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Title	Rheumatoid arthritis and atherosclerosis: common pathogenic mechanisms explored
Authors	Stack, John
Publication date	2020-02-18
Original Citation	Stack, J. R. 2020. Rheumatoid arthritis and atherosclerosis: common pathogenic mechanisms explored. MD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2024-04-26 04:29:03
Item downloaded from	https://hdl.handle.net/10468/9939

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Rheumatoid Arthritis and Atherosclerosis: Common Pathogenic Mechanisms Explored

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for the degree of

Doctor of Medicine

University College Cork

School of Medicine

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2020

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Acknowledgements

Firstly I would like to thank my supervisor Prof Mick Molloy for his continued support and encouragement throughout this process. I would like to thank ICARE which funded this research. I would also like to thank the staff at UCC who helped me at various stages. These people include Dr Grainne Murphy who helped me with the confocal microscopy and immunohistochemistry, Dr Kevin Hegarty for his advice regarding genotyping and the genetic case control study, Dr Liam Fanning for allowing me use of the MagNA Pure LC instrument in order to facilitate DNA extraction and Mary Daly, CNS who assisted in collecting patient blood samples for DNA extraction. I would also like to thank Dr Grace O'Callaghan for her help and support whilst I was based at the clinical research centre. For the final chapter I am grateful to Prof Geraldine McCarthy and Prof Dermot Kenny for their support and guidance with the GPVI study and to Anne Madigan , clinical research nurse who helped me with sample and data collection. Finally I would like to thank my family and friends who have supported me throughout this process and encouraged me to see this project through to end.

Chapter 1: Introduction

1.1 Rheumatoid Arthritis- Definition and Basic Pathology

Rheumatoid arthritis (RA) is an autoimmune disease that characteristically manifests with a symmetrical arthritis targeting the small joints of the hands and feet. It has an estimated incidence of 1%.[1] A combination of environmental and genetic factors leads to a breakdown in immune tolerance, resulting in activation of both the adaptive and the innate immune response. This in turn gives rise to: hyperplasia and inflammatory changes within the synovium, antibody production (in the form of rheumatoid factor and anti-citrullinated protein antibody [ACPA]), and ultimately cartilage and bone destruction.[1]

1.2 Increased Risk of Atherosclerosis among subjects with RA

Patients with RA carry an increased risk of cardiovascular disease (CVD) which is comparable to that of diabetes.[2] Patients with RA are twice as likely to die from sudden cardiac death as their disease free counterparts and myocardial infarction remains the leading cause of death among subjects with RA.[3]

The increased risk of atherosclerosis among RA subjects remains incompletely understood. Traditional Framingham risk factors such as dyslipidaemia, hypertension and smoking are increased among patients with RA.[4–6] Systemic inflammation promotes a pro-atherogenic lipid profile in RA resulting in high levels of LDL cholesterol and low levels of HDL, which in turn contribute to the increased risk of atherosclerosis.[4] Smoking is an important risk factor for the development of both RA and atherosclerosis. It

is associated with a poorer outcome for patients with RA both in terms of joint destruction and cardiovascular morbidity and mortality. Individuals who possess the shared epitope and who smoke have an increased risk of developing seropositive RA with a tendency towards aggressive, erosive disease.[7] There is also data to suggest that individuals who possess the shared epitope are at increased risk of developing atherosclerotic plaques early in the course of their disease.[8] Insulin resistance appears to be increased in patients with RA promoting the release of adipokines which in turn serve to promote inflammation and atherosclerosis.[9] RA has also been shown to be associated with hypertension.[10] Whilst these factors contribute to the overall increased CVD risk, there is evidence to suggest that other pathological mechanisms may also play a role.

Cadaver studies have shown that the atherosclerotic plaque burden in subjects with RA vs. controls is similar, but that subjects with RA carry an increased amount of unstable plaque. This would suggest that subjects with RA are at an increased risk of plaque instability, likely driven by pro-inflammatory mechanisms that are common to both RA and atherosclerosis.

1.3 Atherosclerosis- An Inflammatory Disease

Atherosclerosis is a dynamic disease process of the arteries, the hallmark of which is the atherosclerotic plaque. Such plaques consist of focal thickenings of the inner most layer of the artery wall, the intima.[11] They originate from fatty streaks, which are an accumulation of lipid-laden macrophages and T

Cells. Fatty streaks are prevalent in young people and may ultimately disappear or progress to form plaques.[12]

Similar to rheumatoid synovium, atherosclerotic plaques are comprised of an inflammatory cell infiltrate, which include: T cells, macrophages, mast cells and lipid-laden macrophages (foam cells). This composite of cells, lipids and debris is covered by a fibrous cap consisting of smooth muscle cells and collagen rich matrix. It is the breakdown of this cap that leads to plaque rupture, extrusion of plaque contents and ultimately thrombosis or embolisation.[13]

A combination of dyslipidaemia and haemodynamic 'shear stress' is thought to form the initial insult that provokes the onset of plaque formation and progression. Excess LDL particles migrate through the vessel wall to the intima where they become oxidised leading to the formation of phospholipids which in turn activate endothelial cells, provoking the inflammatory insult necessary for plaque formation.[14,15]

Endothelial cells, once activated, engage with circulating platelets leading to further endothelial cell activation and upregulation of adhesion molecules (e.g. VCAM-1). In the presence of a chemokine gradient, activated endothelial cells attract circulating monocytes and lymphocytes to the vessel wall where they subsequently migrate into the subendothelial space.[16]

Activated endothelial cells then facilitate the transmigration of monocytes into the endothelial space where they are differentiated by macrophage colony-stimulating factor to become macrophages.[17] These macrophages ingest surrounding oxidised LDL, cell debris, and bacterial endotoxin evolving into a lipid laden macrophage or foam cell, the prototypical cell of

the atherosclerotic plaque. Macrophages and foam cells in turn become activated by toll like receptors (TLRs) on the cell surface in response to a variety of antigens to become the main source of cytokine production within the atheromatous plaque.[18]

As with RA, T Cells also play an important role in the pathogenesis of atherosclerosis. $CD4^+$ T cells are always present in atherosclerotic plaques. Once triggered by the appropriate antigen, $CD4^+$ T cells elicit a Th1 response producing an array of cytokines including tumour necrosis factor alpha (TNF-α), interleukine-1 (IL-1) and interferon gamma (INF-γ). Production of these cytokines leads to a pro- inflammatory cascade which promotes atherosclerosis and the production of other cytokines downstream e.g. c-reactive protein (CRP) and interleukin-6 (IL-6) which can be detected systemically.[18]

Whilst a universal association between patients with atherosclerosis and all inflammatory diseases exists, a specific inflammatory component of RA contributing to cardiovascular disease risk has recently been identified. $CD4^+$ T Cells lacking the surface CD28 molecule ($CD4^+$ $CD28^-$ T Cells) have been shown to be upregulated in both RA and unstable angina.[3] This particular subset of T cells appears to possess marked inflammatory and tissue damaging properties. They secrete an abundance of pro-inflammatory cytokines which in turn promotes endothelial injury and are thought to be key proponents of plaque rupture.[19] These findings suggest that an immunopathogenic mechanism exists promoting atherosclerosis that is unique to RA.[20]

It is therefore apparent that many similarities between the pathogenesis of RA and atherosclerosis exist, with both diseases sharing similar immune mechanisms. These shared mechanisms could help to explain the increased CVD risk that is evident in RA.

For the purpose of this thesis I decided to explore this concept further by focusing on two separate immune mechanisms common to both RA and atherosclerosis. The first of these mechanisms involves the chemokine receptor fractalkine (FKN) and its receptor, CX3CR1. The second mechanism involves platelet activation and the platelet specific glycoprotein VI receptor (GPVI).

1.4 Fractalkine and CX3CR1- Structure and Function

CX3CR1 is a specific chemokine receptor for the chemokine, fractalkine (FKN). FKN belongs to a family of small 8-10 kDa proteins known as chemokines which function mainly as chemoattractant cytokines.[21] Chemokines are named according to their structure, based on the number and spacing of the first 2 cysteins within a conserved cystein motif.[22] More than 40 chemokines have been identified to date.[22] There are four families of chemokines: C, CC, CXC and CX3C. Fractalkine is the sole member of the CX3C family and is also named CX3CL1 (CX3C ligand 1). It is the largest of the chemokines consisting of 373 amino acids and can exist in both soluble and membrane bound form.[23] Membrane bound FKN consists of a cystein containing extracellular domain attached to the cell surface by a mucin-like stalk followed by a single transmembrane spanning domain.[24] The stalk can

be cleaved by proteolysis to yield a soluble form of FKN.[25,26] FKN may therefore function as an adhesion molecule when in its membrane bound form and as a chemoattractant molecule when in its soluble form.

CX3CR1, the unique receptor for FKN, is similar in structure to other chemokine receptors. It has a serpentine structure with 7 transmembrane spanning domains. Signalling occurs via the G protein pathway.[27]

FKN displays a number of unique characteristics that distinguishes it from other chemokines and makes it an interesting molecule to study in terms of the pathogenesis of RA.

It differs from the other chemokines in that it demonstrates remarkable specificity for binding to its receptor, CX3CR1 and is able to bind to its receptor with high affinity under both static and high flow conditions.[28] Notably, its binding properties are much more potent than other well known adhesions molecules such as VCAM-1 or VLA-4.[29]

It also possesses powerful chemoattractant properties and has been demonstrated to be a potent chemoattractant molecule for T cells and monocytes expressing CX3CR1, thus facilitating the recruitment of these cells to the rheumatoid synovium.

In addition to its cell adhesion and chemoattractant properties, the interaction of FKN/ CX3CR1 facilitates a number of functions that are

important in the pathogenesis of RA. These include: the transmigration of inflammatory cells to the synovium, neoangiogenesis and the upregulation of osteoclasts and MMPs which in turn are important mediators of joint destruction.[28,30–32]

1.5 FKN/ CX3CR1 – Role in RA

FKN is a potent chemoattractant molecule that facilitates the transmigration of inflammatory cells through the blood vessel wall into the rheumatoid synovium. Macrophages, dendritic cells, T Cells, NK cells and osteoclast precursors expressing CX3CR1 all migrate to the synovium in response to the presence of FKN and do so in a dose dependent manner. All of these cells form part of the inflammatory milieu that characterises synovitis. SF depleted of FKN demonstrates a 32% reduction in chemotactic capability, illustrating its importance as a chemoattractant molecule.[33] The effects of the FKN/CX3CR1 on the various cells making up the inflammatory infiltrate in RA are now discussed separately.

T Cells

T cells and macrophages predominate the inflammatory infiltrate that is present in RA.[33–35] In peripheral blood taken from patients with RA, CX3CR1 expression on CD4+ and CD8+ T cells is increased when compared with healthy controls.[36] CD4+ T and +CD8+ T cells expressing CX3CR1 secrete higher levels of INFγ, TNFα, granzyme A and perforin when compared to T

cells negative for CX3CR1.[36] Hence, CX3CR1 may be considered a marker of T cell cytokine producing and cytotoxic activity.

Interestingly senescent CD4+ T cells which lack CD28- are expanded in patients with RA, also aberrantly express CX3CR1.[37] These cells overexpress INF-Y, are resistant to apoptosis and show increased cytolytic activity. Conditions for these cells to enter the cell cycle are enhanced in the presence of FKN.[38] Therefore the FKN/CX3CR1 axis not only augments the cytotoxic function of these senescent T cells, but also enhances their replicative potential. FKN/CX3CR1 may therefore contribute to the breakdown in self-tolerance and chronic inflammation that occurs in RA.

Macrophages

CX3CR1 is highly expressed among macrophages and monocytes in rheumatoid synovium. In particular, a subtype of monocytes that express both CD14 and CD16 (CD14+CD16+) are increased in both the serum and synovial tissue (ST) of patients with RA.[34] These monocytes express CX3CR1 more readily than 'conventional' CD14++CD16- monocytes and appear to be more 'proinflammatory' as they express higher high levels of class II MHC molecules, adhesion molecules and chemokine receptors and are hence more efficient at producing inflammatory cytokines such as TNFα.[39] Furthermore these monocytes secrete less of the anti-inflammatory cytokine, IL-10. The expression of CX3CR1 on monocytes may therefore again be considered a marker of their inflammatory potential.

Fibroblast-like Synoviocytes

FKN/ CX3CR1 also plays a role in FLS proliferation. FLS are key mediators of pannus formation and joint destruction within the rheumatoid joint and are a major source of FKN.[1] They are resistant to apoptosis and secrete proinflammatory proteases and cytokines that contribute to joint destruction. FLS proliferation has been demonstrated to be regulated by CD4+ CD28- senescent T cells in vitro.[40] FLS expressing mFKN bind strongly to CD4+CD28- T Cells expressing CX3CR1. This interaction stimulates a FLS 'autocrine growth loop', thus expanding the population of FLS.[38] FLS –T Cell interaction also stimulates T cell production of TNFα which in turn also amplifies this growth loop.

Osteoclasts

The FKN/ CX3CR1 axis also plays a role in osteoclast differentiation and function. CX3CR1 is expressed on osteoclast precursors whilst FKN is expressed on osteoblasts.[41] This is not surprising given that these cells are derived from the same lineage of cells as monocytes and macrophages, which also readily express CX3CR1 as, discussed. FKN on osteoblasts functions as an adhesion molecule for osteoclast precursors. Blocking of the FKN/CX3CR1 axis by anti-FKN mAb inhibits osteoblast-induced differentiation of osteoclasts in vitro.[41] Bone resorption is also reduced in the presence of anti-FKN mAB, further highlighting the importance of the FKN/CX3CR1 axis in the pathogenesis of RA and its potential as a therapeutic target. Anti-FKN mAb could therefore help prevent the evolution of boney erosions- the hallmarks of RA.

Angiogenesis

Angiogenesis, the growth and proliferation of new blood vessels is a key factor in the pathogenesis of RA. Pannus formation characterised by highly vascularised, tumour like expansion of the synovium is pathognomic of the disease. This new vessel formation facilitates the extravasation of inflammatory cells into the synovium. New vessel formation requires the sequential processes of: endothelial cell migration, proliferation, elongation, orientation and differentiation resulting in lumen formation, basement-membrane re-formation and anastomosis with other vessels to create new vascular networks.[42] FKN has been demonstrated to facilitate this process and can induce the proliferation, migration and tube formation of human umbilical vein endothelial cells in both in vitro and in vivo experiments.[43] Furthermore SF deplete of FKN shows substantially reduced angiogenic activity, further evidence of its pro-angiogenic properties.[44]

These examples therefore illustrate the importance of FKN/CX3CR1 in the pathogenesis of RA via their adhesion, chemoattractant and angiogenic properties.

1.6 FKN/ CX3CR1- Role in Atherosclerosis

The effects of the fractalkine/CX3CR1 signalling pathway on atherosclerosis has been demonstrated in both human and animal studies. Wong *et al.*, showed that CX3CR1 was highly expressed among foam cells and smooth muscle cells of human atherosclerotic arteries but not healthy control arteries.[45] Combadiere and Lesnick were able to show independently that pro-atherogenic ApoE$^{-/-}$ mice deficient in CX3CR1 were less likely to develop atherosclerotic plaques than mice able to express CX3CR1.[46,47] Both (ApoE$^{-/-}$, CX3CR1$^{-/-}$) and (ApoE$^{-/-}$, CX3CR1$^{+/-}$) mice were able to demonstrate a resistance to developing atherosclerosis compared to mice with both copies of the CX3CR1 gene (ApoE$^{-/-}$, CX3CR1$^{+/+}$). Lesnick et al were able to further demonstrate that CX3CR1 deficient mice had reduced recruitment of macrophages to the vessel wall. More recently the effects of pharmacological inhibition of CX3CR1 in Apo E -/- mice have been demonstrated. Apo E-/- mice treated with a CX3CR1 antagonist develop reduced levels of atherosclerosis and reduced levels of monocyte recruitment. These results add support to the role of fractalkine and monocyte recruitment in the pathogenesis of atherosclerosis.

In summary atherosclerosis is an inflammatory disease. Numerous immune mechanisms that are involved in the pathogenesis of RA are involved in the pathogenesis of atherosclerosis, including the FKN/ CX3CR1 pathway.

As part of this thesis I chose to explore the role of FKN/CX3CR1 in RA further. The first study presented is a genetic case control study, which tests for association between 2 SNPs (rs3732378 and rs3732379) located within the

coding sequence of *CX3CR1.* The SNPs have been previously shown to a protective association against developing atherosclerosis. Given the similar pathogenic mechanisms that occur in relation to CX3CR1 and RA and also CX3CR1 and atherosclerosis we hypothesized that a similar protective association would be observed between these 2 SNPs and RA.

The second study examines the co-localisation of monocytes and CX3CR1 expression in human RA synovium using immunohistochemistry. In doing so we attempt to provide further evidence of the potent chemotactic properties of CX3CR1/FKN in RA.

1.7 Platelet Activation in RA

The final part of this thesis focuses on the role of platelets and the role of platelet activation in RA. Platelets are well known for their role in thrombosis and haemostasis, however until recently, their inflammatory potential remained less well explored. Platelet activation results in the release of biologically active microparticles (MP) which can promote the expression of a host of inflammatory cytokines.[48] In the setting of thrombosis, enhanced platelet activity results in the release of α-granules containing adenosine diphosphate (ADP), serotonin, P-selectin, chemokines and cytokines.[49] Release of these pro-thrombotic substances ultimately culminates in activation of the platelet specific integrin αIIbβ3, enhanced platelet aggregation and thrombus formation. Included amongst the array of cytokines released are IL-1, IL-6, IL-8 and TNFα, all of which are known for their pro-inflammatory properties.[48] Excessive platelet activation can result

in vascular inflammation, atherosclerotic plaque instability and plaque rupture.[50,51] Platelet activation has also been shown to occur in RA and could potentially help to explain the increased cardiovascular disease risk that exists among these patients.

Platelet activation in RA is a relatively new concept. It was first described by Boilard et al., who showed that platelet MPs were present in abundance within human RA synovial fluid (SF), and were capable of inducing cytokine release from synovial fibroblasts.[52] Using a K/BxN serum transfer mouse model of RA they went on to demonstrate that platelet depleted mice displayed a markedly reduced inflammatory phenotype. Using a combination of pharmacological blockade and *Gp6* $^{-/-}$ mice they further went on to show that signaling via GPVI was the predominant pathway through which platelet MP generation was mediated.

1.8 Soluble GPVI- A Useful Biomarker of Platelet Activation

GPVI is a ~64 kDa transmembrane glycoprotein which is found exclusively on platelets and acts as the predominant platelet receptor for collagen. It contains 2 extracellular immunoglobulin (Ig)- like domains, a mucin domain, transmembrane domain and a cytoplasmic tail of approximately 50 residues. Within the cytoplasmic tail portion of the dimerized Fcγ chain are 2 immunoreceptor-tyrosine based active motifs (ITAM)s. Signal transduction occurs when ligand engages with GPVI resulting in phosphorylation of the ITAM motif and proteolytic cleavage of the GPVI receptor at the platelet surface. A 55kDa ectodomain fragment, soluble GPVI (sGPVI) is shed leaving

behind a 10kDa remnant. Shedding is rapid and irreversible and is likely to function as a rate-limiting step in collagen mediated thrombin generation.[53] GPVI shedding can however also be precipitated by a variety of other mechanisms including engagement with the low affinity immunoglobulin G (IgG) receptor (FcγRIIa) on platelets, with which GPVI is closely associated. [54]

Recently it was demonstrated by Habets et al., that platelet activation is associated with the presence of ACPA and that blockade of the low affinity platelet IgG receptor, FcγRIIa *in vitro*, inhibited ACPA-mediated platelet activation.[55] FcγRIIa was therefore identified as the ligand through which ACPA-mediated platelet activation occurs. FcγRIIa signaling is known to occur in conjunction with GPVI such that ligand binding to FcγRIIa results in 'dual signaling' of FcγRIIa and GPVI pathways and shedding of GPVI.[54] This mechanism is not unique to ACPA and has been demonstrated in other diseases such as immune mediated thrombocytopenia (ITP) in which anti-platelet antibodies derived from a patient with ITP were able to activate GPVI via FcγRIIa.[56,57] Similarly, circulating immune complexes derived from patients with systemic lupus erythematosis (SLE) have been shown to activate platelets *in vitro* via FcγRIIa.[58] In a separate study, anti-GPVI IgG antibodies were detected in a patient with SLE resulting in impaired platelet response to collagen. [59]

Therefore for the final part of this thesis I investigated whether sGPVI would be increased among subjects with seropositive RA vs. seronegative RA and healthy controls as a consequence of antibody mediated platelet activation.

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Chapter 2: Association between a polymorphism in the fractalkine receptor, CX3CR1 and rheumatoid arthritis: a genetic case control study

2.1 Background: Genetics of Rheumatoid Arthritis

2.1.1 The Genetic Basis of RA

The exact cause of RA remains unknown however it is believed to arise from a complex interplay of environmental and genetic factors. A genetic predisposition to developing RA is well established.[1] Familial studies have demonstrated that first degree relatives of patients with RA are approximately twice as likely to develop RA when compared to their healthy control counterparts.[2] Twin studies have also helped to determine the genetic component of RA. They have estimated the disease heritability for RA, which is defined as the discordance in monozygotic (identical) compared to dizygotic twins, to be approximately 65%. [3]

No single genetic mutation determines the genetic predisposition to RA. Rather, numerous risk alleles located across the entire genome all contribute to the overall risk of developing RA. In identifying at risk loci the SNP (single nucleotide polymorphisms) has become the standard genetic marker. [4]

2.1.2 SNPs and RA

SNPs are areas of genetic variability that occur frequently among the general population. They usually involve a single nucleotide substitution but occasionally can arise from single nucleotide insertions or deletions. Most SNPs are common SNPs in that the variant allele occurs in > 5% of the total

population. These SNPs do not give rise to disease themselves unless they alter a coding sequence or occur at an important regulatory sequence. More often, SNPs are associated with common mutations within the region that give rise to functional changes in nearby genes thereby leading to alterations of genetic phenotype which in turn cause disease. [4]

Whilst many SNPs remain undiscovered, advances in genotyping technology, together with the completion of the human genome project have led to the identification of 101 RA associated SNPs among European and Asian populations.[5–7]

2.1.3 The MHC and HLA-DRB1 Locus

The discovery of association between the (human leukocyte antigen) HLA-DRB1 locus and RA in the 1970s remains perhaps the most important discovery in our modern day understanding concerning the genetic basis of RA. Researchers examining mixed lymphocyte cultures noticed that lymphocytes derived from different individuals with RA tended to express similar HLA genes within the major histocompatibility complex (MHC) region, the protein responsible for antigen presentation.[8,9] Multiple risk alleles for RA were subsequently identified at the HLA-DRB1 locus.[10] Molecules encoded by these alleles share a conserved amino acid sequence arranged around the antigen binding groove, known as the 'shared epitope. The shared epitope has been to shown to be associated with anti-citrullinated peptide antibody (ACPA) positive and to a much lesser extent ACPA negative RA accounting for 18% and 2.4% of RA heritability respectively.[11] This association has given rise to the 'shared epitope

hypothesis' which describes the SE facilitating the binding of the MHC to citrullinated peptides, thus triggering a T cell response and breakdown in immune tolerance that ultimately manifests with RA.[10] Whilst much evidence supports the 'SE hypothesis' controversy remains due to a failure to unequivocally identify an 'arthritogenic peptide' and the more recent discovery that ACPA negative RA is also associated with the SE.[5]

2.1.4 Other Notable SNPs

Whilst numerous SNPs associated with RA have been discovered, the functionality of many of these SNPs remains unknown. A number of SNPs have however have been experimentally linked to function. These include: *PTPN22*, *PADI4*, and *CCR6*.

PTPN22

PTPN2, a non-synonymous SNP in the tyrosine-protein phosphate non-receptor type 22 (known as PTPN22 and encoded by *PTPN22*) remains the most studied SNP associated with RA. It has the strongest association with an odds ratio of 1.8 for ACPA +RA.[12] PTPN22 down-regulates T cell receptor (TCR) signaling by dephosphorylating Src family kinases such as Lck or Fyn. Evidence suggests that the risk allele for PTPN22 leads to a loss-of-function whereby the variant phosphatase is degraded more readily thereby leading to increased differentiation and activity of T cells, B cells and dendritic cells, all of which feature prominently in the pathogenesis of RA.[13]

PADI4

A risk allele located at protein-arginine deiminase type 4, (*PADI4*), was the first SNP associated with RA to be identified outside of the HLA.[14] Unlike many other SNPs, which are often associated with multiple autoimmune diseases, *PADI4* is specifically associated with RA. PADI4 mediates the citrullination of peptides.[14] The RA susceptibility haplotype has been shown to stabalise PADI4 mRNA transcripts, thus increasing the amount of circulating citrullinated peptides which act as autoantigens. These, in turn readily bind to the HLADRB1 shared epitope facilitating the initiation of the adaptive immune response that characterizes RA.[1516,17]

CCR6

A polymorphism for the chemokine receptor CCR6, which is expressed by T helper 17 (Th17) cells has also been associated with RA. The presence of this polymorphism correlates with increased expression of IL-17 among patients with RA emphasising the importance of the Th17 pathway in the pathogenesis of RA.[18]

2.1.5 Combining Environmental and Genetic Factors

Genetics alone do not fully explain the aetiology of RA. Environmental factors also make up a significant contribution.[19] Many of these factors remain to be discovered. Smoking and periodontal disease have both been established as independent risk factors for the development of RA and both are associated with increased expression of ACPAs.[20,21] *Porphyromana gingivalis*, a causative bacterium of periodontal disease, expresses PAD (PPAD), which is capable of citullinating human peptides.[21] Similarly

smokers demonstrate increased expression of PADI4.[22] Smoking and periodontal disease may therefore facilitate the production of ACPAs via human PADI4 and PPAD respectively. Chronic exposure to ACPAs may then in turn, lead to a breakdown in immune tolerance perpetuating an autoimmune inflammatory response that characterizes RA.[19]

2.2 Genetic association studies

Whether or not a SNP is associated with a particular disease can be determined through the use of genetic association studies. These studies test the hypothesis that the frequency of a variant allele (of a particular gene of interest) will differ significantly among 2 groups that have differing traits (cases and controls) i.e. that the differences in genotype frequencies observed will relate to the characteristic or outcome measured.

2.2.1 Alleles and Haplotypes

SNPs usually give rise to 2 alleles, which are alternative forms of the same gene. The frequencies at which they occur among a given population are termed the major and minor allele frequencies, with the major allele being the more frequently occurring allele. Up to 10 million SNPs are thought to occur within the human genome. This is thought to account for approximately 90% of the genetic variation evident among the population, with the remaining 10% due to rare genetic variant.[23–25]

Each SNP usually arises from a single incident mutation event. Such events are rare however, occurring at rates of 10^8 per site per generation. When a new allele does arise it becomes associated with the other alleles lying in

close proximity to it on the chromosome and they are passed on to the next generation as a set of alleles. This set of alleles is known as a haplotype.

2.2.2 Recombination and Linkage Disequilibrium

Rarely, alleles lying within the same haplotype can become separated via meiotic crossing –over in cells undergoing meiosis. This process is known as recombination. Recombination among alleles is more likely to occur the further apart they are within the haplotype block. This can be measured via linkage disequilibrium (LD), which measures the strength of an association between 2 SNPs within a haplotype bock. Alleles that are close together and therefore rarely undergo recombination are said to be in strong LD. The result is that much of the genetic variation seen in humans is attributed to SNPs that are in strong LD contained within haplotype blocks.

The principle of LD can be exploited to allow efficient and comprehensive searching of the human genome for areas of genetic variation that have important functional consequences. A comparatively small number of well chosen 'tag SNPs' allows one to predict the remainder of SNPs within a particular region. LD therefore allows us to predict a vast number of SNPs (estimate 10 million) by genotyping a much smaller number of tag SNPs (200,000 to 1,000,000).[23,26]

2.2.3 Utilising LD in the study of human genetics

This concept has been put to effective use by way of the International HapMap Project. This is a multi-national not-for-profit collaborative project that has utilised the concept of LD to create a 'haplotype map' of the human genome. The data derived from the project allows researchers to study

haplotype associations and identify markers of specific diseases which have genetic determinants, ultimately providing an invaluable resource which can be accessed free and openly.[23]

2.2.4 GWAS and Candidate SNP Approach

Two types of genetic association study employ different strategies in order to identify SNPs associated with disease: genome wide association studies (GWAS) and candidate gene association studies.

GWAS

GWAS use an unbiased approach to identify at risk loci.[4] Large case-control cohorts are combined with high-throughput genotyping technology to genotype between 100,000 to 1000,000 SNPs allowing the investigator to capture 65-70% of common variation across the human genome. In doing so numerous SNPs associated with a particular disease can be rapidly identified.[27,28] Due to the effects of multiple testing across multiple loci, very high levels of significance are required before a true association between a SNP and a particular disease can be confirmed by GWAS. Typically p values < 5 x 10^8 are needed in order to report a true association.[7] As a result, GWAS usually necessitate worldwide large-scale collaboration between investigators in order to achieve the large numbers of cases and controls that must be recruited.

Candidate SNP association studies

Candidate SNP case control studies are usually performed on a much smaller scale than GWAS. In contrast to GWAS which effectively "mine" the human genome for areas of common variation, candidate SNP studies take a more

targeted approach.[29] SNPs with an increased probability of association are identified based upon prior known function or genetic information. For example, several SNPs associated with type 1 diabetes have also been shown to be associated with RA: CTLA4, AFF3 and 4q27.[30–32] Similarly a SNP of the SH2B3 gene has been shown to be associated with both RA and coeliac disease.[33]

Various methods can be used to select genes for association studies that will increase the likelihood of a true association being discovered. For example, a known biological function of a protein encoded by a gene that has relevance to a particular disease clearly offers major advantages over another gene of which the biological function is unknown. This concept is known as biological plausibility and adds weight behind the power of a particular study when it exists. Similarly, studies demonstrating functional outcomes associated with gene dysfunction in animals can guide similar testing of genes among humans. Previous association studies showing association are also of relevance and provide strong indicators that a particular gene has an aetiological role. Genome wide association studies can also provide indicators of potential candidate genes by pointing towards areas of high LD associated with a high probability of containing a susceptibility gene.[34]

2.2.5 Factors to Consider in Genetic Association Study Design

Whilst genetic association studies are deceptively simple in their objective they are susceptible to numerous errors and inconsistencies in terms of their design and implementation that can ultimately lead to erroneous

interpretation. Inadequately powered studies, population stratification, genotyping errors and a poorly matched control group are all examples of study design flaws that should be avoided when implementing a genetic case control study.[34]

Sampling strategies-Appropriate selection of cases and controls

If the disease being studied is common, the task of collecting cases for the study is relatively easy. In contrast, the collection of the control group can be somewhat more difficult. Reasonable effort must be made to ensure the accuracy of case and control cohorts. Controls must be selected from the same population as cases and population stratification must be addressed.[35]

Population stratification

Population stratification is the presence within a population of ethnic sub groups among which allele frequencies and disease risks differ. In the context of genetic association studies, this can lead to observed differences in allele frequencies, which can be erroneously attributed to the presence of disease rather than the underlying population stratification that is present. Adequate description of ethnicity and population origin of cases and controls is therefore important. If stratification is thought to be present then the additional genotyping of unlinked markers can be used as way of reducing false positive results created by population stratification. If stratification is present, then the unlinked markers should also show association with the phenotype.[36,37]

Ireland differs from other countries in that up until recent times there has been very little population admixture and little ethnic diversity. Therefore

population stratification should be minimal in association studies originating from Ireland.

Study size and power

Adequate study size is of major importance when designing a genetic association study. Many earlier studies that reported positive associations used only modest sample sizes (typically 100-200 cases). These results of which were not reproducible in many of the subsequent studies. It is likely that many of these positive associations were due to random or spurious outcomes.[35]

The power of a study is determined by a number of other factors in addition to sample size. Measures of LD, major and minor allele frequencies and the incidence of the disease in question all impact on the power of a study. These factors have formed the basis of genetic power calculators that are available freely on the internet which are useful tools for estimating sample size.[38]

Genotyping error

Even highly regarded laboratories using the latest genotyping technologies report some level of genotyping error, which can often be up to 3%. As the rate of error will ultimately affect the power of the study, such error rates should always be included in the study. Other important quality control measures that should be included are: whether or not internal validation of the genotyping took place, the blinding of the laboratory personnel to clinical data, study hypothesis etc., methods for establishing duplicate samples and

whether or not the genotype frequencies of the control population conform to Hardy-Weinberg equilibrium.[36]

Multiple testing effect

When multiple testing of the same population takes place the chances of generating a type I error (the probability of rejecting the null hypothesis when it is true) increases, leading to the discovery of associations that are due to chance. All tests carried out should therefore be reported, even if non-significant.[35]

Publication bias

Selective reporting of positive studies is an ongoing problem in the field of genetics. Well designed negative studies should be given equal consideration for publication.[36]

2.3 Association between a polymorphism in the fractalkine receptor, CX3CR1 and rheumatoid arthritis

For this study we chose 2 candidate SNPs (rs3732378 and rs3732379) located within the coding sequence of *CX3CR1* to test for association with RA based upon previous known association with atherosclerosis. Both SNPs (rs3732378 and rs3732379) are missense, conservative SNPs, located within the sixth and seventh transmembrane domains of CX3CR1 respectively. rs3732378 results in an amino acid change from valine to isolueucine at codon 249 (G/A = V249I) and rs3732379 results in a change

from threonine to methionine at codon 280 (C/T = T280M). The SNPs are common within the Caucasian population and are located on chromosome 3, position 3p21. They are in almost complete linkage disequilibrium such that only 3 haplotypes commonly occur: V249T280, I249T280 and I249M280.[39]

The SNPs have been extensively studied in the field of atherosclerosis with 7 case control studies reported in the literature to date. A recent meta-analysis of these studies found the I249M280 haplotype to be significantly more common among healthy controls compared to individuals with atherosclerosis.[40] This has led some authors to speculate that the I249M280 haplotype may confer a protective effect against the development of atherosclerosis.[41] This hypothesis is supported by some in vitro evidence which demonstrates reduced FKN binding among peripheral blood mononuclear cells (PBMCs) derived from patients expressing the I249M280 haplotype.[41,42]

No association between rs3732378 and rs3732379 has previously been reported with RA. Given the similar pathogenic mechanisms that exist in both RA and atherosclerosis, particularly concerning CX3CR1 we hypothesised that a similar association might exist between RA and CX3CR1 polymorphisms.

2.3.1 Aim

The aim of this study was to test for genetic association between rs3732378 and rs3732739 and RA by means of a candidate SNP, case control approach.

2.3.2 Methods

The study was conducted at Cork University Hospital. Prior approval with Cork Research Ethics Committee was sought for all aspects of the study.

Selection of Cases and Controls

Cases *(n=384)* were recruited between July 2009 and May 2011 and were selected based upon an established diagnosis of RA. Recruitment took place at the rheumatology outpatient departments at Cork University Hospital and Kerry General Hospital. Patients over 18 years of age who met the 1987 ARA classification criteria for a diagnosis of RA were eligible for inclusion in the study.

Patients who did not fulfil the 1987 ARA classification criteria were excluded from the study. Other exclusion criteria included: the coexistence of another autoimmune diseases and an unwillingness or incapacity to provide written informed consent for the study.

In addition to 1987 ARA classification criteria, the following variables were also recorded: date of birth, sex, anti-CCP status, family history of RA, history of ischaemic heart disease

Controls *(n=483)* comprised of an anonymised, readily available collection of DNA that had been previously donated by patients with a diagnosis of primary osteoporosis. The patients consisted mainly of postmenopausal women in whom RA had previously been excluded.

Patients were asked to provide written detailed informed consent prior to inclusion in the study.

Sample collection and storage prior to DNA extraction

Each patient included in the study was asked to provide a sample of blood which was collected in a 3ml EDTA vial.

Samples were then coded, anonymised and stored in a secured alarmed freezer at -20 degrees Celsius prior to DNA extraction.

DNA extraction

DNA was purified from whole blood through the use of the 'MagNA Pure LC Total Nucleic Acid Isolation Kit' and MagNA Pure LC instrument and software.

This machine used magnetic-based technology to extract DNA. Samples were lysed by incubation with a lysis buffer containing a choatropic salt and Proteinase K. Magnetic Glass Particles (MGPs) were added and DNA binded to their surfaces. Unbound substances were removed by several washing steps. Purified DNA was then eluted with a low-salt buffer.

1. 250ul of whole blood was pipetted into the wells of the sample cartridge.

2. 300ul of lysis/binding buffer was added to each sample resulting in complete cell lysis and release of nucleic acids. Nucleases were denatured.

3. Proteinase K was added to the samples resulting in digestion of proteins.

4. DNA binded to the silica surfaces of the added MGPs due to the chaotropic salt conditions, isopropanol and the high ionic strength of the lysis/binding buffer.

5. MGPs with bound DNA were magnetically separated from the residual lysed sample.

6. MGPs with bound DNA were washed repeatedly with wash buffer to remove unbound substances (e.g. proteins (nucleases) cell membranes, and PCR inhibitors such as heparins or haemoglobin) and to reduce chaotropic salt concentration.

7. MGPs with bound DNA were magnetically separated from the wash buffer containing residual sample debris.

8. The purified DNA was eluted from the MGPs in the wells of the elution cartridge. MGPs were retained in the reaction tip and discarded.

Genotyping and Statistics

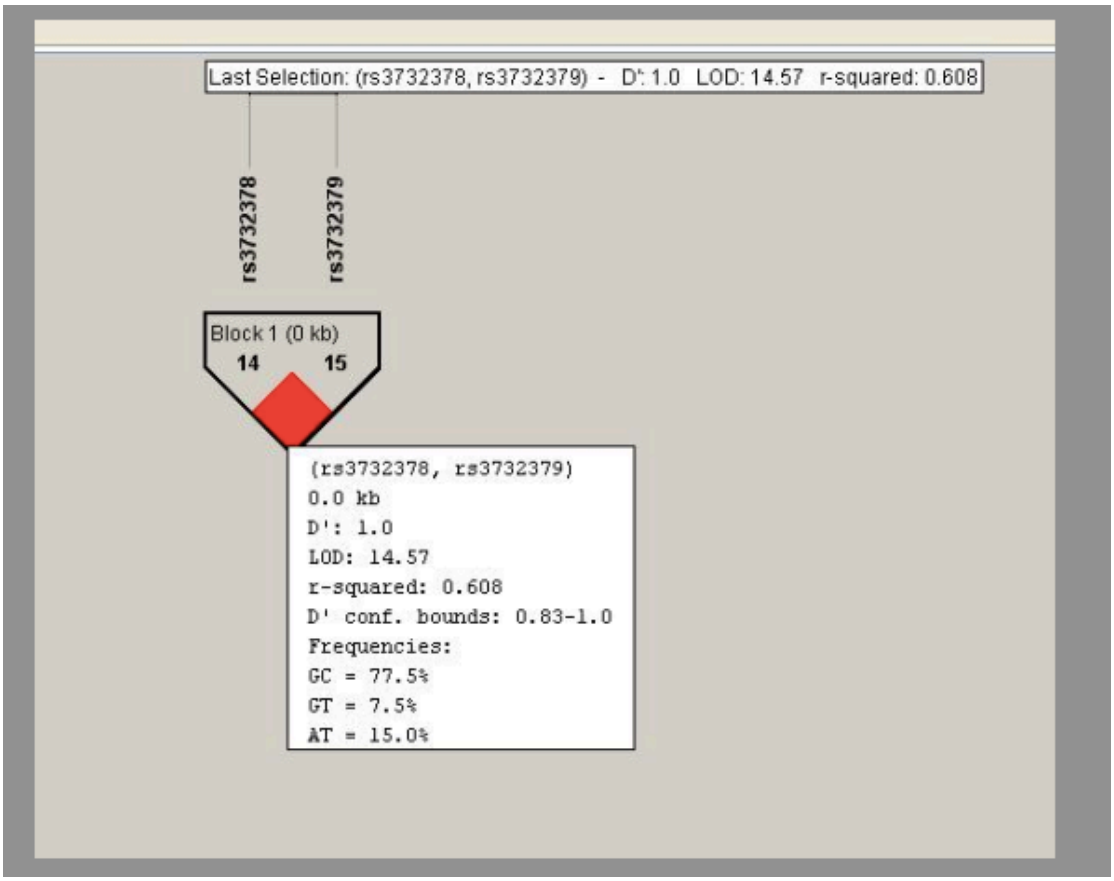
All genotyping was performed blind to case control status by KBioscience. SPSS and PLINK were used for statistical analysis. Genotype and allelic frequencies were calculated using chi-square –analysis- of -contingency tables. Odds ratios were calculated with 95% confidence intervals. Sample

size was estimated using Quanto version 2.1 under the log additive model with 80% power and a significance level of 5%. The minor allele frequency was set at 0.3 (based on previous frequencies reported in a large meta-analysis). The population frequency of RA was set at 1%. Results were generated with 1000 permutations to control for multiple testing.

2.3.3 Results

384 RA cases were recruited to the study. All of which were Caucasian and of Irish ancestry. Out of the total number of cases, 251 were female and 178 cases were ACPA positive. All of the controls *(n= 483)* were female, Caucasian and of Irish ancestry. The genotype distributions did not deviate from HWE ($P > 0.05$). *CX3CR1* SNPs were in strong but incomplete LD ($D' = 1.0$, $r^2 = 0.53$). (See Figure 2.1).

Figure 2.1



Significant association was observed between rs3732378 and RA risk under a dominant model for the minor allele following 1000 permutations (II+VI vs. VV; OR: 1.18 (95% CI, 1.18 – 1.36); *P* = 0.017). There was a higher frequency of AA or AG carriers in the controls (53.6%) versus the cases (45.4%). There was a higher frequency of rs3732378 A alleles in the controls (31.0%) versus cases (26.6%), however, this was not significant (OR: 0.81, (95% CI, 0.65-1.00); *P* = 0.055).

No association was observed between rs3732379 and RA risk (*P* > 0.05). There was no association observed between *CX3CR1* haplotypes and RA risk (*P* > 0.05). (See Table 2.1)

Table 2.1

SNP	Genotype	Cases (n=384)	Controls (n=483)	OR	95%CI	P
rs3732378	**V249I**					
	II	31	40	1.18^	(1.18-1.36)	0.017
	VI	142	205			
	VV	204	207			
rs3732379	**T280M**					
	MM	11	14	1.17*	(0.87-1.57)	0.281
	TM	107	141			
	TT	261	292			

^ Adjusted OR associated with the II+VI versus the VV genotype

* Adjusted OR associated with the MM+TM versus TT genotype

2.3.4 Discussion

In this study, individuals with one or more copies of the rs3732378 variant allele had a significantly reduced risk of RA compared to individuals that were homozygous for the wild-type allele. Similar findings have previously been reported in case control studies relating to patients with HIV and also atherosclerosis.[39–42]

CX3CR1 polymorphisms were first identified by a team of French investigators studying the functional outcomes of *CX3CR1* SNPs on CX3CR1 expression in patients with HIV.[39] (In HIV, CX3CR1 functions as a co-receptor for the virus). They found that individuals expressing the I249M280 haplotype progressed more rapidly to AIDS than individuals expressing the V249T280 or I249T280 haplotypes. The authors went on to demonstrate that FKN binding was reduced among PBMCs derived from HIV infected patients expressing the I249M280 haplotype and that total number of FKN binding sites was also reduced. These findings led the authors to speculate that reduced CX3CR1 expression and FKN binding could 'compromise normal immune function' and 'lead to accelerated disease progression'.

Subsequently the same authors went on to demonstrate a reduced risk of coronary artery disease among individuals heterozygous for the I249 allele.[41] Whilst the numbers included in this study were small (151 cases and 249 controls) the authors were able to support their findings again with

functional studies demonstrating a 40% reduction in FKN binding among PBMCs derived from patients expressing the I249 allele.

In another study McDermott et al demonstrated a similar finding, reporting a reduced risk of atherosclerosis among patients heterozygous for the I249M280 haplotype under a dominant genetic model (OR 0.6; p=0.008).[42] Again, reduced FKN binding was demonstrated in vitro among PBMCs derived form patients expressing the I249M280 haplotype.

Whilst subsequent case control studies have been inconsistent, a meta-analysis which included 2000 coronary artery disease patients and 2841 controls also found the I249M280 haplotype to be significantly more common among controls compared to subjects with coronary artery disease.[42]

These findings have led some authors to speculate that the I249M280 haplotype could confer a protective effect against the development of atherosclerosis. This hypothesis is supported by studies measuring atherosclerotic plaque formation in, CX3CR1-/- knock out mice. Liu et al demonstrated that guide-wire induced endothelial injury of mouse femoral arteries upregulated endothelial FKN expression. CX3CR1-/- mice failed to recruit monocytes to the intima despite expression of FKN. In contrast wild type mice expressing CX3CR1 demonstrated robust monocyte recruitment to the intima.[43] Similarly both Lesnick and Combadiere found a significant reduction in atherosclerotic plaque formation in CX3CR1-/- ApoE-/- double

knockout mice compared to mice with either or both copies of the CX3CR1 gene. A significant reduction of macrophage recruitment to the vessel wall was also demonstrated in mice deficient in CX3CR1.[44,45]

These studies therefore support the hypothesis that reduced or altered CX3CR1 expression could be protective against the development of atherosclerosis via reduced FKN binding and monocyte recruitment to the vessel wall.

As with atherosclerosis, FKN/CX3CR1 is also highly expressed in RA (see Chapter 1). It is interesting therefore, that we found a similar association between RA and rs3732378 in our study. It is tempting to speculate that attenuated CX3CR1 expression resulting from expression of the I249 allele, could in theory lead to reduced monocyte recruitment to the synovium and be protective against the development of RA. However such an effect is likely to be very modest and rather than being a risk loci for the development of RA, the polymorphism is more likely to be indicative of strong associations between FKN- induced pathways and RA. Further studies examining the effects of FKN binding among PBMCs derived from RA patients expressing the I249 allele could investigate this effect further.

This study has a number of limitations. The control cohort was provided from a biobank of DNA donated by post-menopausal women between 1996 and 2005. Although RA was excluded from these patients at the time of DNA extraction, it is impossible to know whether or not any of these patients

subsequently went on to develop RA (as the samples were anonymised). The biobank provided us with a convenient, readily available sample of DNA, however a control cohort collected prospectively would have improved the strength of this study. A prospective cohort could also have included male controls which was lacking from this study.

This study represents a case control study performed on a much smaller scale compared to that of the larger (Genome wide association studies) GWAS case control studies that are more commonly reported in the current literature. Whilst this study may lack the statistical power derived from such larger studies, it is supported by functional studies previously reported elsewhere demonstrating the effects of altered CX3CR1 expression on monocyte recruitment to sites of inflammation. These studies lend biologic plausibility to the association reported in our study.

Our study included a mix of ACPA positive and ACPA negative RA patients. Some experts now argue that these 2 subgroups represent genetically different phenotypes and should be grouped together separately to ensure homogeneity of the case population being studied.[2] This suggestion is largely based on what was once considered an almost exclusive association between the HLA-DB1 shared epitope and ACPA-positive RA. We now know however that ACPA- negative RA is also associated with the shared epitope.[5,46] This finding therefore casts doubt on the notion that ACPA-positive and ACPA-negative RA are 2 genetically different diseases.

In summary we found an association between the SNP rs3732378 and subjects with RA with the variant allele being more common among controls than subjects with RA. There is some evidence to suggest that this SNP may confer a protective effect by means of altered FKN binding and reduced monocyte recruitment. FKN binding with CX3CR1 in rs3732378 allele specific RA patients will need to be investigated following an independent replication of these association results.

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Chapter 3: Co-localisation of CX3CR1 and CD16+ and CD14+ mononuclear cells in rheumatoid synovium

3.1 Introduction

As discussed in Chapter 1, the FKN/CX3CR1 axis serves as a powerful chemoattractant. For this chapter I decided to explore the effects of FKN/ CX3CR1 on monocytes in human RA synovium further by means of immunohistochemistry. Human blood monocytes are a heterogenous population. They can be divided into 3 different subsets: classical (CD14++ CD16-), intermediate (CD14++CD16+) and nonclassical (CD14+CD16++).[1] Classical monocytes make up 90% of the population and are the main producers of the anti-inflammatory cytokine IL-10.[2]

In contrast, non-classical monocytes expressing high levels of CD16 are pro-inflammatory and can produce high levels of TNF, IL1B and Il-6. They also have increased antigen presentation capacity expressing increased levels of MHC class 2 antigens, ICAM-1 and VLA 4.[1,3,4] IL-10 expression is low or absent from these monocytes.

CD16+ monocytes are expanded in patients with active RA.[5]

Increased levels have been detected in the peripheral blood and synovial fluid in patients with RA. They have also been detected in the lining layer of RA synovial tissue.[6]

Given that FKN/CX3CR1 serves as a chemoattractant and adhesion molecule for monocytes, it would be of interest to study monocyte expression of CX3CR1 in RA synovium.

3.2 Aim

To examine the co-localisation of CX3CR1 with CD 16+ and CD14+ mononuclear cells in rheumatoid synovium using immunohistochemistry techniques.

3.3 Methods

3.3.1 Synovial tissue sample collection

Paraffin embedded samples of RA synovial tissue (ST) were obtained from the pathology department at Cork University Hospital. These samples were then coded, anonymised and blinded to disease status.

Control ST was obtained from patients undergoing knee replacement surgery for OA at St Mary's Orthopaedic Hospital, Cork. All patients provided written informed consent.

3.3.2 Fixing samples H and E

3.3.3 Depariffinization

The slides were placed in a rack and deparaffinized and rehydrated using the following washes:

1. Histoclear (Sigma): 5 mins x 2
2. 95% ethanol: 5 mins
3. 70% ethanol: 5 mins
4. Double distilled water (ddH2O): 10 mins

3.3.4 Antigen Retrieval

Heat mediated antigen retrieval was performed by placing the rack of slides in a steamer with 1mM EDTA (pH 8.0) buffer for 15 minutes followed by dipping twice in phosphate buffered saline.

3.3.5 Immunohistochemical staining

Non-specific binding was inhibited by the addition of 10% normal goat serum (NGS) in 0.2% Triton X in PBS for 25 mins at room temperature.

Primary anitbodies were then added using the following dilution (in 10% NGS, 0.02% Triton X):

Antibody	Manufacturer	Host	Dilution
CD14	Novocrasta	Mouse IgG2a	1/100
CD16	Novocrasta	Mouse IgG2a	1/100
CX3CR1	Torrey Pines Biolab	Rabbit	1/75

All samples were stained with CX3CR1, then co-stained with either CD14 or CD16 using the same dilutions.

After co-staining, samples were incubated at room temperature for 65 minutes, then washed 3 times, for 5 minutes each, with PBS under gentle agitation.

For fluorescent detection, fluorochrome conjugated secondary antibodies were added using the following dilutions (in 10% NGS in 0.02% Triton X):

Primary Stain	Antibody	Fluorochrome	Dilution
CD14	Goat anti-mouse (Invitrogen)	Alexa Fluor 546	1/500
CD16	Goat anti-mouse (Invitrogen)	Alexa Fluor 546	1/500
CX3CR1	Goat anti-rabbit (Invitrogen)	Alexa Fluor 488	1/500

Again all samples were stained with the secondary antibody for CX3CR1, then co-stained with the appropriate secondary antibody (either CD14 or CD16).

For identification of nuclei, DAPI (Invitrogen) staining diluted in 1/2000 PBS was employed. Samples were developed for 10 minutes prior to one final was with PBS.

3.3.6 Isotype controls

In order to estimate the contribution of non-specific antibody binding, isotype controls were employed using serum from an unimmunized animal

of the same species as the primary antibody (i.e. rabbit or mouse as appropriate).

3.3.7 Slide Analysis

Slides were analysed using a NIKON confocal microscope and EZ-CI software. Slides were analysed in a dark room in order to minimise the effects of photo bleaching.

For each section, 3 high power fields (hpfs) of both the synovial lining and sub-lining were acquired. The number of cells co-expressing CX3CR1 and CD14 or CX3CR1 and CD16 were calculated and then recorded as percentages of the total cell population.

3.4 Results

ST was obtained from 9 individuals with RA and from 3 individuals with OA. The percentage of cells co-expressing: CX3CR1 and CD14 and also, CX3CR1 and CD16 was calculated for the lining and sublining regions using IHC and confocal microscopy.

Substantially more CD14+ cells were present in RA synovium compared to OA synovium. The majority of these cells were found in the lining region with some scattered cells distributed among the sublining area. The vast majority of CD14+ cells co-expressed CX3CR1, particularly in the lining region compared to the sublining region (95.23% +/- 1.2) vs. (82% +- 3.8), p=0.01. Fewer CD14+ cells were present in OA synovium. Again, the majority these cells co-expressed CX3CR1. There was no significant difference in CX3CR1 co-expression between cells in the lining and sublining regions [(94.8% +/- 4) and (90.5% +/- 6.1) respectively] p=0.6.

Representative images are outlined in Figure 3.1.

CD16+ cells were less populous than CD14+ cells. 90% of RA sections analysed detected cells expressing CD16 compared to 33% of OA sections. Again the majority of CD16+ cells detected in RA synovium co-expressed CX3CR1 in both the lining and sublining of RA synovium regions (97.7% +/- 1.3) and (96.4% +/- 2) respectively. All of the detectable CD16+ cells in OA synovium co-expressed CX3CR1.

Representative images are outlined in Figure 3.2.

RA OA Isotype

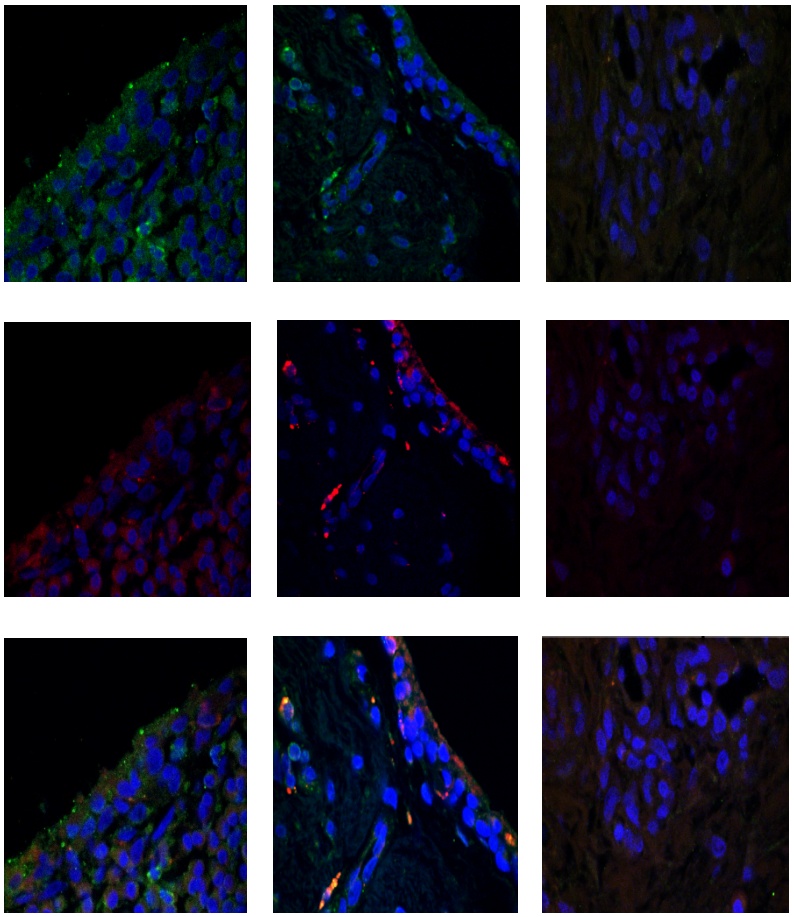

Figure 3.1 Representative Images of CD14 and CX3CR1 Expression in RA and OA ST

Paraffin-embedded synovial tissue samples from individuals with RA (Column on the left) and OA (Middle) were stained with specific antibodies for CD14 and CX3CR1 or the appropriate isotype control (Column to the right). Representative sections of samples stained for CX3CR1 are outlined in the 1st Row, CD14 in the 2nd row and the merged images in the 3rd row.

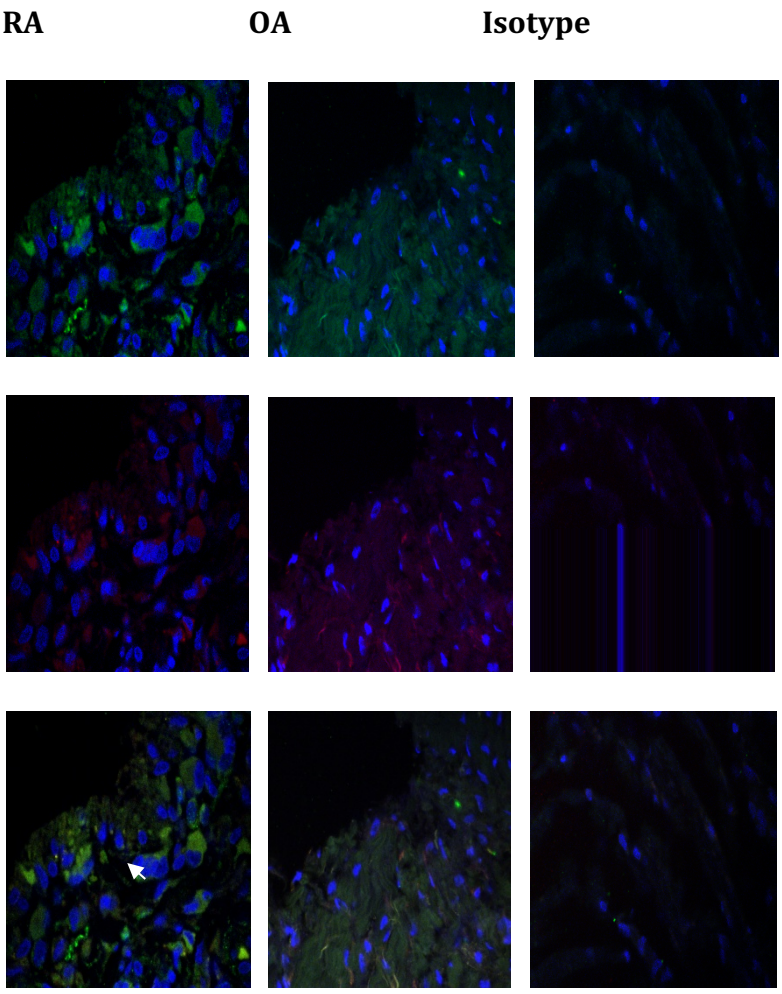

Figure 3.2 Representative Images of CD16 and CX3CR1 Expression in RA and OA ST

Paraffin-embedded synovial tissue samples from individuals with RA (Column to the left) and OA (middle column) were stained with specific antibodies for CX3CR1, CD16 or the appropriate isotype control (Column to the right). Representative images of sections stained for CX3CR1 are outlined in the top row, CD16 in the middle row and the merged images in the bottom row. Minimal CD16 immunoreactivity was identified in samples from OA ST while in individuals with RA CD16 appeared to co-localize with CX3CR1 (arrowhead).

3.5 Discussion

In this study we were able to demonstrate that CD14 monocytes expressing CX3CR1 were present in abundance within the lining and sublining of rheumatoid synovium. CD16+ mononuclear cells were also present albeit in fewer numbers and were also found to express CX3CR1.

In contrast, fewer monocytes were present within OA synovium. Most of these mononuclear cells were CD14 + monocytes, again expressing CX3CR1. Very few monocytes in OA synovium stained positive for CD16.

Yano et al previously demonstrated that CD16+ mononuclear cells localise to the lining and sublining layer of rheumatoid synovium and co-express CX3CR1.[6] This was a small study however, including ST from only 3 RA patients and did not include a comparison cohort of OA samples. Furthermore the study did not examine for the presence of CD14+ cells and CX3CR1 expression.

Our study corroborates Yano et als findings in a larger cohort of patient samples and also compares the differential distribution of CD14+ and CD16+ mononuclear cells between rheumatoid and OA synovium.

FKN is highly expressed in RA in both membrane and soluble form.[7] The concentration of FKN in RA synovial fluid is greater than that of serum FKN, therefore providing a chemokine gradient down which CX3CR1 expressing monocytes may migrate.[8] Given the high expression of CX3CR1 on circulating monocytes and also in synovial tissue, it is highly likely that the FKN/CX3CR1 axis functions as a key mechanism by which monocytes may transmigrate from serum to ST.[6,8]

In addition to facilitating the trafficking of monocytes, FKN has also been shown to induce the secretion of IL-1B and IL-6 from isolated whole blood monocytes in vitro.[6] This provides further evidence of the inflammatory potential of monocytes under the influence of FKN.

Whilst this study helps to clarify the phenotype of mononuclear cells present in RA synovium, it was only able to detect the presence or absence of CD14 or CD16. Due to limitations of IHC we were not able to identify in ST the presence of specifically, mononuclear cells belonging to the 'intermediate' subset. (CD14+CD16+).[9] Less is known about these monocytes. They have been shown to express surface receptors at intermediate levels compared to classical and inflammatory populations. This has led to the suggestion that these are monocytes going through a transitioning stage. Interestingly they have been shown to be associated with negative outcomes in chronic kidney disease and HIV. [9–11] Their exact role in RA remains to be fully elucidated.

Despite the limitations of IHC it is probable that the increased CD16 staining in RA ST represents monocytes of the CD14+ CD16++ subset. These monocytes have previously been demonstrated to be increased in the peripheral blood and synovial compartment of patients with RA.[5]

Kawanaka et al, using flow cytometric analysis demonstrated that CD16 expression was significantly increased in RA patients (11.7%, n=105) versus healthy controls (9.5%). Furthermore RA disease activity and the expression of IL-10, M-CSF and TGF B1 were all increased in proportion to increased CD16 expression. The authors also showed a reduction in CD16 expression in RA patients who responded clinically to a 3 month trial of 10mg weekly methotrexate in combination with low dose prednisolone.

FKN has also been demonstrated to preferentially mediate the arrest and migration of these CD16+ 'inflammatory' monocytes *in vitro,* highlighting importance of the FKN/CX3CR1 pathway in inflammatory conditions such as RA.[12]

Interestingly CD14+ monocytes present in OA synovium also expressed CX3CR1. It would therefore appear that FKN/ CX3CR1 functions as a chemoattractant for all subclasses of monocytes. The differential expression of CD16 versus CD14 monocytes may lead to inflammatory or anti-inflammatory outcomes respectively.

In summary, we demonstrated that CD14+ and CD16+ mononuclear cells co-localise with CX3CR1 in the lining and sublining of RA synovium, helping to further define the expression of FKN/CX3CR1 in RA.

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Chapter 4: Soluble glycoprotein VI, a specific marker of platelet activation is increased in the plasma of subjects with seropositive rheumatoid arthritis.

4.1 Introduction

The final part of this thesis shifts the focus from FKN/CX3CR1 to that of platelet activation and RA. As previously described, arterial thrombosis is a major cause of mortality in rheumatoid arthritis (RA).[1] Patients with RA have an increased risk of cardiovascular disease (CVD) similar to that of diabetes mellitus and have twice the risk of sudden cardiac death when compared to the general population.[2,3] CVD accounts for >50% of deaths among patients with RA.[2] The risk is especially increased for patients who are positive for anti-citrullinated protein antibodies (ACPA) or rheumatoid factor (RF).[4–7] Whilst the reasons for this increased risk remain incompletely understood, recent studies point towards platelet activation and early vascular inflammation as possible mechanisms.

Anti-citrullinated protein antibodies (ACPA) have been shown to cause platelet activation *in vitro*, through the low-affinity immunoglobulin G (IgG) receptor (FcγRIIa) on platelets. Platelet activation via engagement of FcγRIIa results in proteolytic cleavage and shedding of platelet specific glycoprotein VI (GPVI) which can be detected in the plasma as soluble GPVI (sGPVI). We hypothesized that plasma levels of sGPVI would be increased among patients

with seropositive RA as a consequence of antibody-induced platelet activation and GPVI shedding.

4.2 Methods

Subjects

84 patients with RA (65 seropositive and 19 seronegative) were recruited prospectively at the Mater Misericordiae University Hospital (MMUH) Dublin from 2012 until 2016. They satisfied both the 1987 American College of Rheumatology (ACR) and the 2010 ACR/European League Against Rheumatism (EULAR) criteria. Controls (n=67) comprised of 17 patients with osteoarthritis also attending the MMUH and 50 healthy volunteers working at the Royal College of Surgeons of Ireland (RCSI). All subjects provided written informed consent for blood sample donation and the study was approved by the research ethics committee of the MMUH and RCSI. The study was conducted according to the principles expressed in the Declaration of Helsinki. Demographic and clinical data were collected for all participants. Characteristics of seropositive vs seronegative RA are presented in Table 1.

Blood Sampling and Plasma Preparation

Blood was collected into vacutainers containing 0.106 nM sodium citrate as anticoagulant (10% vol/vol). Platelet poor plasma was prepared by centrifugation of whole blood at 2000g for 10 minutes. Plasma was aliquoted and stored -80°C until analysis.

Measurement of Soluble GPVI

sGPVI levels were measured by immunoassay. 96 well standard binding plates from MesoScale Discovery (MSD, Rockville, MD) were coated overnight at 4°C with 4 □g/mL sheep anti-human GPVI polyclonal antibody (R&D Systems, Abingdon, UK). The plate was blocked with 5% MSD Blocker A for 1 hour at RT, washed x3 with 150 □L PBS / 0.05% Tween (PBST) and 25 □L of undiluted platelet poor plasma added to duplicate wells. Samples were incubated at RT with vigorous shaking for 1 hour. The plate was washed x3 with PBST. Biotinylated sheep anti-human GPVI antibody was diluted to 1 □g/mL in 1 % MSD Blocker A and 25 □L added to each well. The plate was incubated at RT for 1 hour with shaking at 650 rpm then washed x3 with PBST. 150 □L 2x read buffer was added to each well and the plate read on a MesoScale Quickplex SQ120 Plate Scanner according to the manufacturer's instructions.

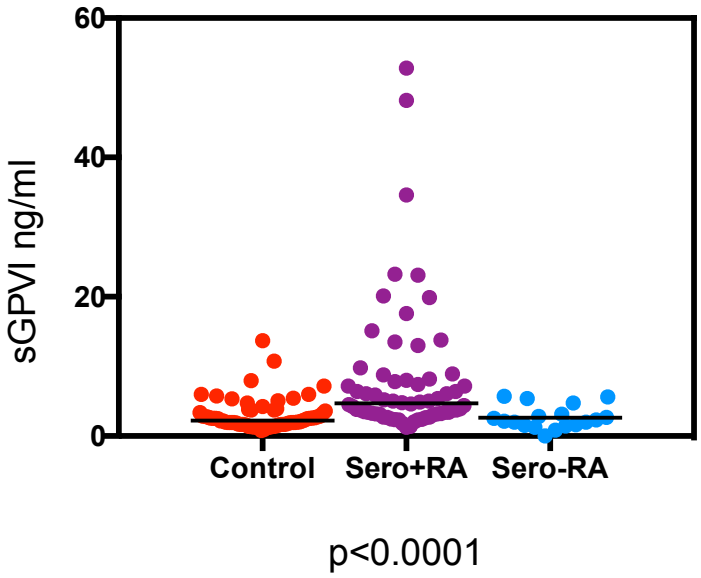
Statistical Analysis

The Kolmogorov-Smirnov test was used to determine whether data sets were parametric or non-parametric. Results were expressed as mean +/- standard deviation (SD) or median +/- interquartile range (IQ) depending on whether they were derived from parametric or non-parametric data respectively. Mann-Whitney U test and Kruskal–Wallis test was used to compare groups. Spearman's Rank Correlation Coefficient was used to assess for associations between sGPVI levels and demographic and clinical markers. GraphPad Prism Version 6.05 was used for data analysis.

4.3 Results

Patients with seropositive RA were significantly older, but no signficant correlation was observed between levels of sGPVI and age, CRP, fibrinogen, ESR, platetet count or DAS28-CRP (Table 1). Patients with seropositive RA had significantly higher levels of sGPVI compared to seronegative RA and controls (Fig 1). Median (IQR) sGPVI levels were 4.2 ng/ml (3.2, 8.0) in seropositve RA, 2.2 ng/ml (1.5, 3.5) in seronegative RA and 2.2 ng/ml (1.6, 3.4) in controls ($p<0.0001$). sGPVI levels correlated with ACPA titres ($r=0.32$, $p=0.0026$) and also with RF titres ($r=0.48$, $p<0.0001$).

Figure 1



p<0.0001

Table 4.1 Characteristics of Patients with Seropositive RA vs. Seronegative RA

	Sero + RA	Sero - RA	*P* value
Total Number	65	19	
Female, n (%)	51 (78)	16 (84)	ns
Male, n (%)	14 (21)	3 (15)	ns
Age, yr (median [IQR])	62 [54-71]	48[38-64]	0.01
CRP, mg/l (median [IQR])	8 [4-15]	6 [2-21]	ns
ESR, mm/hr (median [IQR])	21 [11-32]	13 [7-38]	ns
Fibrinogen g/l (mean +/- SEM)	3.33 +/- 1.12	4.06+/-1.92	ns
Platelet Count x 10^9(mean +/- SEM)	281+/- 98	285 +/- 81	ns
DAS28-CRP (Mean +/- SEM)	3.83 +/- 1.49	4.37 +/- 2.2	ns
RF titre, IU/mL (median [IQR])	81 [20-271]	2.3 [1.5-4.7]	<0.0001
CCP titre, u/mL (median [IQR])	212 [100-340]	2.1 [1.1-3.6]	<0.0001

RA rheumatoid arthritis*, CRP* c- reactive protein*, ESR* erythrocyte sedimentation rate*, DAS28* Disease Activity Score in 28 joints*, RF rheumatoid factor, CCP citrullinated c-protein, IQR* interquartile range*, SEM* standard error of the mean, ns not significant.

4.4 Discussion

This is the first *in vivo* study to identify an association between sGPVI, a marker of platelet activation and seropositive RA in humans. RA is largely viewed as a disease driven by T and B cells. However there is a growing appreciation for the role of platelets in RA pathogenesis. Platelets when activated localise to inflammed joints where they transmigrate into the inflammed synovium promoting the release of cytokines such as IL-6 and TNFa, well known mediators of RA pathogenesis. GPVI had previously been shown to be a key receptor for platelet activation in a mouse model of RA however this is the first study to report an in-vivo association between GPVI and seropositive RA in humans.

ACPA have been shown to cause platelet activation in-vitro via engagement with FcγRIIa. Given that ITAM-based signalling via engagement of either GPVI or FcγRIIa results in shedding of GPVI it is possible that the association we observed between sGPVI and seropositive RA is antibody mediated.[11]

In this study both ACPA and RF correlated with sGPVI. The vast majority of seropositive RA patients were positive for both ACPA and RF antibodies therefore we were unable to distinguish whether the association bewteen sGPVI and seropositive RA was due to the presence of ACPA, RF or both. Although platelets lack the Fc-IgM receptor, RF factor can exist in both IgM and IgG form so conceivably RF-IgG could potentially engage with platelets. Interestingly only one patient in the seropositive RA group had undetectable

ACPA but was strongly RF positive. This patient expressed high levels of sGPVI suggesting that the presence of RF alone could lead to shedding of GPVI. However we cannot draw any firm conclusions from this single observation.

Our study has a number of caveats. We did not directly show that elevated sGPVI in our patients was due to Fc□RIIa-mediated pathways and our approach to investigating platelet activation was single-faceted. We did not examine for levels of other known platelet agonists such as TNFα which has also been shown to be involved in platelet activation,[18,19] nor did we compare sGPVI with other measures of platelet activation (eg plasma soluble P-selectin) that could have further defined the degree of platelet activity in patients recruited to the study.[20] However, previous studies have illustrated significant correlation between plasma sGPVI and soluble P-selectin, with sGPVI having advantages over P-selectin in terms of selectivity, specificity and age-dependence.[10,21]

It is interesting to note that a similar association has been observed between sGPVI and acute ischaemic stroke and that studies indicate that blockade of GPVI function may help to reduce the risk of thrombosis without increasing the risk of bleeding.[9] For example, targeting of GPVI with non-activating anti-GPVI monoclonal antibodies is protective against ischaemic stroke and myocardial reperfusion injury in mice without increasing bleeding risk.[22,23] In humans, an early Phase I study of a dimeric GPVI recombinant fusion protein, GPVI-Fc (Revacept; Janssen-Cilag GmbH Neuss, Germany) has been shown to reduce collagen-induced platelet activation ex-vivo.[24] Blocking ITAM

signalling by targeting the tyrosine kinases and adaptor proteins downstream of FcγRIIa and GPVI may also be a potential approach for treatment of RA. Inhibitors such as fostamatinib (against Syk), ibrutinib (Btk inhibitor) and afuresertib (PI-3 kinase inhibitor) all target members of the ITAM signaling pathway and may have utility in RA therapy.[25–27] Some of these reagents are already in trial for the treatment of chronic lymphocytic leukemia, RA, and asthma.[28–30] Further, the Syk inhibitor PRT318 reduces HIT and immune complex–mediated thrombosis in mice genetically engineered to express platelet Fc□RIIa.[31] It is currently unknown what effect using such targeted therapies would have in RA. However given the attenuated effects of inflammatory arthritis that are seen using GPVI blockade in mice, targeting of GPVI in RA in humans may be beneficial.

A role for platelets during the earliest phases of RA pathogenesis has been hypothesized previously.[12,13] Under favourable conditions, enhanced vascular permeabilty could facilitate the transmigration of platelets and auto-antibodies into inflammed joints where platelets subsequently become activated, amplifying further vascular and synovial inflammation. Supporting this hypothesis is the fact that periodontal disease has also been associated with platelet activation.[32,33] The pathogen of this disease, *Porphyromonas gingivalis* can migrate haematogenously from the oral cavity to atherosclerotic plaques where it is capable of citrullinating proteins and generating ACPA and is considered one the predisposing factors for developing RA.[34] The relationship between periodontal disease, ACPA generation and platelet

activation therefore warrants further investigation and may yield important insights into RA pathogenesis.

4.5 Conclusion

Plasma sGPVI, a specific marker of platelet activation is increased among patients with seropositive RA, providing *in vivo* evidence that the GPVI pathway is involved in antibody-mediated platelet activation in humans. Accumulating data suggests an important role for platelet activation in contributing to aggravated CVD risk and also a role in early RA pathogenesis. Studies targeting GPVI in CVD have shown promising results to date. Further investigation targeting GPVI in RA is warranted and may provide important insights into RA pathogenesis and facilitate attempts to reduce CVD mortality among patients with RA.

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Conclusion

This body of work has drawn on parallels between the pathogenesis of RA and atherosclerosis. In doing so I have helped to identify some potential links between RA and atherosclerosis, two diseases which are closely associated. I have shown that a polymorphism of the CX3CR1 gene, which has been shown to have a protective association against atherosclerosis, has a similar protective association against RA. I have also illustrated by means of immunohistochemistry that monocytes co-localise with CX3CR1 in human RA synovium, providing further evidence of the chemotactic effects between monocytes and the FKN/CX3CR1 axis. Finally I have demonstrated an association between seropositive RA and sGPVI, a specific marker of platelet activation, providing further evidence that platelet activation is associated with seropositive RA and is likely antibody mediated.