, Fong D and Cheng L. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science*. 1988;241:215-8.
85. Holvoet P, Perez G, Zhao Z, Brouwers E, Bernar H and Collen D. Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic disease. *J Clin Invest*. 1995;95:2611-9.
86. Binder CJ, Hartvigsen K, Chang MK, Miller M, Broide D, Palinski W, Curtiss LK, Corr M and Witztum JL. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest*. 2004;114:427-37.
87. Freigang S, Horkko S, Miller E, Witztum JL and Palinski W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. *Arterioscler Thromb Vasc Biol*. 1998;18:1972-82.
88. Zhou X, Caligiuri G, Hamsten A, Lefvert AK and Hansson GK. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2001;21:108-14.
89. Fogelman AM, Shechter I, Seager J, Hokom M, Child JS and Edwards PA. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc Natl Acad Sci U S A*. 1980;77:2214-8.
90. Slatter DA, Bolton CH and Bailey AJ. The importance of lipid-derived malondialdehyde in diabetes mellitus. *Diabetologia*. 2000;43:550-7.
91. Yang TC, Chen YJ, Chang SF, Chen CH, Chang PY and Lu SC. Malondialdehyde mediates oxidized LDL-induced coronary toxicity through the Akt-FGF2 pathway via DNA methylation. *J Biomed Sci*. 2014;21:11.

92. Greaves DR and Gordon S. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. *J Lipid Res*. 2009;50 Suppl:S282-6.
93. Canton J, Neculai D and Grinstein S. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol*. 2013;13:621-34.
94. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF and Freeman MW. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem*. 2002;277:49982-8.
95. Boullier A, Friedman P, Harkewicz R, Hartvigsen K, Green SR, Almazan F, Dennis EA, Steinberg D, Witztum JL and Quehenberger O. Phosphocholine as a pattern recognition ligand for CD36. *J Lipid Res*. 2005;46:969-76.
96. Gillotte-Taylor K, Boullier A, Witztum JL, Steinberg D and Quehenberger O. Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. *J Lipid Res*. 2001;42:1474-82.
97. Duryee MJ, Freeman TL, Willis MS, Hunter CD, Hamilton BC, 3rd, Suzuki H, Tuma DJ, Klassen LW and Thiele GM. Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins. *Mol Pharmacol*. 2005;68:1423-30.
98. Shechter I, Fogelman AM, Haberland ME, Seager J, Hokom M and Edwards PA. The metabolism of native and malondialdehyde-altered low density lipoproteins by human monocyte-macrophages. *J Lipid Res*. 1981;22:63-71.
99. Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, Ermolaeva M, Veldhuizen R, Leung YH, Wang H, Liu H, Sun Y, Pasparakis M, Kopf M, Mech C, Bavari S, Peiris JS, Slutsky AS, Akira S, Hultqvist M, Holmdahl R, Nicholls J, Jiang C, Binder CJ and Penninger JM. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*. 2008;133:235-49.
100. Park YM. CD36, a scavenger receptor implicated in atherosclerosis. *Exp Mol Med*. 2014;46:e99.
101. Chang MK, Binder CJ, Torzewski M and Witztum JL. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc Natl Acad Sci U S A*. 2002;99:13043-8.
102. Buono C, Come CE, Witztum JL, Maguire GF, Connelly PW, Carroll M and Lichtman AH. Influence of C3 deficiency on atherosclerosis. *Circulation*. 2002;105:3025-31.
103. Vlaicu R, Niculescu F, Rus HG and Cristea A. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis*. 1985;57:163-77.
104. Weismann D and Binder CJ. The innate immune response to products of phospholipid peroxidation. *Biochim Biophys Acta*. 2012;1818:2465-75.
105. Ehrlich P. Croonian lecture: on immunity with special reference to cell life. *Proceedings of the royal Society of London*. 1899;66.
106. Avrameas S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol Today*. 1991;12:154-9.
107. Mackie TJ. Non-Specific Stimulation of a Natural Antibody. *J Hyg (Lond)*. 1925;24:176-88.
108. Kidd JG and Friedewald WF. A Natural Antibody That Reacts in Vitro with a Sedimentable Constituent of Normal Tissue Cells : Ii. Specificity of the Phenomenon: General Discussion. *J Exp Med*. 1942;76:557-78.
109. Hurst MM, Volanakis JE, Hester RB, Stroud RM and Bennett JC. The structural basis for binding of complement by immunoglobulin M. *J Exp Med*. 1974;140:1117-21.

110. Panda S, Zhang J, Yang L, Anand GS and Ding JL. Molecular interaction between natural IgG and ficolin--mechanistic insights on adaptive-innate immune crosstalk. *Sci Rep*. 2014;4:3675.
111. Zhang J, Koh J, Lu J, Thiel S, Leong BS, Sethi S, He CY, Ho B and Ding JL. Local inflammation induces complement crosstalk which amplifies the antimicrobial response. *PLoS Pathog*. 2009;5:e1000282.
112. Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, Du X and Hoebe K. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu Rev Immunol*. 2006;24:353-89.
113. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol*. 2006;6:33-43.
114. Horn MP, Gerster T, Ochensberger B, Derer T, Kricek F, Jouvin MH, Kinet JP, Tschernig T, Vogel M, Stadler BM and Miescher SM. Human anti-FcepsilonRIalpha autoantibodies isolated from healthy donors cross-react with tetanus toxoid. *Eur J Immunol*. 1999;29:1139-48.
115. Prabhakar BS, Saegusa J, Onodera T and Notkins AL. Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repertoire. *J Immunol*. 1984;133:2815-7.
116. Martin T, Crouzier R, Weber JC, Kipps TJ and Pasquali JL. Structure-function studies on a polyreactive (natural) autoantibody. Polyreactivity is dependent on somatically generated sequences in the third complementarity-determining region of the antibody heavy chain. *J Immunol*. 1994;152:5988-96.
117. Ichiyoshi Y and Casali P. Analysis of the structural correlates for antibody polyreactivity by multiple reassortments of chimeric human immunoglobulin heavy and light chain V segments. *J Exp Med*. 1994;180:885-95.
118. Lieby P, Poindron V, Roussi S, Klein C, Knapp AM, Garaud JC, Cerutti M, Martin T and Pasquali JL. Pathogenic antiphospholipid antibody: an antigen-selected needle in a haystack. *Blood*. 2004;104:1711-5.
119. Lacroix-Desmazes S, Kaveri SV, Mouthon L, Ayouba A, Malanchere E, Coutinho A and Kazatchkine MD. Self-reactive antibodies (natural autoantibodies) in healthy individuals. *J Immunol Methods*. 1998;216:117-37.
120. Bouanani M, Bataille R, Piechaczyk M, Salhi SL, Pau B and Bastide M. Autoimmunity to human thyroglobulin. Respective epitopic specificity patterns of anti-human thyroglobulin autoantibodies in patients with Sjogren's syndrome and patients with Hashimoto's thyroiditis. *Arthritis Rheum*. 1991;34:1585-93.
121. Kroese FG, Ammerlaan WA and Kantor AB. Evidence that intestinal IgA plasma cells in mu, kappa transgenic mice are derived from B-1 (Ly-1 B) cells. *International immunology*. 1993;5:1317-27.
122. Kantor AB and Herzenberg LA. Origin of murine B cell lineages. *Annual review of immunology*. 1993;11:501-38.
123. Hayakawa K, Hardy RR, Parks DR and Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med*. 1983;157:202-18.
124. Kantor AB, Stall AM, Adams S, Herzenberg LA and Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. *Proceedings of the National Academy of Sciences of the United States of America*. 1992;89:3320-4.
125. Hardy RR and Hayakawa K. CD5 B cells, a fetal B cell lineage. *Adv Immunol*. 1994;55:297-339.

126. Hardy RR. B-1 B cells: development, selection, natural autoantibody and leukemia. *Curr Opin Immunol*. 2006;18:547-55.
127. Godin IE, Garcia-Porrero JA, Coutinho A, Dieterlen-Lievre F and Marcos MA. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature*. 1993;364:67-70.
128. Hayakawa K, Hardy RR, Herzenberg LA and Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J Exp Med*. 1985;161:1554-68.
129. Kantor AB, Merrill CE, Herzenberg LA and Hillson JL. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *Journal of immunology*. 1997;158:1175-86.
130. Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, Silver J and Hardy RR. Positive selection of natural autoreactive B cells. *Science*. 1999;285:113-6.
131. Ferry H, Jones M, Vaux DJ, Roberts IS and Cornall RJ. The cellular location of self-antigen determines the positive and negative selection of autoreactive B cells. *J Exp Med*. 2003;198:1415-25.
132. Hayakawa K, Asano M, Shinton SA, Gui M, Wen LJ, Dashoff J and Hardy RR. Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. *J Exp Med*. 2003;197:87-99.
133. Ehrenstein MR and Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol*. 2010;10:778-86.
134. Griffin DO, Holodick NE and Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *The Journal of experimental medicine*. 2011;208:67-80.
135. Rahman M, Sing S, Golabkesh Z, Fiskesund R, Gustafsson T, Jogestrand T, Frostegard AG, Hafstrom I, Liu A and Frostegard J. IgM antibodies against malondialdehyde and phosphorylcholine are together strong protection markers for atherosclerosis in systemic lupus erythematosus: Regulation and underlying mechanisms. *Clinical immunology*. 2016;166-167:27-37.
136. Fiskesund R, Steen J, Amara K, Murray F, Szwajda A, Liu A, Douagi I, Malmstrom V and Frostegard J. Naturally occurring human phosphorylcholine antibodies are predominantly products of affinity-matured B cells in the adult. *J Immunol*. 2014;192:4551-9.
137. Merbl Y, Zucker-Toledano M, Quintana FJ and Cohen IR. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. *The Journal of clinical investigation*. 2007;117:712-8.
138. Frostegard AG, Sjoberg BG, Frostegard J and Norman M. IgM-Antibodies against Phosphorylcholine in Mothers and Normal or Low Birth Weight Term Newborn Infants. *PloS one*. 2014;9:e106584.
139. Gronwall C, Clancy RM, Getu L, Lloyd KA, Siegel DL, Reed JH, Buyon JP and Silverman GJ. Modulation of natural IgM autoantibodies to oxidative stress-related neo-epitopes on apoptotic cells in newborns of mothers with anti-Ro autoimmunity. *J Autoimmun*. 2016;73:30-41.
140. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nature reviews Immunology*. 2011;11:34-46.
141. Mouthon L, Nobrega A, Nicolas N, Kaveri SV, Barreau C, Coutinho A and Kazatchkine MD. Invariance and restriction toward a limited set of self-antigens characterize neonatal IgM antibody repertoires and prevail in autoreactive repertoires of healthy adults. *Proc Natl Acad Sci U S A*. 1995;92:3839-43.

142. Mouthon L, Lacroix-Desmazes S, Nobrega A, Barreau C, Coutinho A and Kazatchkine MD. The self-reactive antibody repertoire of normal human serum IgM is acquired in early childhood and remains conserved throughout life. *Scand J Immunol*. 1996;44:243-51.
143. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E and Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science*. 2003;301:1374-7.
144. Horkko S, Binder CJ, Shaw PX, Chang MK, Silverman G, Palinski W and Witztum JL. Immunological responses to oxidized LDL. *Free radical biology & medicine*. 2000;28:1771-9.
145. Palinski W, Yla-Herttuala S, Rosenfeld ME, Butler SW, Socher SA, Parthasarathy S, Curtiss LK and Witztum JL. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis*. 1990;10:325-35.
146. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP and Witztum JL. Human-derived anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions in vivo. *Arteriosclerosis, thrombosis, and vascular biology*. 2001;21:1333-9.
147. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ and Witztum JL. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest*. 2000;105:1731-40.
148. Palinski W, Miller E and Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A*. 1995;92:821-5.
149. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL and Silverman GJ. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nature medicine*. 2003;9:736-43.
150. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Friedman P, Dennis EA, Curtiss LK, Palinski W and Witztum JL. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *The Journal of clinical investigation*. 1999;103:117-28.
151. Briles DE, Forman C, Hudak S and Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. *The Journal of experimental medicine*. 1982;156:1177-85.
152. Leon MA and Young NM. Specificity for phosphorylcholine of six murine myeloma proteins reactive with Pneumococcus C polysaccharide and beta-lipoprotein. *Biochemistry*. 1971;10:1424-9.
153. Nishinarita S, Sawada S and Horie T. Phosphorylcholine antibodies in pulmonary infection. *Med Microbiol Immunol*. 1990;179:205-14.
154. Frostegard J. Low level natural antibodies against phosphorylcholine: a novel risk marker and potential mechanism in atherosclerosis and cardiovascular disease. *Clinical immunology*. 2010;134:47-54.
155. Su J, Georgiades A, Wu R, Thulin T, de Faire U and Frostegard J. Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension. *Atherosclerosis*. 2006;188:160-6.

156. Su J, Hua X, Concha H, Svenungsson E, Cederholm A and Frostegard J. Natural antibodies against phosphorylcholine as potential protective factors in SLE. *Rheumatology*. 2008;47:1144-50.
157. Gronlund H, Hallmans G, Jansson JH, Boman K, Wikstrom M, de Faire U and Frostegard J. Low levels of IgM antibodies against phosphorylcholine predict development of acute myocardial infarction in a population-based cohort from northern Sweden. *Eur J Cardiovasc Prev Rehabil*. 2009;16:382-6.
158. Gronwall C, Akhter E, Oh C, Burlingame RW, Petri M and Silverman GJ. IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE. *Clinical immunology*. 2012;142:390-8.
159. Gronwall C, Reynolds H, Kim JK, Buyon J, Goldberg JD, Clancy RM and Silverman GJ. Relation of carotid plaque with natural IgM antibodies in patients with systemic lupus erythematosus. *Clinical immunology*. 2014;153:1-7.
160. Frostegard J, Tao W, Georgiades A, Rastam L, Lindblad U and Lindeberg S. Atheroprotective natural anti-phosphorylcholine antibodies of IgM subclass are decreased in Swedish controls as compared to non-westernized individuals from New Guinea. *Nutrition & metabolism*. 2007;4:7.
161. Frostegard J. Prediction and management of cardiovascular outcomes in systemic lupus erythematosus. *Expert review of clinical immunology*. 2015;11:247-53.
162. 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; a journal of cerebral circulation*. 2010;41:607-12.
163. Padilla ND, Ciurana C, van Oers J, Ogilvie AC and Hack CE. Levels of natural IgM antibodies against phosphorylcholine in healthy individuals and in patients undergoing isolated limb perfusion. *Journal of immunological methods*. 2004;293:1-11.
164. Fiskesund R, Su J, Bulatovic I, Vikstrom M, de Faire U and Frostegard J. IgM phosphorylcholine antibodies inhibit cell death and constitute a strong protection marker for atherosclerosis development, particularly in combination with other auto-antibodies against modified LDL. *Results Immunol*. 2012;2:13-8.
165. Thiagarajan D, Frostegard AG, Singh S, Rahman M, Liu A, Vikstrom M, Leander K, Gigante B, Hellenius ML, Zhang B, Zubarev RA, de Faire U, Lundstrom SL and Frostegard J. Human IgM Antibodies to Malondialdehyde Conjugated With Albumin Are Negatively Associated With Cardiovascular Disease Among 60-Year-Olds. *Journal of the American Heart Association*. 2016;5.
166. Karvonen J, Paivansalo M, Kesaniemi YA and Horkko S. Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation*. 2003;108:2107-12.
167. Anania C, Gustafsson T, Hua X, Su J, Vikstrom M, de Faire U, Heimburger M, Jogestrand T and Frostegard J. Increased prevalence of vulnerable atherosclerotic plaques and low levels of natural IgM antibodies against phosphorylcholine in patients with systemic lupus erythematosus. *Arthritis Res Ther*. 2010;12:R214.
168. Chen Y, Khanna S, Goodyear CS, Park YB, Raz E, Thiel S, Gronwall C, Vas J, Boyle DL, Corr M, Kono DH and Silverman GJ. Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol*. 2009;183:1346-59.
169. Ajeganova S, Fiskesund R, de Faire U, Hafstrom I and Frostegard J. Effect of biological therapy on levels of atheroprotective antibodies against phosphorylcholine and

apolipoproteins in rheumatoid arthritis - a one year study. *Clinical and experimental rheumatology*. 2011;29:942-50.
170. Eriksson UK, Sjoberg BG, Bennet AM, de Faire U, Pedersen NL and Frostegard J. Low levels of antibodies against phosphorylcholine in Alzheimer's disease. *J Alzheimers Dis*. 2010;21:577-84.
171. Tsimikas S, Willeit P, Willeit J, Santer P, Mayr M, Xu Q, Mayr A, Witztum JL and Kiechl S. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol*. 2012;60:2218-29.
172. de Faire U, Su J, Hua X, Frostegard A, Halldin M, Hellenius ML, Wikstrom M, Dahlbom I, Gronlund H and Frostegard J. Low levels of IgM antibodies to phosphorylcholine predict cardiovascular disease in 60-year old men: effects on uptake of oxidized LDL in macrophages as a potential mechanism. *J Autoimmun*. 2010;34:73-9.
173. Frostegard J. Immunity, atherosclerosis and cardiovascular disease. *BMC Med*. 2013;11:117.
174. Turunen SP, Kummu O, Harila K, Veneskoski M, Soliymani R, Baumann M, Pussinen PJ and Horkko S. Recognition of Porphyromonas gingivalis gingipain epitopes by natural IgM binding to malondialdehyde modified low-density lipoprotein. *PLoS One*. 2012;7:e34910.
175. Gronwall C, Amara K, Hardt U, Krishnamurthy A, Steen J, Engstrom M, Sun M, Ytterberg AJ, Zubarev RA, Scheel-Toellner D, Greenberg JD, Klareskog L, Catrina AI, Malmstrom V and Silverman GJ. Autoreactivity to malondialdehyde-modifications in rheumatoid arthritis is linked to disease activity and synovial pathogenesis. *J Autoimmun*. 2017;84:29-45.
176. Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J and Barletta R. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. *The Journal of experimental medicine*. 1981;153:694-705.
177. Huck DM, Okello E, Mirembe G, Ssinabulya I, Zidar DA, Silverman GJ, Getu L, Nowacki AS, Calabrese LH, Salata RA and Longenecker CT. Role of Natural Autoantibodies in Ugandans With Rheumatic Heart Disease and HIV. *EBioMedicine*. 2016;5:161-6.
178. Alberts B. *Molecular biology of the cell.* Sixth edition. ed.
179. Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, Xu Y, Autenrieth IB, Schulze-Osthoff K, Belka C, Stuhler G and Wesselborg S. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell*. 2003;113:717-30.
180. Ravichandran KS. "Recruitment signals" from apoptotic cells: invitation to a quiet meal. *Cell*. 2003;113:817-20.
181. Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA and Witztum JL. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *The Journal of experimental medicine*. 2004;200:1359-70.
182. Panda S and Ding JL. Natural antibodies bridge innate and adaptive immunity. *J Immunol*. 2015;194:13-20.
183. Chen Y, Park YB, Patel E and Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol*. 2009;182:6031-43.
184. Quartier P, Potter PK, Ehrenstein MR, Walport MJ and Botto M. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by

murine bone marrow-derived macrophages in vitro. *European journal of immunology*. 2005;35:252-60.
185. Ogden CA, Kowalewski R, Peng Y, Montenegro V and Elkon KB. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. *Autoimmunity*. 2005;38:259-64.
186. Shaw PX, Goodyear CS, Chang MK, Witztum JL and Silverman GJ. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells. *Journal of immunology*. 2003;170:6151-7.
187. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A and Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97:1184-9.
188. Ehrenstein MR, Cook HT and Neuberger MS. Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. *The Journal of experimental medicine*. 2000;191:1253-8.
189. Sun J, Lundstrom SL, Zhang B, Zubarev RA, Steuer J, Gillgren P, Rahman M, Ajeganova S, Liu A and Frostegard J. IgM antibodies against phosphorylcholine promote polarization of T regulatory cells from patients with atherosclerotic plaques, systemic lupus erythematosus and healthy donors. *Atherosclerosis*. 2018;268:36-48.
190. Raynal P and Pollard HB. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim Biophys Acta*. 1994;1197:63-93.
191. Reutelingsperger CP, Hornstra G and Hemker HC. Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. *Eur J Biochem*. 1985;151:625-9.
192. Tait JF, Frankenberry DA, Shiang R, Murray JC, Adler DA and Disteche CM. Chromosomal localization of the human gene for annexin V (placental anticoagulant protein I) to 4q26----q28. *Cytogenet Cell Genet*. 1991;57:187-92.
193. Matsuda R, Kaneko N, Horikawa Y, Chiwaki F, Shinozaki M, Abe S, Yumura W, Nihei H and Ieiri T. Measurement of urinary annexin V by ELISA and its significance as a new urinary-marker of kidney disease. *Clin Chim Acta*. 2000;298:29-43.
194. Gonzalez-Conejero R, Corral J, Roldan V, Martinez C, Marin F, Rivera J, Iniesta JA, Lozano ML, Marco P and Vicente V. A common polymorphism in the annexin V Kozak sequence (-1C>T) increases translation efficiency and plasma levels of annexin V, and decreases the risk of myocardial infarction in young patients. *Blood*. 2002;100:2081-6.
195. Vermes I, Steur EN, Reutelingsperger C and Haanen C. Decreased concentration of annexin V in parkinsonian cerebrospinal fluid: speculation on the underlying cause. *Mov Disord*. 1999;14:1008-10.
196. Gerke V, Creutz CE and Moss SE. Annexins: linking Ca2+ signalling to membrane dynamics. *Nat Rev Mol Cell Biol*. 2005;6:449-61.
197. Cederholm A and Frostegard J. Annexin A5 in cardiovascular disease and systemic lupus erythematosus. *Immunobiology*. 2005;210:761-8.
198. Cederholm A and Frostegard J. Annexin A5 as a novel player in prevention of atherothrombosis in SLE and in the general population. *Annals of the New York Academy of Sciences*. 2007;1108:96-103.
199. Kietselaer BL, Reutelingsperger CP, Heidendal GA, Daemen MJ, Mess WH, Hofstra L and Narula J. Noninvasive detection of plaque instability with use of radiolabeled annexin A5 in patients with carotid-artery atherosclerosis. *The New England journal of medicine*. 2004;350:1472-3.

200. Rand JH. Molecular pathogenesis of the antiphospholipid syndrome. *Circulation research*. 2002;90:29-37.
201. Cederholm A, Svenungsson E, Jensen-Urstad K, Trollmo C, Ulfgren AK, Swedenborg J, Fei GZ and Frostegard J. Decreased binding of annexin v to endothelial cells: a potential mechanism in atherothrombosis of patients with systemic lupus erythematosus. *Arterioscler Thromb Vasc Biol*. 2005;25:198-203.
202. van Tits L, de Graaf J, Toenhake H, van Heerde W and Stalenhoef A. C-reactive protein and annexin A5 bind to distinct sites of negatively charged phospholipids present in oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol*. 2005;25:717-22.
203. Wan M, Hua X, Su J, Thiagarajan D, Frostegard AG, Haeggstrom JZ and Frostegard J. Oxidized but not native cardiolipin has pro-inflammatory effects, which are inhibited by Annexin A5. *Atherosclerosis*. 2014;235:592-8.
204. Jara LJ, Medina G, Vera-Lastra O and Amigo MC. Accelerated atherosclerosis, immune response and autoimmune rheumatic diseases. *Autoimmun Rev*. 2006;5:195-201.
205. Packard RR and Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clinical chemistry*. 2008;54:24-38.
206. Mok CC and Lau CS. Pathogenesis of systemic lupus erythematosus. *Journal of clinical pathology*. 2003;56:481-90.
207. Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK and Witztum JL. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest*. 1996;98:800-14.
208. Ren Y, Tang J, Mok MY, Chan AW, Wu A and Lau CS. Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils in systemic lupus erythematosus. *Arthritis Rheum*. 2003;48:2888-97.
209. Gaipl US, Munoz LE, Grossmayer G, Lauber K, Franz S, Sarter K, Voll RE, Winkler T, Kuhn A, Kalden J, Kern P and Herrmann M. Clearance deficiency and systemic lupus erythematosus (SLE). *J Autoimmun*. 2007;28:114-21.
210. Urowitz MB, Bookman AA, Koehler BE, Gordon DA, Smythe HA and Ogryzlo MA. The bimodal mortality pattern of systemic lupus erythematosus. *The American journal of medicine*. 1976;60:221-5.
211. Avina-Zubieta JA, To F, Vostretsova K, De Vera M, Sayre EC and Esdaile JM. Risk of Myocardial Infarction and Stroke in Newly Diagnosed Systemic Lupus Erythematosus: A General Population-Based Study. *Arthritis Care Res (Hoboken)*. 2017;69:849-856.
212. Amaya-Amaya J, Montoya-Sanchez L and Rojas-Villarraga A. Cardiovascular involvement in autoimmune diseases. *Biomed Res Int*. 2014;2014:367359.
213. Soltesz P, Kerekes G, Der H, Szucs G, Szanto S, Kiss E, Bodolay E, Zeher M, Timar O, Szodoray P, Szegedi G and Szekanecz Z. Comparative assessment of vascular function in autoimmune rheumatic diseases: considerations of prevention and treatment. *Autoimmun Rev*. 2011;10:416-25.
214. Thompson T, Sutton-Tyrrell K, Wildman RP, Kao A, Fitzgerald SG, Shook B, Tracy RP, Kuller LH, Brockwell S and Manzi S. Progression of carotid intima-media thickness and plaque in women with systemic lupus erythematosus. *Arthritis Rheum*. 2008;58:835-42.
215. Lopez-Mejias R, Castaneda S, Gonzalez-Juanatey C, Corrales A, Ferraz-Amaro I, Genre F, Remuzgo-Martinez S, Rodriguez-Rodriguez L, Blanco R, Llorca J, Martin J and Gonzalez-Gay MA. Cardiovascular risk assessment in patients with rheumatoid arthritis:

The relevance of clinical, genetic and serological markers. *Autoimmun Rev*. 2016;15:1013-1030.
216. da Cunha VR, Brenol CV, Brenol JC and Xavier RM. Rheumatoid arthritis and metabolic syndrome. *Rev Bras Reumatol*. 2011;51:260-8.
217. Solomon DH, Karlson EW, Rimm EB, Cannuscio CC, Mandl LA, Manson JE, Stampfer MJ and Curhan GC. Cardiovascular morbidity and mortality in women diagnosed with rheumatoid arthritis. *Circulation*. 2003;107:1303-7.
218. Gonzalez-Juanatey C, Llorca J, Testa A, Revuelta J, Garcia-Porrua C and Gonzalez-Gay MA. Increased prevalence of severe subclinical atherosclerotic findings in long-term treated rheumatoid arthritis patients without clinically evident atherosclerotic disease. *Medicine (Baltimore)*. 2003;82:407-13.
219. Gonzalez-Juanatey C, Llorca J and Gonzalez-Gay MA. Correlation between endothelial function and carotid atherosclerosis in rheumatoid arthritis patients with long-standing disease. *Arthritis Res Ther*. 2011;13:R101.
220. van Halm VP, Nurmohamed MT, Twisk JW, Dijkmans BA and Voskuyl AE. Disease-modifying antirheumatic drugs are associated with a reduced risk for cardiovascular disease in patients with rheumatoid arthritis: a case control study. *Arthritis Res Ther*. 2006;8:R151.
221. Dixon WG, Watson KD, Lunt M, Hyrich KL, British Society for Rheumatology Biologics Register Control Centre C, Silman AJ, Symmons DP and British Society for Rheumatology Biologics R. Reduction in the incidence of myocardial infarction in patients with rheumatoid arthritis who respond to anti-tumor necrosis factor alpha therapy: results from the British Society for Rheumatology Biologics Register. *Arthritis Rheum*. 2007;56:2905-12.
222. Luczaj W, Gindzienska-Sieskiewicz E, Jarocka-Karpowicz I, Andrisic L, Sierakowski S, Zarkovic N, Waeg G and Skrzydlewska E. The onset of lipid peroxidation in rheumatoid arthritis: consequences and monitoring. *Free Radic Res*. 2016;50:304-13.
223. Nguyen TG, McKelvey KJ, March LM, Hunter DJ, Xue M, Jackson CJ and Morris JM. Aberrant levels of natural IgM antibodies in osteoarthritis and rheumatoid arthritis patients in comparison to healthy controls. *Immunol Lett*. 2016;170:27-36.
224. Hannan EL, Wu C, Walford G, Culliford AT, Gold JP, Smith CR, Higgins RS, Carlson RE and Jones RH. Drug-eluting stents vs. coronary-artery bypass grafting in multivessel coronary disease. *N Engl J Med*. 2008;358:331-41.
225. Gotto AM, Jr. The cardiology patient page. Statins: powerful drugs for lowering cholesterol: advice for patients. *Circulation*. 2002;105:1514-6.
226. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, Joyal SV, Hill KA, Pfeffer MA, Skene AM, Pravastatin or Atorvastatin E and Infection Therapy-Thrombolysis in Myocardial Infarction I. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *The New England journal of medicine*. 2004;350:1495-504.
227. Frostegard J, Zhang Y, Sun J, Yan K and Liu A. Oxidized Low-Density Lipoprotein (OxLDL)-Treated Dendritic Cells Promote Activation of T Cells in Human Atherosclerotic Plaque and Blood, Which Is Repressed by Statins: microRNA let-7c Is Integral to the Effect. *J Am Heart Assoc*. 2016;5.
228. Nissen SE, Tuzcu EM, Libby P, Thompson PD, Ghali M, Garza D, Berman L, Shi H, Buebendorf E, Topol EJ and Investigators C. Effect of antihypertensive agents on cardiovascular events in patients with coronary disease and normal blood pressure: the CAMELOT study: a randomized controlled trial. *Jama*. 2004;292:2217-25.

229. Westlake SL, Colebatch AN, Baird J, Kiely P, Quinn M, Choy E, Ostor AJ and Edwards CJ. The effect of methotrexate on cardiovascular disease in patients with rheumatoid arthritis: a systematic literature review. *Rheumatology*. 2010;49:295-307.
230. Cutolo M, Sulli A, Pizzorni C, Seriolo B and Straub RH. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Ann Rheum Dis*. 2001;60:729-35.
231. Funk CD. Leukotriene modifiers as potential therapeutics for cardiovascular disease. *Nat Rev Drug Discov*. 2005;4:664-72.
232. Back M and Hansson GK. Anti-inflammatory therapies for atherosclerosis. *Nat Rev Cardiol*. 2015;12:199-211.
233. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR and Golub TR. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435:834-8.
234. Sardu C, Marfella R, Santulli G and Paolisso G. Functional role of miRNA in cardiac resynchronization therapy. *Pharmacogenomics*. 2014;15:1159-68.
235. Lutgens E, Lievens D, Beckers L, Wijnands E, Soehnlein O, Zernecke A, Seijkens T, Engel D, Cleutjens J, Keller AM, Naik SH, Boon L, Oufella HA, Mallat Z, Ahonen CL, Noelle RJ, de Winther MP, Daemen MJ, Biessen EA and Weber C. Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by skewing the immune response toward an antiinflammatory profile. *The Journal of experimental medicine*. 2010;207:391-404.
236. Jacobsson LT, Turesson C, Gulfe A, Kapetanovic MC, Petersson IF, Saxne T and Geborek P. Treatment with tumor necrosis factor blockers is associated with a lower incidence of first cardiovascular events in patients with rheumatoid arthritis. *J Rheumatol*. 2005;32:1213-8.
237. Rajamaki K, Lappalainen J, Oorni K, Valimaki E, Matikainen S, Kovanen PT and Eklund KK. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One*. 2010;5:e11765.
238. Ridker PM, Howard CP, Walter V, Everett B, Libby P, Hensen J, Thuren T and Group CPI. Effects of interleukin-1beta inhibition with canakinumab on hemoglobin A1c, lipids, C-reactive protein, interleukin-6, and fibrinogen: a phase IIb randomized, placebo-controlled trial. *Circulation*. 2012;126:2739-48.
239. Ridker PM, Thuren T, Zalewski A and Libby P. Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). *Am Heart J*. 2011;162:597-605.
240. Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, Kuder JF, Wang H, Liu T, Wasserman SM, Sever PS, Pedersen TR, Committee FS and Investigators. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *N Engl J Med*. 2017;376:1713-1722.
241. Sabatine MS, Leiter LA, Wiviott SD, Giugliano RP, Deedwania P, De Ferrari GM, Murphy SA, Kuder JF, Gouni-Berthold I, Lewis BS, Handelsman Y, Pineda AL, Honarpour N, Keech AC, Sever PS and Pedersen TR. Cardiovascular safety and efficacy of the PCSK9 inhibitor evolocumab in patients with and without diabetes and the effect of evolocumab on glycaemia and risk of new-onset diabetes: a prespecified analysis of the FOURIER randomised controlled trial. *Lancet Diabetes Endocrinol*. 2017;5:941-950.
242. Mann CJ. Observational research methods. Research design II: cohort, cross sectional, and case-control studies. *Emerg Med J*. 2003;20:54-60.
243. Schlame M, Rua D and Greenberg ML. The biosynthesis and functional role of cardiolipin. *Prog Lipid Res*. 2000;39:257-88.

244. Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA, Osipov AN, Belikova NA, Kapralov AA, Kini V, Vlasova, II, Zhao Q, Zou M, Di P, Svistunenko DA, Kurnikov IV and Borisenko GG. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol*. 2005;1:223-32.
245. Bayir H, Fadeel B, Palladino MJ, Witasp E, Kurnikov IV, Tyurina YY, Tyurin VA, Amoscato AA, Jiang J, Kochanek PM, DeKosky ST, Greenberger JS, Shvedova AA and Kagan VE. Apoptotic interactions of cytochrome c: redox flirting with anionic phospholipids within and outside of mitochondria. *Biochim Biophys Acta*. 2006;1757:648-59.
246. Tuominen A, Miller YI, Hansen LF, Kesaniemi YA, Witztum JL and Horkko S. A natural antibody to oxidized cardiolipin binds to oxidized low-density lipoprotein, apoptotic cells, and atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2006;26:2096-102.
247. Dahlen SE, Bjork J, Hedqvist P, Arfors KE, Hammarstrom S, Lindgren JA and Samuelsson B. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proc Natl Acad Sci U S A*. 1981;78:3887-91.
248. Helgadottir A, Manolescu A, Helgason A, Thorleifsson G, Thorsteinsdottir U, Gudbjartsson DF, Gretarsdottir S, Magnusson KP, Gudmundsson G, Hicks A, Jonsson T, Grant SF, Sainz J, O'Brien SJ, Sveinbjornsdottir S, Valdimarsson EM, Matthiasson SE, Levey AI, Abramson JL, Reilly MP, Vaccarino V, Wolfe ML, Gudnason V, Quyyumi AA, Topol EJ, Rader DJ, Thorgeirsson G, Gulcher JR, Hakonarson H, Kong A and Stefansson K. A variant of the gene encoding leukotriene A4 hydrolase confers ethnicity-specific risk of myocardial infarction. *Nat Genet*. 2006;38:68-74.
249. Helgadottir A, Manolescu A, Thorleifsson G, Gretarsdottir S, Jonsdottir H, Thorsteinsdottir U, Samani NJ, Gudmundsson G, Grant SF, Thorgeirsson G, Sveinbjornsdottir S, Valdimarsson EM, Matthiasson SE, Johannsson H, Gudmundsdottir O, Gurney ME, Sainz J, Thorhallsdottir M, Andresdottir M, Frigge ML, Topol EJ, Kong A, Gudnason V, Hakonarson H, Gulcher JR and Stefansson K. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet*. 2004;36:233-9.
250. Dwyer JH, Allayee H, Dwyer KM, Fan J, Wu H, Mar R, Lusis AJ and Mehrabian M. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N Engl J Med*. 2004;350:29-37.
251. Pinton P, Giorgi C, Siviero R, Zecchini E and Rizzuto R. Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene*. 2008;27:6407-18.
252. Funk CD, Hoshiko S, Matsumoto T, Rdmark O and Samuelsson B. Characterization of the human 5-lipoxygenase gene. *Proc Natl Acad Sci U S A*. 1989;86:2587-91.
253. Haeggstrom JZ and Funk CD. Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chem Rev*. 2011;111:5866-98.
254. Ferucci ED, Johnston JM, Gordon C, Helmick CG and Lim SS. Prevalence of Mixed Connective Tissue Disease in a Population-Based Registry of American Indian/Alaska Native People in 2007. *Arthritis Care Res (Hoboken)*. 2017;69:1271-1275.
255. Reiseter S, Gunnarsson R, Corander J, Haydon J, Lund MB, Aalokken TM, Taraldsrud E, Hetlevik SO and Molberg O. Disease evolution in mixed connective tissue disease: results from a long-term nationwide prospective cohort study. *Arthritis Res Ther*. 2017;19:284.

256. Flam ST, Gunnarsson R, Garen T, Norwegian MSG, Lie BA and Molberg O. The HLA profiles of mixed connective tissue disease differ distinctly from the profiles of clinically related connective tissue diseases. *Rheumatology*. 2015;54:528-35.
257. Rai R and Swetha T. Association of anti-phospholipid antibodies with connective tissue diseases. *Indian Dermatol Online J*. 2015;6:89-91.
258. Mesa A, Somarelli JA, Wu W, Martinez L, Blom MB, Greidinger EL and Herrera RJ. Differential immunoglobulin class-mediated responses to components of the U1 small nuclear ribonucleoprotein particle in systemic lupus erythematosus and mixed connective tissue disease. *Lupus*. 2013;22:1371-81.
259. Bodolay E, Prohaszka Z, Paragh G, Csipo I, Nagy G, Laczik R, Demeter N, Zold E, Nakken B, Szegedi G and Szodoray P. Increased levels of anti-heat-shock protein 60 (anti-Hsp60) indicate endothelial dysfunction, atherosclerosis and cardiovascular diseases in patients with mixed connective tissue disease. *Immunol Res*. 2014;60:50-9.
260. Grant CR, Liberal R, Mieli-Vergani G, Vergani D and Longhi MS. Regulatory T-cells in autoimmune diseases: challenges, controversies and--yet--unanswered questions. *Autoimmun Rev*. 2015;14:105-16.
261. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, Nochy D, Debre P, Piette JC and Gorochov G. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *Journal of immunology*. 2005;175:8392-400.
262. Habibagahi M, Habibagahi Z, Jaberipour M and Aghdashi A. Quantification of regulatory T cells in peripheral blood of patients with systemic lupus erythematosus. *Rheumatol Int*. 2011;31:1219-25.
263. Lawson CA, Brown AK, Bejarano V, Douglas SH, Burgoyne CH, Greenstein AS, Boylston AW, Emery P, Ponchel F and Isaacs JD. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. *Rheumatology*. 2006;45:1210-7.
264. Proto JD, Doran AC, Gusarova G, Yurdagul A, Jr., Sozen E, Subramanian M, Islam MN, Rymond CC, Du J, Hook J, Kuriakose G, Bhattacharya J and Tabas I. Regulatory T Cells Promote Macrophage Efferocytosis during Inflammation Resolution. *Immunity*. 2018;49:666-677 e6.
265. Mallat Z, Gojova A, Brun V, Esposito B, Fournier N, Cottrez F, Tedgui A and Groux H. Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. *Circulation*. 2003;108:1232-7.
266. Mor A, Luboshits G, Planer D, Keren G and George J. Altered status of CD4(+)CD25(+) regulatory T cells in patients with acute coronary syndromes. *Eur Heart J*. 2006;27:2530-7.
267. Potekhina AV, Pylaeva E, Provatorov S, Ruleva N, Masenko V, Noeva E, Krasnikova T and Arefieva T. Treg/Th17 balance in stable CAD patients with different stages of coronary atherosclerosis. *Atherosclerosis*. 2015;238:17-21.
268. Barath S, Sipka S, Aleksza M, Szegedi A, Szodoray P, Vegh J, Szegedi G and Bodolay E. Regulatory T cells in peripheral blood of patients with mixed connective tissue disease. *Scand J Rheumatol*. 2006;35:300-4.
269. Alunno A, Carubbi F, Bistoni O, Caterbi S, Bartoloni E, Mirabelli G, Cannarile F, Cipriani P, Giacomelli R and Gerli R. T Regulatory and T Helper 17 Cells in Primary Sjogren's Syndrome: Facts and Perspectives. *Mediators Inflamm*. 2015;2015:243723.
270. Slobodin G and Rimar D. Regulatory T Cells in Systemic Sclerosis: a Comprehensive Review. *Clin Rev Allergy Immunol*. 2017;52:194-201.

271. Nadkarni S, Mauri C and Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *J Exp Med*. 2007;204:33-9.
272. Lieberman R, Potter M, Mushinski EB, Humphrey W, Jr. and Rudikoff S. Genetics of a new IgVH (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J Exp Med*. 1974;139:983-1001.
273. Frostegard J. Atherosclerosis in patients with autoimmune disorders. *Arterioscler Thromb Vasc Biol*. 2005;25:1776-85.
274. Lopez LR, Simpson DF, Hurley BL and Matsuura E. OxLDL/beta2GPI complexes and autoantibodies in patients with systemic lupus erythematosus, systemic sclerosis, and antiphospholipid syndrome: pathogenic implications for vascular involvement. *Annals of the New York Academy of Sciences*. 2005;1051:313-22.
275. Caligiuri G, Khallou-Laschet J, Vandaele M, Gaston AT, Delignat S, Mandet C, Kohler HV, Kaveri SV and Nicoletti A. Phosphorylcholine-targeting immunization reduces atherosclerosis. *J Am Coll Cardiol*. 2007;50:540-6.
276. Nicoletti A, Paulsson G, Caligiuri G, Zhou X and Hansson GK. Induction of neonatal tolerance to oxidized lipoprotein reduces atherosclerosis in ApoE knockout mice. *Mol Med*. 2000;6:283-90.
277. Turunen SP, Kummu O, Wang C, Harila K, Mattila R, Sahlman M, Pussinen PJ and Horkko S. Immunization with malondialdehyde-modified low-density lipoprotein (LDL) reduces atherosclerosis in LDL receptor-deficient mice challenged with Porphyromonas gingivalis. *Innate Immun*. 2015;21:370-85.
278. Su J, Frostegard AG, Hua X, Gustafsson T, Jogestrand T, Hafstrom I and Frostegard J. Low levels of antibodies against oxidized but not nonoxidized cardiolipin and phosphatidylserine are associated with atherosclerotic plaques in systemic lupus erythematosus. *J Rheumatol*. 2013;40:1856-64.
279. Su J, Hua X, Vikstrom M, Leander K, Gigante B, Hellenius ML, de Faire U and Frostegard J. Low levels of IgM antibodies to oxidized cardiolipin increase and high levels decrease risk of cardiovascular disease among 60-year olds: a prospective study. *BMC Cardiovasc Disord*. 2013;13:1.