Determinants of severity and co-morbidity in rheumatoid arthritis: influence of genetic variation and smoking

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Cc: Director of Postgraduate Research

This thesis is dedicated to my dear mother, Ms. Zhimin Xiang, without whom this achievement would not have been possible.

此论文谨献给我亲爱的母亲，项智敏女士。她使这样的成就成为了可能。

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by chronic synovial inflammation, ultimately leading to joint destruction and permanent disability. Disease expression may be affected by genetic variations and environmental factors such as cigarette smoking. The main objective of this study was to identify some of the main determinants of poor outcome in RA, both in terms of disease severity and co-morbidity. Analysis of possible interaction of genetic factors with smoking was carried out. Candidate genes included GSTM1, GSTT1, VEGFA, eNOS, MMP1, MMP2, MMP3, TGFB1 and PTPN22.

Smoking was associated with seropositive RA, in particular with RF+ RA. It was associated with the development of erosive disease and with more severe functional outcome in seronegative patients.

Promoter polymorphism VEGFA-2578(A/C) (rs699947) was associated with serum VEGF-A level, which may reflect a genotype-specific response to inflammation. This polymorphism was associated with disease activity, which only occurred in non-smokers. The same polymorphism was also associated with the occurrence of IHD and MI, which may be due to an interaction with smoking.

Both MMP1 (rs1799750) and MMP3 (rs679629, rs3025058) polymorphisms were independently associated with serum MMP-1 level, whereas serum MMP-3 was mainly associated with MMP3 polymorphisms. MMP3 SNPs were associated with disease activity, independent of systemic inflammation and serum MMP-3. A haplotype across

the MMP1-3 loci was associated with the development of erosive disease in earlier RA. Evidence of interaction with smoking was also found regarding the above association.

No association of polymorphisms in TGFB1 with serum TGF-ϐ1 level was found. A missense polymorphism TGFB1+868(C/T) (rs1800470) was associated with the occurrence of IHD and MI, which may be explained by an interaction with smoking.

The current thesis highlighted the complexity of factors associated with severity and co-morbidity in RA, and showed the importance of smoking in exacerbating various aspects of disease outcome.

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LIST OF ABBREVIATIONS

AA..... Autoimmune arthritis
ACPA ..... Anti-citrullinated protein/peptide antibodies
ACR ..... The American College of Rheumatology
ADAM ..... Protein containing a disintegrin and a metalloprotease domain
ADAMTS..... Secreted ADAM with thrombospondin domain
Anti-CCP..... Anti-cyclic citrullinated protein antibodies
Anti-CCP2..... Anti-cyclic citrullinated peptides second generation assay
AP ..... Attributable proportion
APC..... Antigen-presenting cells
AR..... Absolute risk
ARN ..... Accelerated rheumatoid nodulosis
BMI..... Body mass index
BSA..... Bovine serum albumin
C5..... Component 5
CBL..... Casitas B-lineage lymphoma
CD..... Cluster of differentiation
CDR ..... Cluster of differentiation receptor
CHRNA5..... Neuronal acetylcholine receptor subunit alpha 5
CI ..... Confidence interval
COX..... Cyclooxygenase
CRN ..... Classic rheumatoid nodules
CRP..... C-reactive protein
CSUM ..... Carstairs score upper-median
CTLA4 ..... Cytotoxic T lymphocyte-associated antigen 4
CVA..... Cerebrovascular accident
CVD..... Cardiovascular disease
DAS..... Disease activity score
DD..... Disease duration
ddH_2O..... Double-distilled water
DMARD..... Disease-modifying anti-rheumatic drug
DNA..... Deoxyribonucleic acid
dNTPs..... Deoxynucleotide triphosphates

ECG..... Electrocardiography

ECM..... Extracellular matrix

ELISA ..... Enzyme-linked immunosorbent assay

ESR ..... Erythrocyte sedimentation rate

EULAR ..... The European League Against Rheumatism

G-CSF ..... Granulocyte colony-stimulating factor

GM-CSF..... Granulocyte-macrophage colony-stimulating factors

GSH..... Glutathione

GST ..... Glutathione S-transferase

GWAS ..... Genome-wide association study

HAQ..... The health assessment questionnaire

HAT..... Histone acetyltransferase

HDAC ..... Histone deacetylase

HGF..... Hepatocyte growth factor

HIF-1Ŭ..... Hypoxia-inducible factor 1Ŭ

HLA ..... Human leukocyte antigens

HWE ..... Hardy-Weinberg equilibrium

IFN-ɔ ..... Interferon gamma

IHD ..... Ischemic heart disease

IL..... Interleukin

ILD..... Interstitial lung disease

IQR..... Interquartile range

IU ..... International unit

LD ..... Linkage disequilibrium

MCP ..... Metacarpophalangeal joint

MHC ..... Major histocompatibility complex

MI..... Myocardial infarction

MJS ..... The mechanical joint score

MLC..... Mixed lymphocyte culture

MMP ..... Matrix metalloproteinase

MTP ..... Metatarsophalangeal joint

MTX..... Methotrexate

NCSS..... Number cruncher statistical system for Windows

NF-kB..... Nuclear factor kappa-light-chain enhancer of activated B cells

NO..... Nitric oxide

NOS..... Nitric oxide synthase

NSAID ..... Nonsteroidal anti-inflammatory drug

OR..... Odds ratio

PADI..... Peptidylarginine deiminase

PBS ..... Phosphate buffered saline

PCR..... Polymerase chain reaction

PDGF-BB..... Platelet-derived growth factor with 2 B chains

PECAM-1..... Platelet endothelial cell adhesion molecule

PIP..... Proximal interphalangeal joint

PTPN22..... Protein tyrosine phosphatase, non-receptor 22

RA..... Rheumatoid arthritis

RASF..... Rheumatoid arthritis synovial fibroblasts

RF..... Rheumatoid factor

RFLP..... Restriction fragment length polymorphism

RN..... Rheumatoid nodulosis

RNA ..... Ribonucleic acid

ROS..... Reactive oxygen species

RR..... Relative risk

SD ..... Standard deviation

SE..... Shared epitope

SNP ..... Single-nucleotide polymorphism

SSZ..... Sulfasalazine

STAT4 ..... Signal transducer and activator of transcription 4

TGF(-Ŭ) ..... Transforming growth factor (alpha)

Th..... T helper cells

TIMP..... Tissue inhibitors of metalloproteinase

TLR..... Toll-like receptor

TNF(-Ŭ)..... Tumour necrosis factor (alpha)

TNFAIP3..... TNF-Ŭ-induced protein 3

TRAF1 ..... TNF receptor-associated factor 1

ULN ..... The upper limit of normal

UTR..... Untranslated region

VEGF(A)..... Vascular endothelial growth factor (A)

LIST OF PUBLICATIONS

Chen Y, Dawes PT, Mattey DL. A specific MMP1-MMP3 haplotype is associated with high levels of MMP-1 and shows interaction with smoking in relation to erosive disease in rheumatoid arthritis [abstract for oral presentation]. Rheumatology 2010;49 (Suppl 1):i9-i10.

Chen Y, Dawes PT, Packham JC, Mattey DL. Interaction between smoking and polymorphism in the VEGF gene is associated with ischaemic heart disease and myocardial infarction in RA [abstract]. Rheumatology 2011;50 (Suppl 3):iii60-iii61.

Chen Y, Dawes PT, Mattey DL. Polymorphism in the promoter region of the vascular endothelial growth factor gene is associated with serum VEGF level and disease activity in RA [abstract]. Rheumatology 2011;50 (Suppl 3):iii60.

Chen Y, Dawes PT, Packham JC, Mattey DL. Interaction between smoking and polymorphism in the promoter region of the VEGFA gene is associated with ischemic heart disease and myocardial infarction in rheumatoid arthritis. The Journal of Rheumatology 2011;38:802-8.

Chen Y, Nixon N, Dawes PT, Mattey DL. Influence of variations across the MMP1 and 3 genes on the serum levels of MMP-1 and -3 and disease activity in rheumatoid arthritis. Genes & Immunity (in press).

CHAPTER 1 INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

1.1.1 History, definition and classification

The first known traces of arthritis date back at least as far as 4500 BC. A text dated 123 AD first described symptoms very similar to rheumatoid arthritis (RA). In 1800, the first recognized description of RA was made by the French physician Dr. Augustin Jacob Landré-Beauvais. The name ñRAò itself was coined in 1859 by the British rheumatologist Dr. Alfred Baring Garrod.

The initial definition was a more general description of rheumatoid disease that has since been classified into a number of separate disease entities. Although the description today is more detailed and specific, there is no exact definition. It is suggested that the present classification of RA may still incorporate more than one disease entity. Generally, based on its clinical feature, RA is described as a systemic autoimmune disease characterised by chronic inflammation of the synovial joints, ultimately leading to joint destruction and permanent disability. The widely accepted classification of RA is the 1987 American College of Rheumatology (ACR) criteria for the diagnosis of RA (Table 1.1.1a). Recently, a new classification criteria was released jointly by the ACR and the European League Against Rheumatism (EULAR) (Table 1.1.1b).

Table 1.1.1a The 1987 ACR criteria for the diagnosis of RA

Criterion	Comments
1. Morning stiffness	Duration > 1 hour, lasting > 6 weeks
2. Arthritis of at least three joints*	Soft tissue swelling/exudation, lasting > 6 weeks
3. Arthritis of hand joints	Wrists, MCPs or PIPs, lasting > 6 weeks
4. Symmetrical arthritis	At least one area, lasting > 6 weeks
5. Rheumatoid nodules	
6. Positive rheumatoid factor (RF)	
7. Radiographic changes	Erosions, particularly wrists, hands and feet

At least 4 criteria must be fulfilled and there are no exclusion criteria; * Possible area: metacarpophalangeal joints (MCPs), proximal interphalangeal joints (PIPs), wrist, elbow, knee, ankle, metatarsophalangeal joints (MTPs).

(Reproduced from Arnett et al, 1988)

Table 1.1.1b The 2010 ACR/EULAR classification criteria for RA

	Score
Target population (Who should be tested?): Patients who	
1) have at least 1 joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease[A]	
Classification criteria for RA (score-based algorithm: add score of categories A ï D; a score of Ó6/10 is needed for classification of a patient as having definite RA)[ÿ]	
A. Joint involvement[§]	
1 large joint[¶]	0
2 ï 10 large joints	1
1 ï 3 small joints (with or without involvement of large joints)[#]	2
4 ï 10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joint)**	5
B. Serology (at least 1 test result is needed for classification) [AA]	
Negative RF and negative anticitrullinated protein/peptide antibodies (ACPA)	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification) [ÿÿ]	
Normal C-reactive protein (CRP) and normal erythrocyte sedimentation rate (ESR)	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms[§§]	
< 6 weeks	0
Ó6 weeks	1

***** The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of RA with a history compatible with prior fulfilment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be

classified as having RA; [À]Differential diagnoses vary among patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis, and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted; [ÿ] Although patients with a score of < 6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time; [§] Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first MTPs are excluded from assessment. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement; [¶] ñLarge jointsò refers to shoulders, elbows, hips, knees, and ankles; [#] ñSmall jointsò refers to the MCPs, PIPs, second through fifth MTPs, thumb interphalangeal joints, and wrists; ** In this category, at least 1 of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g. temporomandibular, acromioclavicular, sternoclavicular, etc.); [ÀÀ]Negative refers to international unit (IU) values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but Ò3 times the ULN for the laboratory and assay; high-positive refers to IU values that are > 3 times the ULN for the laboratory and assay. Where RF information is only available as positive or negative, a positive result should be scored as low-positive for RF; [ÿÿ] Normal/abnormal is determined by local laboratory standards; [§§] Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g. pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

(Reproduced from Aletaha et al, 2010)

1.1.2 Epidemiology

RA afflicts both males and females of all ages, although the peak onset is between 40 ï 50 years of ages. It affects females more often than males, with a female-to-male excess of between 2 and 4 times. Females usually exhibit a more aggressive disease and a poorer long-term outcome. Males, however, are more likely to die from extra-articular complications of RA. The specific reason for these differences is not well understood, although this has been interpreted as suggestive of involvement of sex hormones (Da Silva and Hall, 1992).

RA is a worldwide disease. It affects all races and ethnic groups, although the prevalence of the disease may vary. In Caucasians, the figure for prevalence is approximately 1%. In other nationalities, such as the residents in eastern Asia, rural Africa and Caribbean, the rate is normally lower. In contrast, dramatically increased prevalence has been reported in several American Indian and Alaska Native populations, with the highest record at 7.1% (Ferucci et al, 2005). These data support a genetic role in disease risk. Furthermore, they also imply that the geography and culture related factors including climate, lifestyle and diet may also have an affect.

The family history can be a risk factor for RA. In particular families, the occurrence of the disease is higher, especially with the first- and second-degree relatives compared to the background population (Grant et al, 2001). Twin study has estimated that genetics account for approximately 50 ï 60% towards RA susceptibility (MacGregor et al, 2000).

Permanent remissions of RA are very rare once the joint damage has started. The majority of patients suffer from RA until their death. In most case, the disease is chronic and becomes progressively more severe over time. The life expectancy is reduced by approximately 7 years in males and 3 years in females, which is mainly due to the co-morbidities including infections, cardiovascular, respiratory and renal diseases, and RA itself (Hakim et al, 2006).

1.1.3 Aetiology

Knowledge of the chain of aetiological approaches that lead to the development of RA is unknown, although it is believed that multiple factors, including environmental and genetic ones, are involved. Complicated interactions between these factors are thought to be important in triggering immunologic events that eventually result in the clinical signs of RA. The relative contribution of the genetic component is though to be 50 ï 60% and the remaining risks are attributable to environment, which indicates that both constitute great influence on the development of disease. The role of environmental and genetic factors on the aetiology of RA will be discussed in Sections 1.2 and 1.3 respectively.

It is more likely that each of the individual disease phenotypes results from a number of different combinations of genetic and environmental risk factors. Some risk factors may have a strong effect but only in a small proportion of patients, while others will have weak effects and be present in a larger number of individuals but require the involvement of other risk factors.

1.1.4 Pathology

The fundamental pathological abnormality in RA is synovitis. The synovial membrane in RA becomes hyperplastic and, on direct visualization both the lining and sublining layers of synovium demonstrate characteristic changes in histology. In the lining layer, the number of macrophage-like and fibroblast-like synoviocytes increases, and the thickness of the intima increases from the normal 2~3-cell layers up to 10-cell layers. The sublining becomes highly infiltrated with peripheral blood-derived inflammatory cells that particularly include macrophages, B- and cluster of differentiation (CD)4+T-lymphocytes. Follicles of lymphoid cells may be present, resembling germinal centres of lymph nodes. Neovascularization driven by vascular endothelial growth factor A (VEGFA) and vascular cell adhesion molecule-1 (VCAM-1) develops dramatically, which is an essential feature of the hyperplastic sublining layer.

The nature of the synovium changes along with the disease progression. Hyperplastic synovial lining forms an aggressive front called pannus which ignores the normal tissue boundaries and extends from the joint margins, and erodes the articular cartilage. Extensive erosion of cartilage and bone, with concomitant involvement of surrounding tissue, leads to joint destruction.

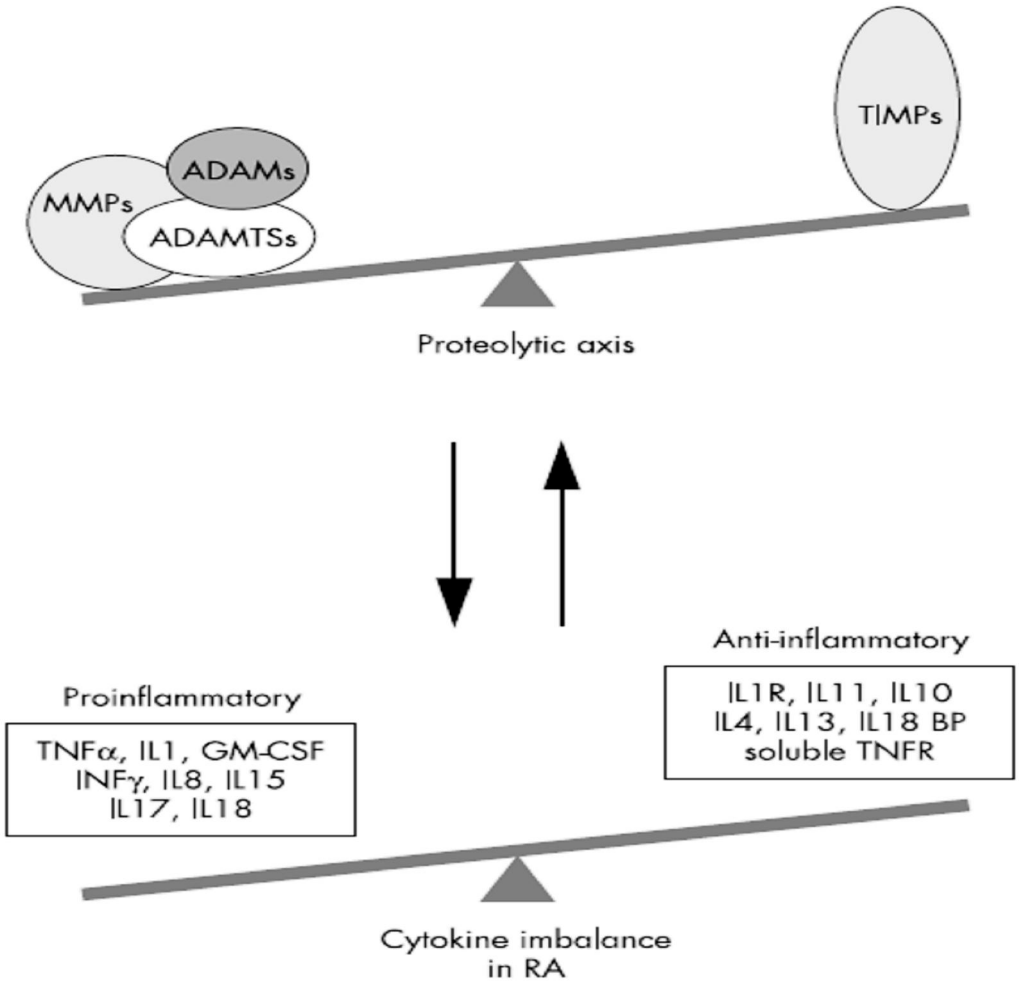
Imbalance of inflammatory mediators may be a central pathogenic mechanism in RA. Increased levels of cytokines are present in the RA synovium. Tumour necrosis factor alpha (TNF-Ŭ) and interleukin (IL)-1 are the prominent cytokines, but many other pro-inflammatory ones, including IL-6, IL-12, IL-15 and IL-18 are also present. The overall spectrum of cytokines present is more consistent with a macrophage rather than with a T-

cell source, although low levels of T-helper 1 (Th1) related interferon gamma (IFN- ɔ), and T-helper 2 (Th2) related IL-4 are detected. Nevertheless, the RA joints also contain anti-inflammatory cytokines, such as IL-10, IL-13 and transforming growth factor alpha (TGF-Ŭ), and high levels of cytokine-neutralizing factors, such as soluble TNF-Ŭ receptors and IL-1 receptor antagonist (IL-1ra). These findings together, however, give a suggestion that cytokine imbalance in favour of pro-inflammatory mediators may play an important role in resulting in RA. Other pro-inflammatory factors present within the RA joints include the pleiotropic mediator nitric oxide (NO), prostaglandins and leukotrienes, and reactive oxygen intermediates. Synovial TNF-Ŭ could influence the expression of other pro-inflammatory mediators such as IL-1 and granulocyte-macrophage colony-stimulating factors (GM-CSF), which implicates its central role in the RA cytokine cascade. Its production may be driven by T-cells. Cell-to-cell contact between activated T-cells and synovial monocytes may be a stimulus to TNF-Ŭproduction (Hakim et al, 2006).

The mechanism behind bone and cartilage destruction in RA is not well understood. However, it is known that a variety of destructive enzymes are secreted by pannus. A superfamily of proteases called metzincins (zinc-dependent metallopeptidases) is prominently involved. In this superfamily, there are the matrixin family of matrix metalloproteinases (MMPs), the transmembrane proteases containing disintegrin and metalloprotease domains (ADAMs) and the secreted ADAMs with thrombospondin domain (ADAMTSs). There are more than 20 members in the MMP and ADAMTS family including the collagenases, gelatinases, stromelysins, some elastases and aggrecanases. These enzymes act on collagen and the proteoglycan matrix, thereby destroying the central structure of articular cartilage, tendon and bone extracellular matrix (ECM) proteins in synovial joint arthritis (Malemud, 2006). Furthermore, MMPs also appear to play an

essential role in the early T-cell mediated phase of RA (Kammer et al, 1985), in the cytokine-induced inflammatory response that promotes progressive ECM protein degradation (Malemud, 2004) and in dysfunctional apoptosis (Malemud et al, 2005), all of which are important features of arthritis pathophysiology. Tissue inhibitors of metalloproteinases (TIMPs) are well known for their MMP inhibitory activity, and there is evidence that some TIMPs also inhibit specific ADAM and ADAMTS enzymes (Mohammed et al, 2003). A balance between destructive and inhibitory metalloproteinases in the microenvironment is thought to be essential to maintain the normality of joints. Metalloproteinase activity is linked to the activation of various cytokines involved in inflammation. The net metalloproteinase activity can shift the balance between some pro- and anti-inflammatory cytokines. Cytokines, in turn, also induce the production and activation of various metalloproteinases. An imbalance in one axis can potentially cause imbalance in the other, perpetuating a loop of destructive activity in RA (Mohammed et al, 2003).

Figure 1.1.4a The projected interactions between the metalloproteinase axis and the cytokine axis in RA



(Reproduced from Mohammed et al, 2003)

1.1.5 Co-morbidity

A co-morbid condition is a medical condition that co-exists along with the disease of interest. Co-morbidity can be further defined in terms of a current or past condition. It may be affected by risk factors also for the primary disease, may be linked to the process and/or

the treatment of the primary disease, or may be completely independent of these. The more co-morbidities a patient has, as may be expected, the greater the utilization of health services, the greater societal and personal costs, the poorer the quality of life, and the greater chances of hospitalization, disability and mortality. Furthermore, co-morbidity usually makes diagnosis and treatment decisions more challenging, leading to greater difficulty in terms of patient care. Therefore, for researchers, it is important to understand the linkage/relationship between the primary disease and its co-morbidities; for physicians, it is essential to recognize such co-morbid illnesses and to account for them in the care of the individual patients.

A RA patient has approximately 1.6 co-morbidities on average (Wolfe and Michaud, 2008), and the number increases with the progress of RA and the patientôs age. RA leads to outcomes in different aspects including mortality, work disability, hospitalization, medical costs, quality of life, happiness, etc. Different co-morbidities influence such outcomes variously. For example, cardiac and pulmonary co-morbidities are mostly associated with the premature death, while depression is thought to be strongly associated with work disability (Gabriel and Michaud, 2009).

Cardiovascular disease

Cardiovascular disease (CVD) is a major co-morbid condition in RA with increased prevalence compared with that in the general population. It has been reported to account for almost 50% of the excess mortality in RA (Naz and Symmons, 2007; van Doornum et al, 2002). The most common CVD is ischemic heart disease (IHD). Adequate studies have demonstrated significantly higher incidence of IHD in RA patients in comparison to

control subjects (Maradit-Kremers et al, 2005; Watson et al, 2003; Wolfe, et al, 2003; Solomon et al, 2003; Fischer et al, 2004; Sodergren et al, 2007; Turesson et al, 2004; del Rincon, 2001). Research also has shown that the clinical presentation of IHD in RA appears to be different to that in the normal population, with RA patients more likely to experience unrecognized myocardial infarction (MI) and sudden death (Maradit-Kremers et al, 2005). An emerging body of literature now indicates that individuals with RA are also at high risk for developing heart failure, especially in RF positive patients (Nicola et al, 2005).

Malignancy

Cancer is the second most common cause of death in RA, just after CVD. Overall, there is only slightly increased incidence rate in RA subjects with the ñstandardized incidence rateò estimated to be 1.05 (95% CI = 1.01 ï 1.09), provided by a meta-analysis study which has summarized 13 recent publications during the past decade (Smitten et al, 2008). This increase appears to be due to only a few specific malignancies which may include lymphoma and lung cancer. After reviewing 6 recent studies, Smitten and co-workers has suggested that there is approximately 2-fold increased incidence of lymphoma in RA (Smitten et al, 2008). Another study has further demonstrated that this increased risk is associated with severity and inflammatory activity of the primary disorder (Baecklund, et al, 2006). An increased incidence of lung cancer of approximately 1.6 times has been seen in RA patients (Smitten et al, 2008). This is probably due, at least in part, to the excess exposure of cigarette smoking in both lung cancer and RA populations. Smoking has been strongly associated with the development of both disorders. In contrast, for certain cancer types, the incidence decreases in persons with RA. For example, the incidence of breast

cancer is reduced, although it is the second most common cause of malignancy among RA individuals after lung cancer (Smitten et al, 2008). The risk for colorectal cancer has also been reported to be decreased in RA (Smitten et al, 2008).

Lung disease

Among pulmonary disorders, interstitial lung disease (ILD) is an important complication of RA, and is associated with substantially increased mortality. The rate of ILD in RA varies from one study to another, ranging between 1 to 58% (Carmona et al, 2003; Dawson et al, 2001; Gabbay et al, 1997; Gochuico et al, 2008; Mori et al, 2008; Turesson et al, 2003). This great difference is probably due to the lack of validated classification criteria for ILD, and the plethora of detection methods employed to diagnose ILD. Furthermore, the above studies are based on referral cohorts, since extra-articular disease is thought to be more frequent in patients with more severe RA, the rate of ILD may be over-estimated in some reports. Recently, a population based study has suggested that the lifetime-risk of developing ILD is 7.7% for RA population and 0.9% for subjects without RA, and that ILD contributes approximately 13% to the excess mortality in RA (Bongartz et al, 2010). The cause of ILD in individuals with RA is not well known. However, several drugs have been linked to certain lung diseases and/or ILD, including injectable gold, penicillamine, sulfasalazine (SSZ), methotrexate (MTX), infliximab and leflunomide, with some reports linking infliximab to rapidly progressive and/or fatal ILD (Gabriel and Michaud, 2009). In addition, there has been evidence suggesting cigarette smoking as a co-factor in the development of RA-associated ILD (Patel, et al, 2008).

Infection

Similar to most other inflammatory diseases, RA appears to increase the risk for bacterial, tubercular, fungal, opportunistic and viral infections, with all infections being more common in more active and severe RA. This may be due to the immuno-modulatory effects of RA, or to the agents with immunosuppressive effects applied in its treatment (Doran et al, 2002). Recently, Franklin and co-authors have indicated that there is at least a 2.5 times increased incidence of infection in inflammatory polyarthritis subjects compared to that in the general population within the same community. Furthermore, they also have suggested cigarette smoking, presence of RF and corticosteroid use as significant independent predictors of infection-related hospitalization (Franklin et al, 2007). There is another report revealing that the use of corticosteroid is associated with increased infection in RA, whereas the use of other disease-modifying anti-rheumatic drugs (DMARDs), including MTX, is not (Lacaille et al, 2008). The data with regard to the association of anti-TNF therapy with infection is contradictory, with positive (Bongartz et al, 2006; Curtis et al, 2007; Costenbader et al, 2006) and negative (Schneeweiss, et al, 2007; Wolfe et al, 2006; Dixon et al, 2006) results both existing. Tuberculosis seems to be increased in RA individuals independent of treatment (Brassard et al, 2009; Carmona et al, 2003; Seong et al, 2007), although an American study differed in this regard (Wolfe et al, 2004). Anti-TNF therapy substantially enhances the risk for tuberculosis, notably in patients treated with infliximab (Seong et al, 2007; Wolfe et al, 2004; Keane et al, 2001). Herpes zoster has been increased in RA patients. Although the reason remains unclear, it may be related to functional status as measured by the Health Assessment Questionnaire (HAQ), and to treatment where monoclonal anti-TNFs are used (Wolfe et al, 2006; Strangfeld et al, 2009).

Gastrointestinal ulcer disease

There is no evidence showing that gastrointestinal ulcers are related to a specific process of RA. However, increased incidence of gastrointestinal ulcers occurs in patients with RA, which is probably due to the use of nonsteroidal anti-inflammatory drugs (NSAIDs). Selective cyclooxygenase (COX)-2 inhibitors, named ñcoxibsò, have been associated with less gastroduodenal damage and risk of gastrointestinal bleeding by sparing COX-1 enzyme in the gastric mucosa and in platelets, in comparison with conventional NSAIDs (Coruzzi et al, 2007). However, this reduction is incomplete, and gastrointestinal toxicity still exists. The risk for gastrointestinal ulceration is also associated with corticosteroid use, and increased further by concomitant NSAID usage (Garcia Rodriguez et al, 2001).

Hypertension

Increased rate of hypertension amongst RA patients has been observed, although contradictory reports have been also published (Panoulas et al, 2008). Several studies have demonstrated that hypertension is associated with subclinical atherosclerosis (Gerli et al, 2005; Roman et al, 2006; Dessein et al, 2006) and is an important independent predictor of CVD with relative risk ranging from 1.49 ï 4.3 (Wolfe et al, 2003; Assous et al, 2007; Wallberg-Jonsson et al, 1999), in patients with RA. Overall, the impact of hypertension on cardiovascular outcome is thought to be similar between RA patients and those who do not have RA (Gonzalez et al, 2008). However, since cardiovascular mortality is higher in RA patients compared with the general population, the number of deaths attributed to hypertension may be higher amongst RA subjects (Panoulas et al, 2008).

Anaemia

Anaemia is a common co-morbid condition in individuals with RA. The estimated rate of mild anaemia ranges from 31.5 to 60%, at least 3 times more compared to the general population (Wilson et al, 2004; Wolfe and Michaud, 2006). However, severe chronic anaemia is rare (3.4%) in RA (Wolfe and Michaud, 2006). The literature has suggested that anaemic RA patients are likely to have more severe joint disease, leading to a low quality of life (Wilson et al, 2004). Moreover, successful treatment of anaemia has been shown to reduce the RA symptoms and/or improve the quality of life (Wilson et al, 2004). Inflammatory activity appears to be linked with anaemia, since both ESR and CRP are parameters among the strongest predictors of anaemia (Wolfe and Michaud, 2006). RA patients with co-morbid CVD, diabetes and gastrointestinal disease are more likely to have anaemia (Furst et al, 2009). In addition, renal function, as measured by estimated creatinine clearance, may also have a relationship with anaemia in RA subjects (Wolfe and Michaud, 2006).

Osteoporosis

Osteopenia is considered as a consequence of RA, decreased physical activity and treatment with corticosteroids. Fracture, resulting from osteoporosis, ranks highly among co-morbidities contributing to increased disability, future hospitalization, and mortality. There is approximately a 2-fold increase of fracture among patients with RA, compared to the general population (Gabriel and Michaud, 2009). Although osteoporosis is increased in RA independent of corticosteroids, the usage of this drug substantially increases the risk

(van Staa et al, 2006; Lane et al, 1995; Kroger et al, 1994; Haugeberg et al, 2000; Sinigalia et al, 2000).

1.1.6 Clinical features

RA usually presents as a polyarthritis affecting small joints or small and large joints. In addition, a wide variety of other systems may be affected by the rheumatoid process. Among extra-articular manifestations, rheumatoid nodules are the most common ones. As a disease characterized by systemic auto-immunity, the presence of several autoantibodies including RF and ACPA have been detected in serum and synovial fluid of RA subjects.

Joint damage

The start of the disease is usually insidious but also can be episodic or acute. At first presentation, RA is typically observed in the small peripheral synovial joints such as those of the hands and feet. Examples include the PIPs and MCPs of the fingers, and the MTPs of the toes. Early disease is characterized by pain and other cardinal signs of inflammation such as heat, swelling, functional loss and erythema over joints, but not by damage and deformity. The clinical features of synovitis are particularly apparent in the morning.

If the disease remains active and uncontrolled, the inflammation will usually spread to additional joints, with disease manifesting also in the larger synovial joints such as the wrists, ankles, elbows and knees. The number and particular joints involved in each individual varies. It is unclear what governs the development of disease in specific joints. Gradual irreversible tissue damage (e.g. to tendon, cartilage, bone) may occur during the

disease progress, causing deformity and instability of joints. The most serious long term disability is associated with damage to the larger weight bearing joints.

Although RA predominantly affects peripheral joints, discovertebral joints of the cervical spine are often affected. This may lead to atlantoaxial subluxation or, less commonly, subluxation at lower levels, with subsequent compression of the spinal cord. The earliest and most common symptom of cervical subluxation is pain radiating up into the occiput. Other symptoms include paraesthesia, sudden deterioration in hand function, sensory loss, abnormal gait, and urinary retention or incontinence (Akil et al, 1995).

Extra-articular manifestation

RA is a systemic inflammatory disease, which, in addition to the characteristic peripheral polyarthritis, can involve other tissue and organs. Nodules are the most common extra-articular feature, while many of the other classic features occur in 5% or less normal clinic settings. Currently, there are no reliable predictors for these features in early RA, although they are generally associated with males, smokers, more severe joint disease, worse function, high levels of inflammatory markers, and the presence of RF, antinuclear antibodies (ANA) and the human leukocyte antigens (HLA)-DRB1 shared epitope (SE) (Young and Koduri, 2007). On the other hand, the presence and severity of extra-articular manifestations do not necessarily predict the development of more severe joint course. However, it has been suggested that the occurrence of extra-articular manifestations is associated with excess mortality (Turesson et al, 2002). Severe extra-articular diseases are also associated with an increased risk of first ever cardiovascular events in patients with RA (Turesson et al, 2007), which may further increase the mortality rate. Thus, a prompt

and specific management is needed in order to minimize extra-articular manifestations itself and its impact.

Subcutaneous nodules are often found in patients with RA and occur as classic rheumatoid nodules (CRN), accelerated rheumatoid nodulosis (ARN), or rheumatoid nodulosis (RN). CRNs occur in approximately 20% to 25% of patients with seropositive RA, whereas this rate is significantly lower in seronegative disease. A much greater incidence (75%) is observed in patients with RA-associated Felty syndrome (Sayah and English, 2005). The frequency of CRN correlates directly with RF titer and more aggressive forms of disease, such as joints erosions and rheumatoid vasculitis (García-Patos, 2007). CRNs are relatively frequent in the white population, and affect males more than females. Their occurrence is thought to have a genetic involvement. RA patients with heterozygosity for HLA-DRB1 alleles, specifically *0401 with B1*0404/8 or *0101, are at high risk for nodular disease (MacGregor et al, 1995). Most CRNs are painless, although those found on the palms or soles may feel uncomfortable. ARNs are recognized complication of MTX therapy in patients with RA. They are often painful, small nodules that are found preferably on the hands, feet and ears of male patients with severe RA (Segal et al, 1988; Salaffi et al, 1995; Filosa et al, 1999). Most ARNs are histologically identical to CRNs. HLA-DRB1*0401 allele and RF seropositivity have been associated with MTX-induced ARN (Ahmed et al, 2001; Williams et al, 1998). RNs commonly occur in patients with severe, seropositive RA. However, in some instances, skin nodules are the predominant sign, with minor or absent joint symptoms (García-Patos, 2007). Ginsberg and co-workers considered this disease entity as an unusual variant of RA (Ginsberg et al, 1975). RN patients are generally men in their 30 ï 50 of ages. Unlike traditional RA, RN appears to have a poor association

with the HLA-DRB1 alleles and does not seem to lead to future development of erosive arthritis and systemic manifestations of classical RA (Toussirot et al, 1998).

Other extra-articular manifestations are relatively rare compared to rheumatoid nodules, except for ILD which has been described as a complication of RA (Section 1.1.5). Ever published conditions are listed in Table 1.1.6a.

Table 1.1.6a Published extra-articular features of RA

Extra-articular RA	
Nodules	Glomerulonephritis
Raynaudôs phenomenon	Systemic vasculitis
Secondary Sjögrenôs syndrome	Severe cutaneous vasculitis
ILD ï pulmonary fibrosis	Benign cutaneous and nail-fold vasculitis
Pericarditis	Lymphadenopathy
Pleuritis	Weight loss, cachexia
Feltyôs syndrome	Malaise, fatigue, fever
Polyneuropathy, mononeuropathy, mononeuritis multiplex	Episcleritis, scleritis, kerato-conjunctivitis perforans
Myopathy, polymyositis	Amyloid

(Modified from Young and Koduri, 2007)

Autoantibodies

RA is typically associated with serological evidence of systemic auto-immunity, indicated by the presence of autoantibodies in serum and synovial fluid. Since the initial description of RF (Waaler, 1940), a number of other autoantibodies have been discovered in patients with RA. The autoantigens recognized by these autoantibodies include cartilage components, stress proteins, enzymes, nuclear proteins and citrullinated proteins (Table

1.1.6b), demonstrating that RA is not characterized by only one autoreactivity to a single autoantigen but by accumulated autoreactivities in both B and T cells (Song and Kang, 2009). The spectrum of these self-antigens and immunologically relevant epitopes probably varies during the disease course, and the set of autoantigens in one patient may differ from that in another (Bläss et al, 1999). The clinical importance and pathogenic roles of these antibodies are largely unknown expect for RF and ACPA, whose clinical usefulness has been acknowledged due to their acceptable sensitivities and specificities, and prognostic values.

Table 1.1.6b A summary of well-characterised autoantigens in RA

Citrullinated proteins and peptides	CCP-1 and -2, fibrinogen, fibrin, vimentin, filaggrin, citrullinated peptides of collagen II, citrullinated Ŭ-enolase, citrullinated translation initiation factor 4G1
Components of articular cartilage	Collagen II, IX and XI
Circulating serum proteins	IgG, Fibrinogen, plasminogen, ferritin
Nuclear components	RA33/hnRNP A2, eukaryotic translation elongation factor 1 Ŭ
Stress proteins	HSP-65, -70 and -90, BiP
Inflammatory/immune factors	B7-H1, IL-1 Ŭ, IL-8
Enzymes	Calpastatin, Ŭ-enolase, aldolase-A, dipeptidyl peptidase, osteopontin, glucose-6-phosphate isomerase
Receptors	Lipocortin 1
Anti-neutrophil cytoplasmic antibodies	

(Adapted from Mewar and Wilson, 2006)

RFs are a family of autoantibodies directed to the Fc region of IgG. They are locally produced in RA by B cells present in lymphoid follicles and germinal center-like structures that develop in inflamed synovium (Wernick et al, 1985; Jones et al, 1984). There has been no clear evidence whether RFs are involved in the initial events triggering the disease process of RA or they themselves are triggered by RA. IgM isotype of RFs are the major RF species in RA and are detected in 60 ï 80% of RA patients (Nell et al, 2005). RF has been proven to be a very useful marker for disease diagnosis, as included in ACR classification criteria for RA. However, it is not specific for RA. RF also has been observed in many other autoimmune disorders, such as in systemic lupus erythematosus, mixed connective tissue disease and primary Sjögren syndrome, as well as in some non-autoimmune conditions, such as in chronic infections and old age (Nell et al, 2005). RFs in healthy individuals are distinguished from those in RA subjects. RFs from healthy individuals are synthesised by CD5+ B cells, exhibit low affinity for IgG, polyreactivity and a low ratio of replacement to silent mutations in their cluster of differentiation receptors (CDRs). They are under strict control to prevent the emergence of high affinity RF. However, in rheumatoid synovium, B cells produce high affinity RFs with multiple replacement mutations in their CDRs, in a process dependent on T cell help (Mewar and Wilson, 2006). CD40 signalling has been proven to play a crucial role here (Kyburz et al, 1999). RF specificity to RA is increased at high titers and with IgA isotypes (Nell et al, 2005; Jónsson T et al, 1998). High titer RF and IgA isotypes are also associated with radiologic erosion, extra-articular manifestations and thus, poorer disease outcome (Jónsson T et al, 1995; Jorgensen et al, 1996; Päi et al, 1998). The association of high titer RF with a poor prognosis suggests that RF may have a role in the pathogenesis of RA. Furthermore, RF status is a strong predictor of mortality in RA. Increased rate of early death has been found in patients with RF positive RA. By contrast, patients with

persistently RF negative RA have mortality rate similar to that in the general population (Gonzalez et al, 2008).

ACPAs such as anti-perinuclear factor (APF), anti-keratin antibody (AKA), anti-Sa and anti-cyclic citrullinated protein antibodies (anti-CCP) are an overlapping group of antibodies with a remarkable specificity completely dependent on the citrullination of arginine residues (Mewar and Wilson, 2006). Citrullination is the term used for the post-translational modification of arginine in a protein into citrulline. This reaction is performed by peptidylarginine deiminase (PADI) enzymes during a variety of biologic processes, and needs the presence of sufficient concentration of Ca^{2+}. Apoptotic granulocytes create a working environment for PADI, in which cytosolic Ca^{2+} level rises due to caspase-mediated plasma membrane Ca^{2+} pump cleavage (Vossenaar et al, 2003). Therefore, when the apoptotic cells are not cleared efficiently as in an inflammatory environment, intracellular citrullinated proteins and/or PADI are released into the extracellular space, where the former are taken up by antigen-presenting cells and the latter induces the citrullination of synovial proteins (Song and Kang, 2009). ACPAs are consequently produced in RA joints, where proteins are citrullinated during the inflammatory process.

Although citrullination is a prerequisite to ACPA synthesis, it dose not always induce APCA production. APCA production appears to be limited to subjects with certain genetic backgrounds, among which HLA-DRB1 SE is thought to be the most dominant genetic factor (van Gaalen et al, 2004; Kaltenhäuser S, et al, 2007; Berglin et al, 2004). ACPAs are found in 70 ï 90% of RA patients with high disease specificity over 90% (Schellekens et al, 2000; Suzuki et al, 2003). Accordingly, they are rarely detected in other diseases or in healthy individuals. In general, ACPA has better diagnostic value than RF in terms of

sensitivity and specificity. Furthermore, it has been found that ACPA precedes up to 9 years prior to RA onset (Rantapää-Dahlqvist et al, 2003; Nielen et al, 2004), suggesting the production of these antibodies as the earliest preclinical events in the disease process. Their detection, thus, is of major interest regarding the identification of RA among most recent arthritides. In some RA subjects, however, ACPA sero-conversion occurs after RA onset. ACPA status is a predictor of disease outcome. It is associated with more erosive RA (Kroot et al, 2000; van der Helm-van Mil AH, et al, 2005; Im et al, 2009). Furthermore, it is also greatly associated with increased radiological progression, in the presence of HLA-DRB1 SE (van Gaalen et al, 2004; Berglin et al, 2004). Thus, its prognostic value may lead to early aggressive treatment to prevent irreversible joint damage. Since citrullinated residues are the key antigenic determinants recognised by ACPAs, an anti-cyclic citrullinated peptides second generation assay (anti-CCP2) has been developed as a proxy to test this overlapping heterogeneous group of antibodies.

Both RF and ACPA are present during the preclinical stage of RA, months to years prior to disease onset. The risk of RA development is highest when RF and ACPA are present in conjunction (Rantapää-Dahlqvist et al, 2003; Nielen et al, 2004). The titers of autoantibodies increase as disease onset is approached and most of the negative to positive sero-conversion occur within 3 year prior to the symptoms onset. However, in certain patients, sero-conversion towards either one of the two antibodies continues to occur after RA onset, most likely within the first few years of disease (Nielen et al, 2004). In respect of sensitivity and specificity, ACPA appears to have better diagnostic value. On the other hand, RF is a better predictor of the extra-articular manifestations of RA. However, recently, it has been demonstrated that citrullination occurs in the lung tissues of RA patients with ILD (Bongartz et al, 2007), suggesting ACPA response may be involved in

the pathology of RA-associated ILD. Both autoantibodies are related to high disease activity and bad radiological outcome. They are shown to be independently associated with erosive disease (De Rycke et al, 2004; Berglin et al, 2006). However, it has been suggested the superiority of ACPA over RF in predicting an erosive course. During RA treatment, both RF and ACPA tend to decrease in responsive patients, usually with a more significant reduction in RF (Song and Kang, 2009).

1.1.7 Clinical measurement

Inflammation, disease activity, joint damage and functional outcome can be described by several measurements to provide an indication of disease.

Inflammation level

A raised ESR and the presence of CRP are commonly found in patients with RA, especially when the disease is active. They provide the best information about the acute phase response, and their raised levels reflect the strength of the underlying inflammation. The disease is likely to progress if a raised ESR/CRP persists. Although not specific to RA, they are important laboratory-based parameters.

ESR value tends to rise with age and be generally higher in females. It is also increased in states of anaemia and in black populations. The widely used rule for calculating normal maximum ESR values in adults is given by: ESR (mm/hour) Ò [Age (in years) + 10 (if female)]/2 (Miller et al, 1983). CRP is a circulating protein synthesized by liver. In comparison with ESR, it is influenced by fewer known factors (apart from liver failure).

Although < 1 mg/l is ideal, clinical diagnosis usually identifies a level < 10 mg/l as normal and a level Ó10 mg/l as presentation of inflammation.

Disease activity

Tender joint count, swellen joint count, ESR level and a self-reported visual analogue scale (VAS) of general health are included in a combined index, called the disease activity score (DAS), to measure the disease activity in patients with RA. It has been extensively validated for its use in clinical trails in combination with EULAR response criteria. It provides a number between 1 and 10, indicating how active the RA is. A modified vision which includes 28 joint counts (DAS28) is widely used (including in the present study) (Prevoo et al, 1995). The formulas for DAS28 calculation is shown in Appendix 8.1.1. Recently, CRP levels, as a replacement of ESR, are used in DAS28-CRP (Wells et al, 2009).

Erosive damage

Persistent active disease leads to largely irreversible joint damage which can be seen on X-ray. Abnormalities seen on radiographs are important measurement to assess the course of RA, although it is not commonly evident in patients recently diagnosed. There are several types of methods available to score progressive joint damage ranging from a global score for the whole patient to the more sophisticated methods of scoring erosions and joint space narrowing in a selected number of joints. These latter abnormalities give additive information and are the most important features in scoring radiographs in RA. The Larsen score (Larsen et al, 1977) which gives a global assessment per joint is most commonly

used. Recently, ultrasonography and magnetic resonance imaging provide information about synovitis and erosion in early disease when inflammatory and destructive articular change is typically sub-radiographic, which gives a great advantage in early assessment of RA (Grassi et al, 1998; McQueen et al, 1998).

Functional outcome

The Health Assessment Questionnaire (HAQ) is a disability index (Fries et al, 1982) which was developed as a comprehensive measure of outcome in patients with a wide variety of rheumatoid diseases, including RA. It is a self-reported patient-oriented outcome measurement, and has become a mandated outcome measure for both clinical trials and observational studies in RA worldwide. The HAQ form is presented in Appendix 8.1.2.

The mechanical joint score (MJS) (Johnson et al, 2002) is a newly developed index used to assess the total amount of joint damage and impairment of mechanical function in patients with RA. The examination and scoring system for this clinical index is presented in Appendix 8.1.3.

1.1.8 Treatment

The major therapeutic strategy for RA is to reduce the levels of inflammation and to slow the destructive process in the joints. There are various chemical agents available to combat the disease. These can be divided into 2 broad categories, the NSAIDs and the DMARDs. The NSAIDs are drugs with analgesic, antipyretic and anti-inflammatory (in higher doses) effects. They are used to reduce the levels of inflammation, to diminish symptoms, and to

lessen the pain. The term ñnonsteroidalò is used to distinguish these drugs from steroids which, among a broad range of others effects, have a similar eicosanoid-depressing anti-inflammatory action. The most prominent members of this group of drugs include aspirin, ibuprofen and naproxen. Most of the NSAIDs act as inhibitors of the enzyme COX, inhibiting both the COX-1 and -2 isoenzymes.

The DMARDs are used to help suppress the destructive process in the joint and to slow disease progression. Although within a category, most DMARDs are unrelated, and operate by different mechanisms. The most commonly prescribed DMARDs are MTX and SSZ. MTX is an antifolate agent which impairs the function of folic acids, while SSZ plays several roles including suppression of IL-1 and TNF-Ű, promotion of apoptosis of inflammatory cells and increase of chemotactic factors. MTX is usually the first-line drug of choice, particularly when active RA has been diagnosed. However, combinations of DMARDs are often used together, since each drug in the combination can be used in smaller dosage than if it were given alone, thus reducing the risk of side effects. It is usual that patients receive both NSAIDs and DMARDs.

Recently, the focus of drug development to treat RA has shifted away from the use of chemical agents and has been superseded by the development of various biological agents. This has evolved from an increasing understanding of the pathology within the synovium, in particular of the cells and proteins that appear to have significant roles in the chronic inflammation that resides. The most promising research has focused on TNF-Ű, which is based on the recognition of the role of TNF-Ű as the ñmaster regulatorò of the inflammatory response in many organ systems (Feldmann and Maini, 2003). TNF-Ű is elevated in the serum and synovial fluid of patients with RA (Altomonte et al, 1992; Borzi

et al, 1993; Barrera et al, 1993; Yocum et al, 1989). Since TNF-Ŭ is proinflammatory in nature, measures to neutralise these levels may lead to a therapeutic effect. This neutralization can be achieved with monoclonal antibodies such as infliximab (Remicade), adalimumab (Humira), certolizumab pegol (Cimzia) and golimumab (Simponi), or with a circulating receptor fusion protein such as etanercept (Enbrel). These biological agents have been applied in treatment of RA, with high efficacy. However, side effects have been observed which include lymphoma, infections, congestive heart failure, demyelinating disease, a lupus-like syndrome, induction of autoantibodies, injection site reactions, and systemic side effects (Scheinfeld, 2004).

1.2 ENVIRONMENTAL RISK FACTOR FOR RA

1.2.1 Cigarette smoking

Smoking is one of the most potent and prevalent addictive habits, influencing the behaviour of human beings for over 4 centuries. Tobacco smoke affects multiple organ systems and results in numerous smoking-induced disorders, notably the neoplastic, respiratory and cardiovascular diseases. Cigarette smoking has been associated with several autoimmune diseases, including RA (Table 1.2.1a). It influences the immune system in many ways with different impact according to various autoimmune diseases. In RA and many others, smoking substantially increases the incidence and/or leads to a worse outcome, whereas in ulcerative colitis, Behçetôs disease and aphthous stomatitis, it appears to serve as a beneficial factor.

It is difficult to fully understand how cigarette smoking affects a hostôs immune system, since burning a cigarette creates as many as 6000 different components, including nicotine, polycyclic aromatic hydrocarbons, tobacco glycoprotein and some metals. However, it is known that smoking affects both cell-mediated and humoral immune responses, and is associated with both release and inhibition of pro-inflammatory and anti-inflammatory mediators. Cigarette smoke generally has been shown to augment the release of numerous pro-inflammatory cytokines such as TNF-Ŭ, IL-1, IL-6, IL-8 and GM-CSF, and to suppress the production of anti-inflammatory cytokines such as IL-10 (Arnson et al, 2010). Furthermore, it has also been shown to play a role in driving macrophage and dendritic cell activation (Arnson et al, 2010). On the other hand, however, it has been associated with decrease of many immune mediators regardless of their immunological properties, through certain pathways and/or upon particular cell types, acting as an immunosuppressive agent. Indeed, there are mixed effects of smoking on the function of immune system (Table 1.2.1b).

Table 1.2.1a Influence of smoking on autoimmune diseases

Disease	Manifestations of smoking
Exacerbated by smoking	
RA	Increased prevalence in smokers, mostly men and long-term smokers. Higher rate of rheumatoid nodules and multiple joint involvements. Low response to treatment.
Systemic lupus erythematous	Slightly elevated risk for present smokers. Elevated titers of anti-dsDNA in current and past smokers.
Gravesôhyperthyroidism	Higher disease prevalence in past and current smokers. Increased rates of Gravesô ophthalmopathy.
Crohnôs disease	Increased prevalence in past and current smokers. Higher prevalence of ileal disease and a lower prevalence of colonic involvement in smokers. Greater likelihood for complicated disease.
Goodpastureôs syndrome	Smoking predisposes to pulmonary hemorrhage regardless of anti-GBM titers.
Buergerôs disease	Prevalent almost entirely in young, male smokers.
Primary biliary cirrhosis	Increased disease prevalence in smokers.
Systemic sclerosis	Higher rates of digital ischemia in smokers.
Multiple sclerosis	Higher incidence of disease in smoking women, worse clinical progression.
Firbomyalgia	More pain, numbness, disease severity and functional difficulties in smokers.
Improved by smoking	
Ulcerative colitis	Reduced risk in current smokers. Slightly elevated risk in former smokers. More benign disease course in smokers. Symptom exacerbating during smoking cessation.
Behçetôs disease and aphthous stomatitis	Fewer oral aphthae in smokers.

(Modified from Arnson et al, 2010)

Table 1.2.1b The mixed effects of smoking on the function of immune system

Pro-inflammatory effects	Immunosuppressive effects
Effects on dentritic cells and antigen-presenting activity	
Activation of dendritic cell-mediated adaptive immunity.	Suppression of dendritic cell maturation and cytokine release.
Action on neutrophils and macrophages	
Increased circulatory levels of polymorphonuclear neutrophils.	Suppression neutrophil-mediated inflammatory actions. Depressed polymorphonuclear neutrophils migration and chemotaxis. Reduced macrophage activity against intracellular organisms.
Action on the T-cell lymphocyte population	
Polyhenol-rich glycoprotein stimulates the proliferation of peripheral T-lymphocytes. Increased circulatory levels of T-lymphocytes. Abnormal CD4+/CD8+ ratio. Favored activity of the Th2 allergic pathway.	Nicotine inhibits the antibody-forming cell response, impairs antigen-mediated signalling in T-cells, and induces T cell anergy.
Action on B cell lymphocyte population	
Augmentation of auto-reactive B cells.	
Effects on humoral immunity	
	Reduced circulating levels of immuno-globulins.
Action on inflammatory markers and mediators	
Chronic smoking increases levels of acute phase proteins and pro-inflammatory cytokines, especially TNF-Ǔ, TNF-Ŭreceptors and IL-6.	Inhibition of IL-1ƃ, IL-2, IL-10, TNF-Ǔ, and IFN-ɔ release. Inhibition of endothelial cell release of IL-8.
Other general non-specific mechanisms	
Exposure and release of autoantibodies. Release of intracellular antigens via tissue hypoxia or toxin-mediated cellular necrosis. Increased free radicals, which interact with DNA.	Attenuation of IFN signalling.

(Adapted from Arnson et al, 2010)

The first observation showing the linkage between smoking and RA was demonstrated by Vessey et al in the 1980s, which was a serendipitous finding from an investigation focusing on the effects of sex hormones in RA (Vessey et al, 1987). Since then, numerous studies have confirmed smoking as the best-known environmental risk factor for RA, in respect of both disease susceptibility and outcome. It has been suggested that both intensity and duration of smoking are directly related to the risk, with prolonged effects (10 ï 20 years) after cessation (Costenbader et al, 2006). In studies evaluating intensity and duration separately, it appears that duration is more important (Stolt et al, 2003; Karlson et al, 1999). However, a cumulative dose of exposure (intensity multiplied by duration) may be the best predictor of the risk.

Smoking has a stronger influence in males than in females, on the development of RA. A recent meta-analysis reviewing this issue has indicated that the risk for male smokers of developing RA is about twice that of male non-smokers. For females, this figure drops to 1.3. However, for heavy smokers (20 pack-years or more), the risk is equally high for both genders (Sugiyama et al, 2009).

Smoking has been identified as a risk factor for seropositive RA. Several studies have even identified an association between smoking and RF in subjects without RA (Hayahara et al, 2010; Jonsson et al, 1998; Mathews et al, 1973; Tuomi et al, 1990), supporting a role for this exposure very early during the development of clinical disease. In this regard, it has been proposed that exposure to tobacco smoke may stimulate RF production, thereby contributing to the clinical onset of RA. Strong association of smoking with anti-CCP has also been found in RA subjects. It appears that the risk for anti-CCP presence positively increases with the number of pack-years of smoking, in particular among individuals

whose exposure of smoking is over 20 pack-years. An interaction between smoking and HLA-DRB1 SE may be involved in the production of anti-CCP, since smoking has been found to be a risk factor for anti-CCP positive RA only in SE positive subjects (Linn-Rasker, 2006). Recently, Mahdi and co-workers have demonstrated that the combined effect of smoking, HLA-DRB1 SE and protein tyrosine phosphatise, non-receptor 22 (PTPN22) is strongly and preferentially associated with the presence of antibodies to the immunodominant citrullinated Ŭ-enolase CEP-1 epitope (anti-CEP-1), a subset of anti-CCP detected in 43 ï 63% of the anti-CCP positive individuals (Mahdi et al, 2009). The literature has generally concluded the existence of association between smoking, RF and anti-CCP. However, most studies are based on white populations. One study indicated that such smoking-autoantibodies association was not seen in African Americans with recent-onset RA (Mikuls et al, 2008).

RA severity can be defined according to the degree of structural damage and to the occurrence of extra-articular manifestations or cardiovascular involvement. The presence of RF and anti-CCP is known to be associated with a more significant radiological progression and structural damage. Ever smokers with RA have a disease characterized by a greater proportion of autoantibody positivity, thereby expecting a more severe radiographic outcome. In the same serological group, however, recent studies conducted in Icelandic and German cohorts with recent-onset RA and the Swiss population-based registry of RA included in the Swiss Clinical Quality Management program for RA (SCOM-RA) all failed to show an influence of smoking on radiographic outcome (Manfredsdottir et al, 2006; Westhoff et al, 2008; Finckh et al, 2007). The Icelandic study suggested that smoking promoted the disease activity but did not significantly increase the structural damage of joints (Manfredsdottir et al, 2006). Meanwhile, the German study

found that although there was no worse joint damage, smokers usually felt worse (Westhoff et al, 2008). Thus, more aggressive treatment and/or better care may be applied amongst smokers in response to high disease activity and more pain, which may mask the negative impact from cigarette smoking.

A smoking involved interaction with glutathione S-transferase mu 1 (GSTM1) polymorphism has been suggested as a predictor of structural damage. Mattey and co-workers indicated that female RA patients who carried the GSTM1-null polymorphism (lack of the locus) and had ever smoked had significantly higher Larsen scores, compared with the remaining combinations. Since GSTM1 gene has the ability to detoxify chemicals and potential carcinogens in cigarette smoke, this observation provided a potential mechanism relative to smoking effects on RA (Mattey et al, 2002).

Smoking seems to contribute significantly to the occurrence of extra-articular manifestations in many ethnic backgrounds including European, African-American and Korean populations. Several studies have seen that smokers, especially with RF positive disease, have an increased risk of rheumatoid nodules (Mattey et al, 2002; Nyhall-Wahlin et al, 2006; Mikuls et al, 2008; Kim et al, 2008). Furthermore, tobacco exposure appears to be associated with ILD in patients with RA (Gochuico et al, 2008; Patel et al, 2008).

The majority of mortality studies concerning RA subjects have identified excess mortality in RA cohorts compared to that in the general population, and many have highlighted RF as a strong predictor. On the other hand, it also has been known that this excess mortality is mainly due to cardiovascular and respiratory diseases, and severe RA itself. Since cigarette smoking is closely linked with production of RF, is associated with heart and lung diseases,

and is probably connected with more severe RA, it should be considered as a risk relative to excess mortality in RA. In respect of the increased death from CVD particularly, study has found that an interaction between smoking, HLA-DRB1 SE alleles and anti-CCP is involved (Farragher et al, 2008).

Smoking status has been associated with RA treatment responsiveness. Patients with RA who smoke show a greater need for DMARDs, with non- and light-smokers having a significantly higher probability of improvement than heavy smokers (20 pack-years or more) (Westhoff et al, 2008). RA patients with a history of smoking have been found to show a poor response to treatment with TNF antagonists, irrespective of smoking status at initiation of anti-TNF therapy (Mattey et al, 2009). This may be due to smoking having a long-term effect on TNF-Ŭ release (Glossop et al, 2006). Most recently, there is a report indicating that smoking is associated with less response to both MTX and anti-TNF treatment, with a significant increased number of non-responders amongst current smokers, compared with non-smokers (Wedren et al, 2009).

1.2.2 Other environmental factors

Although cigarette smoking is the only well-established environmental risk factor for RA, a number of other environmental agents are probably also involved.

Drink and diet

The consumption of coffee has been considered as risk factor for RA. A linkage between caffeine and RF positive RA, but not RF negative RA, has been observed (Heliovaara et al,

2000). Interestingly, decaffeinated coffee has also been associated with an increased risk of RA. The authors suggest that the use of industrial solvents during the decaffeination process may play a role in the disease development (Mikuls et al, 2002). Nevertheless, there is research revealing no association of caffeine/coffee intake with RA (Karlson et al, 2003).Tea has been shown to have a protective effect, which may be due to its anti-inflammatory and antioxidative properties (Mikuls et al, 2002). A decreased rate of RA, benefited by alcohol consumption, has been reported (Pedersen et al, 2006; Källberg et al, 2009).

High consumption of red meat and meat products has been associated with increased risk of inflammatory polyarthritis (Pattison et al, 2004). However, such association was not seen in a more recent study (ito-Garcia et al, 2007). The so-called ñMediterranean dietò has been linked with health benefits for a number of diseases and it is also true for RA (Skoldstam et al, 2003; Linos et al, 1999). Therefore, in order to prevent RA, consumption of olive oil, oil-rich fish, fruit and vegetables, and vitamin D is highly recommended.

Infection

There have been a large number of infectious agents that have been implicated in RA, including Epstein-Barr virus, retroviruses, parvovirus B19, Mycobacterium tuberculosis, Escherichia coli and Proteus mirabilis (Edwards and Cooper, 2005). These associations have been supported by increased antibody titres to the infectious organism being present in RA, or the possibility of molecular mimicry. However, no particular microbe has been directly identified as a causative factor of RA.

Occupational exposure

An increased frequency of RA has been observed in several job categories. Many such occupations involve certain exposures. Studies have consistently reported that exposure of silica is associated with RA (Klockars et al, 1987; Steenland et al, 1995; Steenland et al, 2001; Calvert et al, 2003). Exposure to mineral oil has also been suggested as a risk factor, particularly for the seropositive diseases (Sverdrup et al, 2005). Animal studies have shown that mineral oils can induce polyarthritis in rat, and it has been suggested that they may act as so-called adjuvants enhancing immune reactions (Kleinau et al, 1994; Sverdrup et al, 1998; Svelander et al, 2001). A similar mode of action has been introduced for silica as well (Parks et al, 1999). Other occupational exposures associated with RA include organic solvents (Lundberg et al, 1994), asphalt and flour (Olsson et al, 2004).

Non-genetic host factor: hormonal and reproductive factor

Like most autoimmune disorders, RA has a predisposition for females, which has led to considerable effort on examining the role of hormonal and reproductive factors in disease occurrence/outcome. It has been hypothesized, based on these trends in incidence, that female sex hormones may be involved in the RA development, whereas androgens may play a suppressive role (Da Silva and Hall, 1992). Several observations have supported this hypothesis. For example, pregnancy is associated with a period of remission in a substantial proportion of female patients with RA, whereas the postpartum period and breastfeeding after a first pregnancy are associated with increased risk of the disease (Silman and Pearson, 2002). Furthermore, there is evidence suggesting that the use of oral contraceptives is associated with reduced incidence and severity of RA (Brennan et al,

1997; Jorgensen et al, 1996). In addition, it is worth mentioning that, in men, male sex hormones, particularly testosterone, are lower in patients with RA compared to non-RA subjects (Silman and Pearson, 2002). Although much effort has been devoted to the elucidation of hormonal pathways in the development of RA, little has been discovered.

Environmental and non-genetic host factor: other

Other non-genetic risk factors that have been associated with the development of RA include blood transfusion, obesity (Symmons et al, 1997), high birth weight (Mandl et al, 2009) and stress (Cutolo and Straub, 2006). Low socioeconomic status has been associated with RF positive disease (Bengtsson et al, 2005; Pedersen et al, 2006). Furthermore, a sexually transmitted agent with a higher male-to-female than female-to-male transmission rate might theoretically explain the female predominance in RA, but only a few studies have examined sexual behaviour and venereal diseases as possible risk factors (Yli-Kerttula et al, 1985; Drevlow et al, 1996).

1.3 GENETIC INVOLVEMENT IN RA

1.3.1 Identification of genetic risk

The early evidence for a genetic component to RA was derived from familial and twin studies. In certain families, the incidence of RA is increased compared to the general population. It was observed that first- and second-degree relatives of a RA patient have an increased risk of RA (Grant et al, 2001). Twin studies provide best estimates of heritability. The monozygotic twin concordance rate for RA is about 4 times greater than the dizygotic

twin concordance rate, suggesting a heritability of 50 ï 60% (Lawrence, 1969; Aho et al, 1986; Silman et al, 1993). However, the absence of complete concordance indicates that RA aetiology is not solely genetic. Also, among individuals the content of genetics contributing to RA may vary from one to another.

Currently, over 40 genetic loci have been convincingly associated with risk for RA. Apart from the HLA locus, the majority of the susceptibility genes have been identified in the last 5 years, thanks to the rapidly advancing genotyping technologies and genome-wide association studies (GWASs). However, the function and role in disease pathogenesis of the newly defined risk genes are largely unknown. A large proportion of genetic risk for RA still remains to be identified. Some of this ñmissing heritabilityò will likely be due to rare genetic variation (Gregersen 2010), thus larger sample size and extensive resequencing in future GWAS is required. Since the genetic regulation of a gene is complex, it is not easy to identify the actual causative variation (or combination of variants) responsible for an observed association. In addition, interaction between genes may play an important role relative to a disease phenotype. Indeed, it is still a very long way to go before we understand the genetic influence on RA.

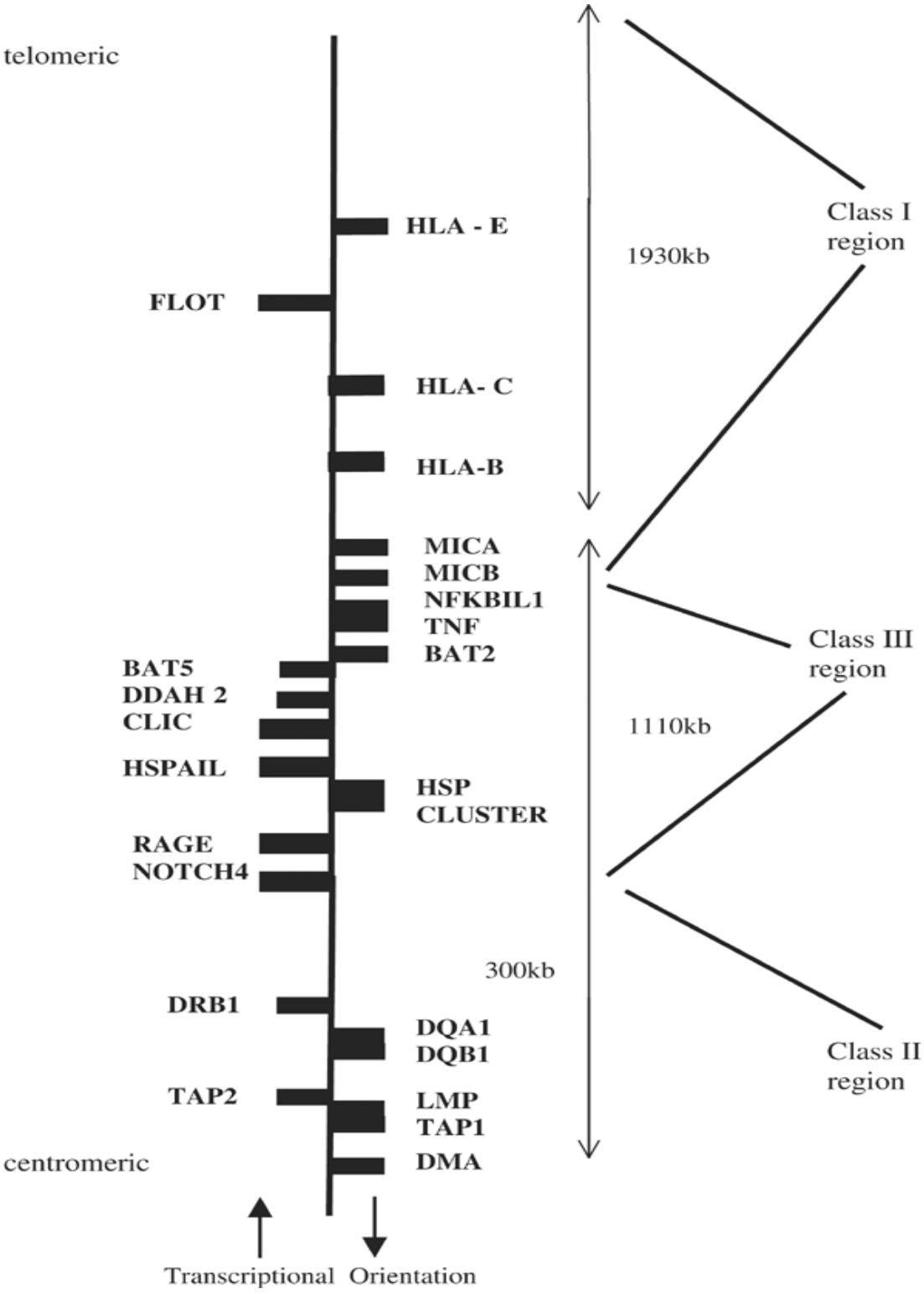
1.3.2 The major histocompatibility complex

The discovery of the major histocompatibility complex (MHC) was a significant breakthrough in the understanding of the immune response and related biological processes. It was first identified and named precisely after its significant role in transplant rejection between mice from different endogamic strains. The MHC is a complex of numerous genes that encode proteins important in the assembly of antigen-presenting

MHC molecules, the processing of peptide antigens, and the presentation of these to T lymphocytes. It also houses genes for a number of other proteins that have essential roles in the immune and inflammatory responses. Therefore, the MHC is critical for the ability of the host immune system to recognise self from non-self and to elicit the appropriate immune response in the latter case.

In humans, the MHC is also referred to as the HLA. It is located on chromosome 6 (6p21.3), and extends over 3.6Mb between flanking genetic markers myelin oligodendrocyte glycoprotein (MOG) and collagen alpha-2(XI) chain (COL11A2). It is the most gene dense region and a highly polymorphic region. The HLA is divided into three subgroups, class I, class II and class III. The class I region, at the telomeric end of the HLA, contains the HLA class I genes, HLA-A, -B, -C, ï E, -F and -G. In the class II region there are the HLA-DR, -DP, -DQ, -DM and -DO loci. The class III region lies between the class I and II regions.

Figure 1.3.2a Genetic location and organization of the MHC, termed the HLA in humans



The class I F and G sub-region, and the class II DP and DO are not displayed.

(Figure reproduced from Newton et al, 2004)

The class I and II region house the HLA genes mainly responsible for production of the cell-surface proteins (classic HLA molecules) that present antigens to T lymphocytes. For example, the HLA-A, -B and -C genes code for the Ŭ chain of the HLA class I molecule whose function consists in antigen presentation to the T8 lymphocytes, and genes of the HLA-DR, -DP and -DQ loci code for components of the HLA class II molecule whose role is to present peptides to T4 lymphocytes. However, the HLA-E, -F and -G genes in class I or the HLA-DM and -DO loci of class II do not contribute to produce classic HLA molecules. Instead, the HLA-E, -F and -G genes code for non-classic molecules that have functions interacted with inhibitory receptors in nature killer cells, whereas the molecules encoded by HLA-DM and -DO are intracellular proteins. The class III region does not house HLA genes but contains numerous individual genes encoding for other immune components such as complement components (e.g. C2, C4, factor B), cytokines (e.g. TNF-Ŭ, LTA, LTB) and heat shock protein 70 (Hsp70) (Milner and Campbell, 2001).

Association with RA susceptibility

Since RA is characterised by auto-immunity and pronounced inflammatory response, analysis of genes involving in the immune- and inflammation-related pathways is of most value for identifying genetic aetiological and pathogenetic factors. Accordingly, the HLA has become the most thoroughly investigated genetic locus in RA because of a significant proportion of its 200+ genes that function in the immune system.

In the early 1970s, weak and variable associations between RA and HLA class I region genes were described using the then available serological regents. When techniques became available for testing HLA class II antigens, an association of RA with HLA-Dw4

was firstly reported in 1976, using mixed lymphocyte culture (MLC) (Stastny, 1976). Thereafter, a serologically defined marker, HLA-DR4, was found to be association with RA. Combining MLC and serological techniques distilled the RA association to a few specific alleles, namely DR4 Dw4, DR4 Dw14.1, DR4 Dw14.2 and DR5 Dw15. In 1990s, various deoxyribonucleic acid (DNA)-based techniques started to provide much greater precision in studies, thus the MLC and serological techniques were superseded. It was recognized that the MLC and serological markers listed above represented alleles in the HLA-DRB1 locus. These alleles are subsequently renamed as HLA-DRB1*0401, *0404, *0408 and *0405 respectively (Hochberg et al, 2008).

The development of higher resolution HLA-DRB1 genotyping led to the demonstration that different HLA-DRB1 alleles are not equally associated with RA. Some alleles, such as DRB1*0401 and *0404, appear to confer a much greater degree of risk than others, for example *0101. In particular, certain combination, such as DRB1*0401/*0404, appear to confer an especially high risk (Wordsworth et al, 1992; MacGregor et al, 1995). There is also demonstration that not all HLA-DRB1 specificities are associated with the development of RA. In contrast, there are alleles that are negatively associated with RA and therefore provide a protective role. These alleles include DRB1*0103, *0402, *0802 and *1302 (Milicic et al, 2002).

In different ethnic groups the predominant RA-associated alleles vary considerably: *0401 and *0404 are the predominant RA risk alleles in Caucasians, *0405 in eastern Asians, *1402 in native American Indians, and *0101 and *1001 in Israeli Jews, Greeks and Spaniards (Hochberg et al, 2008). Association of RA with DRB1*09 also has been reported

in Koreans, Chileans, Japanese and UK Caucasians (Lee et al, 2004; Gonzalez et al, 1992; Wakitana et al, 1998; Milicic et al, 2002).

The shared epitope hypothesis

Gregersen and co-workers first reported a unifying hypothesis for the association of different HLA-DRB1 specificities associated with RA, termed the SE hypothesis. They demonstrated that RA was associated with specific DRB1 alleles that encode a conserved sequence of amino acids (QRRAA, RRRAA or QKRAA) at residues 70 ï 74 in the third hyper variable region (HVR3) of the DRß1 chain. These residues constitute an Ŭ helical domain forming one side of the antigen binding site, a site likely to affect antigen presentation (Gregersen et al, 1987). The alleles carrying the SE sequence are DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1402, *1001, *09, etc. More recently, Tezena and co-workers introduced a revision of the SE hypothesis that the amino acid sequence RAA at 72 ï 74 residues was the key region in increasing susceptibility to RA, whereas the amino acid sequence at positions 70 and 71 was a modulator for the RAA sequence (Tezena et al, 2005). Mattey and co-workers developed another revised system focusing on the function of amino acid residue encoded at position 70. It was demonstrated that the presence of SE-containing alleles encoding a glutamic acid at position 70 conferred the greatest risk of RA. Alternatively, 2 non-SE alleles encoding an aspartic acid at position 70 were associated with the lowest risk. The results suggest that the strength of the association of DRB1 genotypes with RA is dependent not only on SE status but also on which amino acid residues are encoded at position 70, and that the presence of an aspartic acid residue at position 70 protects against development of RA (Mattey et al, 2001). The alleles encoding

an aspartic acid at position 70 are DRB1*0103, *0402, *0802, *1302, *1102, *1103, *1201, etc.

The function of the HLA-DR molecule is to bind peptide antigens for presentation to T lymphocytes. The SE motif constitutes a helical domain forming one side of the antigen-binding site, which suggests functional importance of the SE and an ability to influence the nature of potentially arthritogenic peptides. Although it is not clear how SE works in RA, there are 3 models that have been proposed. The first model is based on T cell selection. HLA molecules are very important in the selection and establishment of the antigen-specific T cell repertoire. Interactions with HLA molecules in the self-peptide context instruct the immature T cell to differentiate between self and non-self. Normally, T cells that characterize self-reactivity are deleted or inactivated, while others are positively selected to establish capability of recognizing foreign antigens. However, it is thought that in some way the SE assists the positive selection of auto-reactive T cells, and consequently breaks self-tolerance. Thus, the immune system reacts against the bodyôs own constituent parts. The second model is based on the principle that polymorphic determinants in the HLA molecule on antigen-presenting cells (APC) determine the binding and presentation of the antigenic peptide. A potential arthitogenic peptide, for example from the joint, might be targeted, and presented to mature T cell by these óSE-determinedô APC. However, to date, no peptide related to RA has been identified. In the third model, a mechanism called ómolecular mimicryô may occur in which similarities between the SE and foreign protein might break self-tolerance and generate auto-reactive T cell clones that eventually lead to RA (Hochberg et al, 2008).

Despite incomplete details, the SE theory reasonably explains a possible mechanism in the pathogenesis of RA related to the HLA region genes. However, there are still a number of observations that do not fit comfortably with this theory. For example, in African-Americans the SE was shown to have no significant association with RA (Alarcon et al, 1983; Karr et al, 1980), and in Arabs RA is associated with DR markers that do not possess the SE (Sattar et al, 1990 (1, 2)). The presence of the SE is neither necessary nor sufficient for disease to occur. Although the relative risk (RR) for developing RA in SE positive individuals is high, the absolute risk (AR) however is relatively low. In Newtonôs review, it is summarized that carriage of single DRB1*0401 allele has a RR of 4.7, for single *0404 allele the RR = 5.0, for *0401/*0401 homozygotes the RR = 18.8, and for *0401/*0404 heterozygotes the RR = 31.0. In contrast, the AR for developing RA if carrying an SE-encoding allele is 1/35 for *0401, 1/20 for *0404, 1/80 for *0101 and 1/7 for *0401/*0404 heterozygotes (Newton et al, 2004). Furthermore, modelling of the HLA susceptibility to RA has suggested that there are other susceptibility gene(s) within the HLA region, since simple dominant/additive/recessive model could not explain the inheritance pattern (Génin et al, 1998). Considerable research has gone into trying to identify the further HLA genes which are likely to be involved in RA. The most widely studied loci are HLA-DQ which is next to DRB1, and TNF genes located in the HLA class III region and approximately 1000 kb away from DRB1. However, there is no definitive evidence that alleles in HLA-DQ influence susceptibility to RA, and there is compelling evidence against a direct role for polymorphisms in the TNF gene contributing to RA susceptibility.

Association with RA phenotype

Polymorphisms in the HLA-DRB1 locus have been associated with RA susceptibility, with the SE alleles associated with increased incidence of RA. The SE alleles may also be related to clinical features of RA. Several studies have observed that HLA-DRB1 SE alleles are associated with disease onset at an earlier age in Caucasian RA patients (MacGregor et al, 1995; Wu H et al, 2004; Karlson et al, 2008). Recently, this association was confirmed in Japanese patients (Furuya et al, 2007). HLA-DRB1 SE alleles are associated with autoantibodies in RA, and this has been shown to interact with smoking and the PTPN22 gene (Padyukov et al, 2004; Klareskog et al, 2006; Linn-Rasker et al, 2006; Kallberg et al, 2007; Morgan et al, 2009; Mahdi et al, 2010). There was a further suggestion that the association between SE and RF+ was primarily due to DRB1*0401 allele (Mattey et al, 2002). In addition, a recent study, which analyzed 2,221 single-nucleotide polymorphisms (SNPs) spanning 10.7 Mb from 6p22.2 to 6p21.31 across the HLA, indicated that HLA-DRB1 was the only independent risk locus for ACPA positive RA within the human MHC (Ding et al, 2009).

HLA-DRB1 SE alleles were shown to be associated with erosive status of RA. However, recent evidence indicated that this apparent association may be due to the prior association of DRB1 SE with anti-CCP positivity (Karlson et al, 2008). Mattey and co-workers reported an association of HLA-DRB1 SE alleles with greater radiographic damage in RA patients. Interestingly, this association was only observed in RF- patients, but not in RF+ patients. The authors suggested that this does not necessarily mean that the SE has no influence on RF+ subjects but any effect may be masked by the greater influence of seropositivity, since the study also found RF+ as an independent risk for worse

radiographic outcome (Mattey et al, 2001). Among HLA-DRB1 SE alleles, the association with the risk of developing worse disease of RA seems to vary, with particular combinations being associated with certain disease outcome. For example, *0401/*0404 combination has been reported to be associated with worse clinical outcome (MacGregor et al, 1995). Homozygosity for the *0401 allele has been associated with major organ involvement (Weyand et al, 1992), whereas both homozygosity and heterozygosity of 2 HLA-DRB1 SE alleles have been associated with rheumatoid nodules and vasculitis (Weyand et al, 1992; Perdriger et al, 1997; Mattey et al, 2002; Gorman et al, 2004). A Spanish report recently demonstrated that HLA-DRB1*04 SE alleles were associated with endothelial dysfunction, and thus may predict increased CVD in RA (Gonzalez-Juanatey et al, 2003).

Association with RA mortality

Mortality rate is increased in RA patients compared with the general population. Two reports published in 2007 independently demonstrated that HLA-DRB1 genotypes may be involved. Mattey and co-workers showed that the presence of 2 SE alleles was associated with increased risk of mortality from IHD and malignancy. Analysis of specific SE genotypes revealed that the *0101/*0401 and *0404/*0404 combinations were the strongest predictors of mortality from IHD, while *0101/SE+ was particularly associated with the death from malignancy (Mattey et al, 2007). Gonzalez-Gay and co-workers, in their report, indicated that the presence of single SE was sufficient to be associated with increased cardiovascular mortality. However, the SE was restricted to DRB1*04 alleles, in particular the *0404 allele (Gonzalez-Gay et al, 2007).

1.3.3 Other RA susceptibility genes

Calculations based on monzygotic twin concordance rates and HLA haplotype sharing in affected sibling pairs indicate that HLA genes contribute approximately 30 ï 50% of the total genetic risk for RA (Deighton et al, 1989). This leaves considerable room for other genes that are outside the HLA region to contribute to RA. It is suggested that no other individual region has such a powerful role as the HLA. Thus, it is more likely that the remaining genetic risk is distributed among several loci on different chromosomes. Since the discovery of PADI4 as a susceptibility gene in 2003, over 40 risk genes/loci have been found in recent years, which is a great achievement in this research field despite the fact that the mechanisms behind the association are largely unknown.

Peptidylarginine deiminase 4

The genome-wide linkage study of RA sibling pairs conducted by the European Consortium of RA Families (ECRAF) and Japan found evidence of linkage in the 1p36 locus encompassing all the known genes that encode citrullinating enzymes (Cornelis et al, 1998; Shiozowa et al, 1998). These genes are called PADIs, and there are 4 members in humans. Since the presence of ACPA is the most specific serological marker for RA, PADIs become candidate genes to be analysed for their association with RA. A Japanese case-control study first reported that functional haplotypes in PADI4 gene were associated with susceptibility to RA, whereas no polymorphisms in the other PADI genes were associated (Suzuki et al, 2003). Thereafter, similar results were consistently observed in other eastern Asian populations such as Koreans and Chinese (Kang et al, 2006; Fan et al 2008). However, data based on European populations were conflicting, with positive

results obtained in French and German studies and negative results in British and Spanish studies (Gandjbakhch et al, 2009; Hoppe et al, 2006; Burr et al, 2010; Martinez et al, 2005). Recent meta-analysis suggested that PADI4 was a susceptibility gene in both eastern Asian and Caucasian population, but its role in eastern Asian populations is much more significant (Lee et al; 2007; Iwamoto et al, 2006). Most recently, a report using both Japanese and Dutch samples and in consideration of sex and smoking status indicated that PADI4 polymorphism highly predisposes male smokers to RA, and that the genetic heterogeneity observed between Asian and European populations may be partly explained by differences in smoking prevalence among men (Kochi et al, 2010). The relationship of PADI4 polymorphisms with the presence of anti-CCP and disease severity (erosive status or functional outcome) has been investigated, with negative results in three reports (Barton et al, 2005; Suzuki et al, 2006; Nishimoto et al, 2008). However, in a study investigating the relationships between PADI4 polymorphism, anti-PADI4 autoantibodies and joint destruction, it was shown that PADI4 polymorphism was associated with anti-PADI4 autoantibodies, and these autobodies were associated with severe joint destruction (Harris et al, 2008).

Cytotoxic T lymphocyte-associated antigen 4

T cells play a major role in the pathogenesis of RA. Based on the success of anti-T-cell therapy on animal models and association with HLA genes, it has been proposed that RA is a T-cell-mediated autoimmune disease (Panayi et al, 1992). Genes involved in the regulation of T cell responses are, therefore, strong candidate genes for RA susceptibility. Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) (located at 2q33), also known as CD152, is a T cell negative regulator. Genetic polymorphism within this gene has been

found to be associated with a variety of T-cell-mediated disorders (Kristiansen et al, 2000). A recently described CT60 A/G dimorphism (rs3087243) has been reported to associated with a variety of autoimmune diseases, with the CT60 A allele being protective and the G allele increasing susceptibility (Ueda et al, 2003; Torres et al, 2004). The G allele of CT60 was associated with lower messenger ribonucleic acid (mRNA) levels of soluble CTLA4 isoform, which could increase T cell activation and, therefore might have an important role in determining susceptibility to autoimmune diseases (Ueda et al, 2003).

The first evidence of association between CTLA4 polymorphism and RA appeared in a Japanese study. However, this observed association was restricted to patients carrying the susceptible HLA allele (HLA-DRB1*0405) (Yanagawa et al, 2000). In 2005, CTLA4 as an independent susceptibility gene to RA was established by several Chinese studies (Lee et al, 2003; Cai et al, 2005; Han et al, 2005). The association has been also observed in Caucasian populations in larger studies (Plant et al, 2010; van der Helm-van Mil et al, 2010), implying the influence may be weaker in Caucasians. No report relative to the severity of RA has been published.

Signal transducer and activator of transcription 4

The North American RA Consortium (NARAC) recently reported a new RA linkage region at chromosome 2q33 (Amos et al, 2006). Gregersenôs group then tested SNPs in and around 13 candidate genes within the linked chromosome 2q region for association with RA. A variant allele (rs7574865) of signal transducer and activator of transcription 4 (STAT4), located on the third intron of this gene, was found to be associated with RA within this region in both white American and Korean populations (Remmers et al, 2007;

Lee et al, 2007). STAT4 encodes a transcription factor that transmits signals induced by several key cytokines including IL-12, IL-23 and type 1 interferons. It is a latent cytosolic factor that, after activation by cytokines, is phosphorylated and accumulates in the nucleus. Activated STAT4 stimulates transcription of specific genes including IFN-ɔ, a key indictor of T cells differentiation into Th1 cells. Thus, STAT4-dependent signalling plays a very important role in the development of Th1 cell response (Remmers et al, 2007). Variant STAT4, in this signalling pathway, may lead to an alterative T cell response, ultimately resulting in RA. To date, numerous publications have demonstrated that STAT4 is a consistent RA risk gene across Whites and eastern Asians (Kobayashi et al, 2008; Orozco et al, 2008; Zervou et al, 2008; Lee et al, 2010; Kelley et al, 2010; Morgan et al, 2010; van der Helm-van Mil et al, 2010; Plant et al, 2010), except for one suggesting that this gene is only associated with the presence of RF, but not with the development of disease in Han Chinese (Li et al, 2009). Furthermore, STAT4 has been also associated with RA in Colombians (Palomino-Morales et al, 2008). In contrast, in Black Americans, no such relationship has been seen (Kelley et al, 2010). No reports regarding the relationship with disease activity/severity have been published.

Oligodendrocyte lineage transcription factor 3-TNF-α-induced protein 3

The first published case-control GWAS, analyzing the genetics of seven complex human diseases, identified a moderate association of 6q23 region with susceptibility to RA (Wellcome Trust Case Control Consortium, 2007). Subsequently, this candidate region was re-genotyped in a larger independent UK cohort of 5063 cases and 3849 controls. Strong evidence of association was detected with SNP (rs6920220) in this region (Thomson et al, 2007). Independently, a US study reported that there was another SNP (rs10499194) at

6q23 associated with RA (Plenge et al, 2007). The associations involving these 2 SNPs were independent of each other, and of HLA-DRB1 and PTPN22 genes (Thomson et al, 2007; Plenge et al, 2007).

The 6q23 is an intergenic region flanked by oligodendrocyte lineage transcription factor 3 (OLIG3) and TNF-Ŭ-induced protein 3 (TNFAIP3). The TNFAIP3 gene acts as a negative feedback regulator of the transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) in response to TNF- and toll-like receptor-, but not IL-1ɓ-induced activation (Boone et al, 2004; Wertz et al, 2004). It is now considered as a central regulator of immunopathology (Vereecke et al, 2009; Criswell 2010). It is possible that genetic variants could influence the function of the adjacent TNFAIP3 gene.

To date, the association of genetic variants at 6q23 with RA has been established in European, eastern Asian and African-American populations (Hughes et al, 2010; van der Helm-van Mil et al, 2010; Lodolce et al, 2010; Morgan et al, 2010; Shimane et al, 2010; Stark et al, 2009). Recently, evidence of association between rare polymorphism within the TNFAIP3 gene and RA was seen (Bowes et al, 2010), further supporting TNFAIP3 as the responsible gene. Interestingly, another report suggested that the association signals from 6q23 intergenic region and TNFAIP3 gene locus were interdependent, which may imply a very complex mechanism of gene regulation (Dieguez-Gonzalez et al, 2009). Scherer et al reported that polymorphism in the 6q23 region was associated with the rate of joint destruction in ACPA positive RA (Scherer et al, 2010). SNPs such as rs6920220, rs6933404 and rs6927172 have been shown to influence the transcription of TNFAIP3, with evidence of differential transcription factor binding to rs6927172 alleles (Elsby et al, 2010).TNFAIP3 protein (as known as A20) has been shown to suppress inflammatory

responses and bone destruction in human fibroblast-like synoviocytes and in mice with collagen-induced arthritis (Hah et al, 2010).

TNF receptor-associated factor 1-complement component 5

A SNP (rs3761847) on chromosome 9q33.2 located in linkage disequilibrium (LD) with 2 genes, TNF receptor-associated factor 1 (TRAF1) and complement component 5 (C5), relevant to chronic inflammation was identified to be associated with susceptibility to RA in 2007 (Plenge et al, 2007). Independently, another study identified several other SNPs that were associated with RA in this region, and furthermore provided evidence of association with disease severity measured by radiological damage (Kurreeman et al, 2007). The TRAF1 gene encodes an intracellular protein that mediates signal transduction through TNF receptors 1 and 2, and through CD40. TNF is a critical cytokine in the pathogenesis of RA, and its antagonists are an effective treatment for RA (Elliott et al, 1994; Weinblatt et al, 1999). TRAF1 knockout mice shows exaggerated T cell proliferation and activation in response to TNF, or when stimulated by the T-cell-receptor complex, suggesting that TRAF1 acts as a negative regulator in these signalling pathways (Tsitsikov et al, 2001).

C5 encodes complement component 5. The complement pathway has been implicated in the pathogenesis of RA for over 30 years (Zvaifler, 1973; Cooke et al, 1975). Thus, the causal polymorphism could possibly act either through TRAF1 or C5 gene. To date, several publications have confirmed the association of TRAF1/C5 with susceptibility to RA in Caucasians (Chang et al, 2008; Zervou et al, 2008; Plant et al, 2010; Morgan et al, 2010;Stark et al, 2009; Kurreeman et al, 2008). However, in other populations such as

Koreans and Colombians, the results were negative for such association (Palomino-Morales et al, 2008; Lee et al, 2009; Han et al, 2009). Most recently, it was suggested that SNP markers within the TRAF1/C5 locus could predict erosive disease at year 1 and year 5 in patients with early inflammatory polyarthritis (Plant et al, 2011). In addition, a study has also suggested that this locus is associated with mortality, particularly from malignancy or sepsis, in patients with RA (Panoulas et al, 2009). However, a later investigation found no such associations (van Nies et al, 2010).

Newly identified genes

The number of novel genes/loci susceptibility to RA increased dramatically in the last 3 years, with the great help from GWAS. On average, approximately 10 genes/loci were discovered in each year. The summarized susceptibility genes/loci for RA, if not being described, are listed in Table 1.3.3a. Further effort is needed to validate these associations in larger and different populations, to evaluate the influence of these genes/loci on disease outcome/phenotype, to investigate the biological mechanisms behind them, and to develop the solutions to these genetic deficiencies. Also, it is important to emphasize that the recent GWAS are primarily designed to detect genetic variation that is relatively common (e.g. frequency Ó 5%), thus rare polymorphism, that may be also importantly involved, is missed. Recently, Surolia et al reported that rare genetic variants causing a functionally defective enzyme of sialic acid acetylesterase represent a strong association with susceptibility in common autoimmune disorders including RA (Surolia et al, 2010).

Table 1.3.3a Summary of novel identified susceptibility genes to RA*

Location	Gene	Variant	Population	Reference
4q27	IL2-IL21	rs13119723, rs6822844	European; Argentina	Stahl et al, 2010; Maiti et al, 2010
6p21.32	HLA-DQA1-A2	rs6457617	European	Julia et al, 2008
20q13.12	CD40	rs4810485	European	Raychaudhuri et al, 2008; Stahl et al, 2010; Plant et al, 2010; Orozco et al, 2010
9p13.3	CCL21	rs2812378, rs951005	European; Asian	Raychaudhuri et al, 2008; Stahl et al, 2010; Freudenberg et al, 2011; Orozco et al, 2010
1q23.1	CD244	rs3766379, rs6682654	Asian	Suzuki et al, 2008
12q13.3	KIF5A-PIP4K2C	rs1678542	European	Raychaudhuri et al, 2008; Plant et al, 2010
10p15.1	IL2RA	rs706778, rs2104286	European	Stahl et al, 2010; Kurreeman et al, 2009
10p15.1	PRKCQ	rs4750316	European	Raychaudhuri et al, 2008; Stahl et al, 2010
22q13	IL2RB	rs743777	European	Kurreeman et al, 2009
2q11.2	AFF3	rs11676922, rs10865035, rs1160542	European; Asian	Stahl et al, 2010; Plant et al, 2010; Freudenberg et al, 2011
1p36.32	MMEL1-TNFRSF14	rs3890745	European	Raychaudhuri et al, 2008; Stahl et al, 2010
2p16.1	REL	rs13017599, rs13031237	European	Stahl et al, 2010; Gregersen et al, 2009; Eyre et al, 2010
8p23.1	BLK	rs2736340	European; Asian	Gregersen et al, 2009; Freudenberg et al, 2011
6q25.3	TAGAP	rs394581	European	Raychaudhuri et al, 2009
2q33	CD28	rs1980422	European	Raychaudhuri et al, 2009
11p12	TRAF6-RAG1	rs540386	European	Raychaudhuri et al, 2009
1q31	PTPRC	rs10919563	European	Raychaudhuri et al, 2009
1q23	FCGR2A	rs12746613	European	Raychaudhuri et al, 2009

6q21	PRDM1	rs548234	European	Raychaudhuri et al, 2009
1p13	CD2-CD58	rs11586238	European	Raychaudhuri et al, 2009
6p12	IL-17A	rs2275913	European	Nordang et al, 2009
1q31	IL-10	rs1800872	Asian	Ying et al, 2011
11q22.2	IL-18	rs1946518	Asian	Ying et al, 2011
11q24	SIAE	Variants causing defective enzyme	European	Surolia et al, 2010
2p14	SPRED2	rs934734	European	Stahl et al, 2010
4p15.2	RBPJ	rs874040	European	Stahl et al, 2010
6q27	CCR6	rs3093023, rs3093024	European; Asian	Stahl et al, 2010; Kochi et al, 2010
7q32.1	IRF5	rs10488631, rs3757385, rs2004640, rs10954213	European	Stahl et al, 2010; Dawidowicz et al, 2011
3p14.3	PXK	rs13315591	European	Stahl et al, 2010
5q11.2	ANKRD55-IL6ST	rs6859219	European	Stahl et al, 2010
18q23	SALL3	rs2002842	European	Julia et al, 2008
7q21.2	CDK6	rs42041	European	Raychaudhuri et al, 2008
14q24.3	BATF	rs7155603	European	Stahl et al, 2010
5q21.1	C5orf30	rs26232	European	Stahl et al, 2010
1q24.2	CD247	rs840016	European	Stahl et al, 2010
17q12	IKZF3	rs2872507	European	Stahl et al, 2010
15q23	KIF3	rs17374222	European	Stahl et al, 2010
1p34.3	POU3F1	rs12131057	European	Stahl et al, 2010
12q24.12	SH2B3	rs3184504	European	Stahl et al, 2010
21q22.3	UBASH3A	rs11203203	European	Stahl et al, 2010
1q21	FCRL3	rs7528684	Asian	Lee et al, 2010; Wu et al, 2010
11q24	FLI1	NA	Asian	Freudenberg et al, 2011
3p21	ARHGEF3	NA	Asian	Freudenberg et al, 2011
5q33.1	LCP2	NA	Asian	Freudenberg et al, 2011
1q42	GPR137B	NA	Asian	Freudenberg et al, 2011
12q15	TRHDE	NA	Asian	Freudenberg et al, 2011
6q14	CGA1	NA	Asian	Freudenberg et al, 2011

* Genes/loci described elsewhere in the present thesis are not included; NA, not available.

1.3.4 Genes studied in the present research

Glutathione S-transferase

Living organisms are continuously exposed to foreign chemical species. These xenobiotics may interact deleteriously with an organism, causing toxic effects (Ames et al, 1990). Along with strategies such as sequestration, scavenging and binding, catalytic biotransformation evolved as a significant biochemical protection mechanism against toxic chemical species. This enzymic detoxification of xenobiotics has been classified into 3 stages which act in a tightly integrated manner. Phase 1 and 2 involve the conversion of a lipophilic, non-polar xenobiotic into a more water-soluble and therefore less toxic metabolite. In phase 3, these water-soluble metabolites can then be removed more easily from the cells (Sheehan et al, 2001). The GSTs are major phase 2 detoxification enzymes found mainly in the cytosol. They catalyse the conjugation of activated xenobiotics to an endogenous water-soluble substrate, glutathione (GSH) (in reduced form). Importantly, reduced GSH is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species (ROS) such as free radicals and peroxides (Pompella et al, 2003). The GSTs, thus, are thought to be important in the cell detoxification process.

The GSTs comprise a complex and widespread enzyme family that has been subdivided further into an ever-increasing number of classes based on a variety of criteria, including amino acid/nucleotide sequence, and immunological, kinetic and tertiary/quaternary structural properties (Sheehan et al, 2001). To date, in humans, the GST gene family comprises 21 genes in 8 classes (Table 1.3.4a). Polymorphisms in these genes have been

identified. Notably, the null alleles (detection of the gene sequence) have been observed and are common in the GST mu 1 (GSTM1) and GST theta 1 (GSTT1) genes. Homozygous detections of these genes result in a complete loss of enzyme function, which has been implicated in the predisposition of certain individuals to increased risk from toxic xenobiotics (Strange et al, 2000; Hayes and Strange, 2000; Pemble et al, 1994). Several human diseases have been linked with these mutations such as a number of cancers (Sheehan et al, 2001), Parkinsonôs disease, Alzheimerôs disease, atherosclerosis, liver cirrhosis and cataract formation (Dourado et al, 2008).

Table 1.3.4a A list of human GSTs

Class	Members
alpha	GSTA1, GSTA2, GSTA3, GSTA4, GSTA5
kappa	GSTK1
mu	GSTM1*, GSTM1L, GSTM2, GSTM3, GSTM4, GSTM5
omega	GSTO1, GSTO2
pi	GSTP1
theta	GSTT1[A], GSTT2
zeta	GSTZ1
microsomal	MGST1, MGST2, MGST3

* The gene coding for GSTM1 is located on chromosome 1p13.3, within a gene cluster coding for the mu class of enzymes; [A]The gene coding for GSTT1 is located on chromosome 22q11.23, and is approximately 50kb away from the GSTT2 gene.

Vascular endothelial growth factor

VEGF, also known as vascular permeability factor (VPF), is a sub-family of the platelet-derived growth factor family of cystine-knot growth factors. The most important member

is VEGF-A, normally called just VEGF. Other members (in humans) include placenta growth factor (PGF), VEGF-B, VEGF-C and VEGF-D.

VEGF-A is a fundamental promoter of normal and abnormal angiogenesis. It has been shown to stimulate endothelial cell mitogenesis and cell migration (Ferrara and Henzel, 1989; Plouet et al, 1989). It is also a vasodilator and increases microvascular permeability (Ferrara, 1999). In normal condition, the function of VEGF-A is to create new blood vessels during embryonic development, new blood vessels after injury, new vessels in muscle following exercise, new vessels (collateral circulation) to bypass blocked vessels, etc. Overall, it is to restore the oxygen supply to tissues when blood circulation is inadequate. In diseases, however, it contributes to pathological processes. For example, solid cancers cannot grow beyond a limited size without an adequate blood supply, whereas those that can express VEGF-A are able to grow and metastasize.

The members of VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (also called the VEGFRs, containing 3 types: VEGFR-1, -2 and -3) on the cell surface, causing them to dimerize and become activated through transphosphorylation. The VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain. VEGF-A binds to VEGFR-1 and -2. VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF-A, while the function of VEGFR-1 is less well-defined (Holmes et al, 2007).

The human VEGFA gene is localized on chromosome 6p12, and is organized in 8 exons (Wei et al, 1996). At least 6 different isoforms of VEGF-A are generated by alternative

splicing of the VEGFA gene (Ferrara et al, 2003). VEGFA is regulated by a variety of growth factors, cytokines, hormones and hypoxia (Plouet et al, 1989; Klagsbrum and DôAmore, 1996), and is produced by numerous cell types including macrophages, keratinocytes, T cells, and mesangial and smooth muscle cells as well as by various tumour cells (Berse et al, 1992; Iijima et al, 1993; Freeman et al, 1995). Polymorphisms within the VEGFA gene have been identified, and have been reported to be associated with many disorders where angiogenesis may play important role in disease development, including the development of atherosclerosis (Howell et al, 2005).

Nitric oxide synthase

Nitric oxide synthases (NOSs) are a family of enzymes that catalyze the production of NO from L-arginine and molecular oxygen (Wang et al, 1995). NO is an important cellular signalling molecule, having a vital role in many biological processes such as the control of vascular tone and the development of nervous system.

Different members (isoforms) of the NOS family are encoded by separate genes (Taylor et al, 1997). In mammals, there are 3 isozymes, including neuronal NOS (nNOS), cytokine-inducible NOS (iNOS) and endothelial NOS (eNOS). The former 2 are soluble and found predominantly in the cytosol, while eNOS is a membrane-associated protein located at plasma membranes surrounding cells and the membranes of Golgi bodies within cells. In the endothelial cell, NO is synthesised by eNOS. Circulating NO is primarily produced by eNOS and it contributes to the control of vascular tone and blood flow, insulin secretion, airway tone, and the regulation of cardiac function and angiogenesis (Quyyumi et al, 1995; Nava et al, 1995; Nava et al, 1995). The gene encoding for eNOS is mapped on

chromosome 7q36. Polymorphisms in this gene have been suggested to be associated with several conditions, notably the cardiovascular disorders (Gulec et al, 2008; Velloso et al, 2010; Cam et al, 2005; Casas et al, 2004; Aroon et al, 1998; Hibi et al, 1998; Shimasaki et al, 1998).

Matrix metalloproteinases

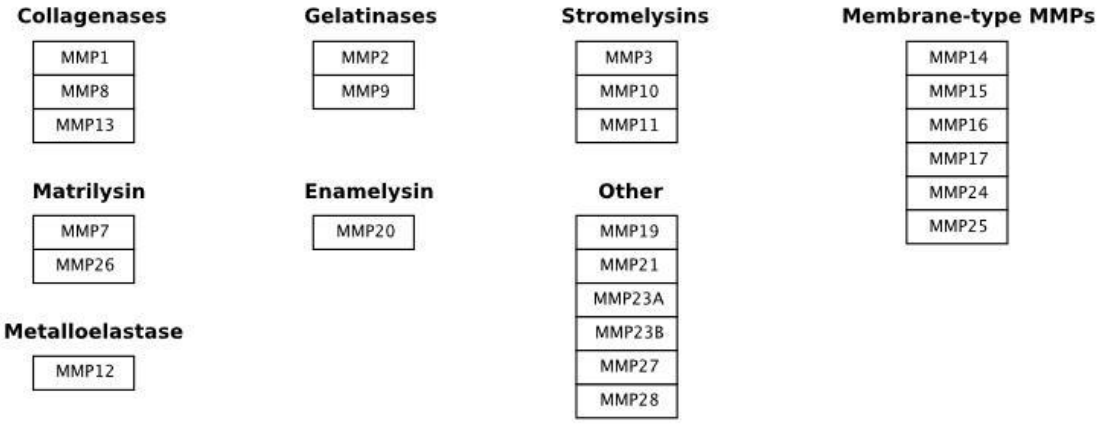
MMPs are a group of zinc- and calcium-dependent proteases that contribute to tissue remodelling and repair during development, morphogenesis, inflammation, etc. They act by specifically degrading all of the components of the ECM, which serves as a scaffold for tissue formation. These proteins also process a number of bioactive molecules, known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (e.g. Fas ligand), and chemokine/cytokine (in)activation (van Lint and Libert, 2007). In addition, they are thought to play a major role in cell behaviour such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defence.

There are at least 24 members of the MMP family in humans, classified into several subgroups according to the substrate specificity or the cellular localization (Figure 1.3.4a). All the MMPs share a common 3-domain structure. It contains the pro-peptide, the catalytic domain, and the haemopexin-like C-terminal domain which is linked to the catalytic domain by a flexible hinge region. The MMPs are initially synthesized as inactive zymogens with a pro-peptide domain that must be removed before the enzyme is active (Pei et al, 2000).

In the synovial joint, MMPs are mainly secreted by fibroblasts, macrophages and chondrocytes. Much of the joint damage in RA is believed to be mediated by abnormal release of MMPs in rheumatoid synovium stimulated by persistent inflammation (MacNaul et al, 1990; Konttinen et al, 1991; Gravallese et al, 1991; Konttinen et al, 1999; Cunnane et al, 2001). MMP-1 (collagenase-1) and MMP-3 (stromelysin-1) are the most widely studied members in RA. MMP-1 is specifically responsible for degradation of type I collagen, a major component of the ECM, as well as other fibrillar collagens such as types II, III, V, IX and X. MMP-3 has a broader substrate specificity with activity against type II, III, IV, IX, X and XI collagens, proteoglycans, fibronectin and laminin. Furthermore, it is able to activate other MMPs such as MMP-1, -2, -9 and -13. Elevated levels of MMP-1 and -3 have been observed in patients with RA in both serum and synovial fluid, and are strongly associated with inflammation and joint destruction (Walakovits et al, 1992; Taylor et al, 1994; Sasaki et al, 1994; Keyszer et al, 1999; So et al, 1999; Cheung et al, 2000; Yamanaka et al, 2000; Cunnane et al, 2001; Klimiuk et al, 2002; Green et al, 2003; Tchetverikov et al, 2004; Fiedorczyk et al, 2006; Ates et al, 2007; Shinozaki et al, 2007).

The genes for MMP1 and 3 are both located on the long arm of chromosome 11 (11q22.3), in a cluster together with seven other MMP members, including MMP7, 8, 10, 12 and 13. Polymorphisms (MMP1 1G/2G and MMP3 5A/6A SNPs) within these genes have been associated with the outcome of RA (Constantin et al, 2002(1); Constantin et al, 2002(2); Lee et al, 2003; Mattey et al, 2004; Dörr et al, 2004; Nemec er al, 2007; Ye et al, 2007; Tsukahara et al, 2008; Scherer et al, 2010). However, the results between studies are conflicting.

Figure 1.3.4a Human MMPs classified into subgroups according to the substrate specificity or the cellular location



Transforming growth factor beta

TGF-ϐ is a protein that controls cell proliferation, cell differentiation, cell motility, apoptosis and other functions in most cells (Blobe et al, 2000). It is a secreted protein that exists in at least 5 isoforms called TGF-ϐ1, TGF-ϐ2 and TGF-ϐ3. TGF-ϐ1, which contains 1 isoform, is encoded by the TGFB1 gene, while TGF-ϐ2 and TGFï ϐ3 each including 2 isoforms are created by alterative splicing of the TGFB2 and TGFB3 genes respectively. The TGF-ϐ family is part of a superfamily of proteins known as the transforming growth factor beta superfamily, which includes inhibins, activin, anti-müllerian hormone, bone morphogenetic protein, decapentaplegic and Vg-1 (Herpin et al, 2004).

The peptide structures of the 3 members of the TGF-ϐ family are highly similar. They are all encoded as large protein precursors, with TGF-ϐ1 containing 390 amino acids and the other ones each containing 412 amino acids. They each have an N-terminal signal peptide of 20 ï 30 amino acids that they require for secretion from a cell, a pro-region (called latency associated peptide) and a 112 ï 114 amino acid (~ 25 kilodaltons) C-terminal

region that becomes the mature TGF-ϐ molecule following its release from the pro-region by proteolytic cleavage (Khalil, 1999; Derynck et al, 1985; Herpin et al, 2004).

TGF-ϐ1 is the founding and the most important member of this family. It is associated with almost all the functions in which TGF-ϐ involves, including cell proliferation, differentiation, motility and apoptosis. The TGF-ϐ1 protein is found throughout the body, and plays roles in development before birth, the formation of blood vessels, the regulations of muscle tissue, body fat development and bone growth, wound healing, immune system functions, etc (Blobe et al, 2000). In humans, the gene for TGF-ϐ1 (TGFB1) is located on the long arm of chromosome 19 at position 13.2 (Ghadami et al, 2000). Polymorphisms within the gene have been identified. It has been suggested that these mutations may be associated with the differential expression/activation of this cytokine, and therefore with diseases, such as heart disease, cancer and diabetes.

Protein tyrosine phosphatase, non-receptor type 22

PTPN22, in humans, is encoded by the gene located on the short arm of chromosome 1 near the telomere (1p13.2). The PTPN22 gene is 57,898 bases in length and encodes a protein of 807 amino acids. There are 24 exons in the gene, and alternative splicing of this gene results in 2 transcript variants encoding distinct isoforms. The encoded protein is a lymphoid-specific intracellular phosphatase that associates with the molecular adapter protein casitas B-lineage lymphoma (CBL), and may be involved in regulating CBL function in the T cell receptor signalling pathway (Gregersen 2005). Mutations in this gene may be associated with a range of autoimmune disorders, including RA, systemic lupus

erythematosus, autoimmune thyroid disease, type 1 diabetes mellitus and Gravesô disease (Matthews et al, 1992; Gregorieff et al, 1998; Cohen et al, 1999).

CHAPTER 2 OBJECTIVES

2.1 RESEARCH OBJECTIVES, AIMS AND REASONING

2.1.1 Objectives, aims and reasoning

RA is a chronic condition which has a prevalence of ~ 1% of the population in industrialised countries. It is affected by both genetic and environmental factors, and has considerable association with permanent disability, morbidity and mortality. The preceding literature in the field of genetic epidemiology mainly investigated the genetic susceptibility to RA. Recently, in case-control designed GWAS, a great number of new genetic loci associated with the occurrence of RA were detected. However, genes associated with disease outcome and disease co-morbidity were less studied. Understanding of both kinds of genetic association is essential to explore the reasons for disease. However, identification of a gene associated with disease severity/co-morbidity is more useful in terms of personalized therapeutic care for RA patients. Susceptibility genes are not necessarily the genes associated with disease outcome, although HLA-DRB1 SE is reported to be associated with both. In the present study, we selected several genes including GSTM1, GSTT1, VEGFA, eNOS, MMP1, MMP2, MMP3, TGFB1 and PTPN22, based on their importance in immunity (TGFB1 and PTPN22), in angiogenesis and atherosclerosis (VEGFA and eNOS), in tissue destruction (MMP1, 2 and 3) or in detoxification of products generated by cigarette smoke (GSTM1 and T1), to investigate the potential associations of these genes with RA outcome and co-morbidities. Smoking is a major environmental risk factor for RA susceptibility, although its role in disease severity and/or co-morbidity remains to be clarified. It is possible that smoking may affect RA through interaction with the above selected genes. Thus, in this study, interaction between smoking and candidate genes were investigated.

The research conducted was divided into a number of specific objectives relating to cigarette smoking and/or each candidate gene. These are listed below. Further description of aims and reasoning for each specific objective is presented in ñBrief Introductionò Sections in the Results Chapter.

List of specific objectives

- To investigate the associations of smoking with autoantibodies, disease clinical measures and co-morbid conditions.

- To investigate the associations of polymorphisms at the GSTM1 and T1 genes with disease clinical measures and co-morbid conditions. To assess whether there is an interaction with smoking with regard to these associations.

- To investigate the associations of polymorphisms at the VEGFA gene with serum levels of VEGF-A, disease clinical measures and co-morbid conditions. To investigate if there is an interaction of VEGFA polymorphism with smoking in respect of these associations.

- To investigate the associations of polymorphism at the eNOS gene with disease clinical measures and co-morbid conditions. To test whether there is an interaction with smoking regarding these associations.

- To investigate the associations of polymorphisms at the MMP1, 2 and 3 genes with serum levels of MMP-1, -2, -3, -8 and -9, serum levels of TIMP-1, -2, -3 and -4, disease clinical measures and co-morbid conditions. To investigate if there is an interaction of these polymorphisms with smoking in terms of these associations.

- To investigate the associations of polymorphisms at the TGFB1 gene with serum levels of TGF-ƃ1, disease clinical measures and co-morbid conditions. To assess whether there is an interaction with smoking according to these associations.

- To investigate the associations of polymorphism at the PTPN22 gene with disease clinical measures and co-morbid conditions. To test if there is an interaction with smoking in respect of these associations.

CHAPTER 3 PATIENTS AND METHODOLOGY

3.1 PATIENT POPULATION

3.1.1 Selection criteria and consent

The study population was consecutively recruited from individuals who were admitted for RA to the Haywood Hospital and who were attending established clinics for drug monitoring and routine RA follow up in the hospital. All patients satisfied the 1987 ACR revised criteria for RA (Arnett et al, 1988), as determined by a consultant rheumatologist. Patients less than 18 years old, or with learning difficulties or dementia were excluded. All of the patients were British Caucasians, resident in North Staffordshire, England. The data for each patient was highly comprehensive, which included clinical measures, laboratory-determined parameters, and a broad set of demographic and lifestyle factors. Prior to inclusion, each patient was informed about the research and its objectives, and given the opportunity to voice any questions. Subsequently, all patients in this study provided written informed consent. The research was approved by the North Staffordshire local research ethics committee (Appendix 8.2).

In total, 431 individuals were included. Since there were specific variables unavailable for some patients, for each investigation the number of samples used and the individuals involved may be slightly different. The demographic, laboratory-based and clinical variables for the whole group and for the specific subsets are presented in the appropriate Results Chapter Sections.

3.1.2 Demographic and smoking information

The patients studied generally represented a typical hospital based population of RA subjects. Of these, 72 were early RA with disease duration (DD) less than 2 years, while the remaining subjects were established RA patients with DD longer than 2 years, at the time of recruitment. Demographic data was given by each patient, including gender, age, weight, height, post code of residence, occupation, etc. Body mass index (BMI) was obtained by BMI = weight (in kilograms)/height^2 (in metres). Socioeconomic status was estimated by the Carstairs score of particular location searched by post code.

Information about cigarette smoking on each patient was carefully recorded. This generally included a history and the current status of smoking. Cigarettes per day indicated the intensity and years of smoking indicated the duration. To measure the total amount someone had smoked, we used pack-years which was calculated by packs (20 cigarettes) per day × years of smoking. The smoking questionnaire used in this study is shown in Appendix 8.3. Patients classified as ever smokers (including past and current smokers) were those who had smoked at least 1 cigarette per day for at least 1 year. This definition was used to ensure that we only included patients with clear evidence of a smoking history. Some people will start to smoke for a short time when they are young but will give up very quickly, so we did not wish to include these as ever smokers.

3.1.3 RA characteristics

A core set of clinical and laboratory-based RA characteristics was recorded at recruitment. This included age at RA onset, RA duration, IgM RF, anti-CCP, levels of ESR and CRP,

DAS28, MJS, HAQ, the presence/absence of erosive and nodular disease, etc. These patients were to be followed up for a period of 60 months. For the follow up visits (every 12 months), DAS28, HAQ, CRP, ESR and the new development of erosive and nodular status were/would be assessed. In Section 1.1.7, the assessments of ESR, CRP, DAS28, MJS and HAQ were described. IgM RF and anti-CCP were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturersô recommendation. To determine nodule status, each patient was examined by a consultant rheumatologist for the presence of subcutaneous nodules. A full examination was conducted on each patient and the presence and site of any nodules were recorded. Erosive evidence was based on radiographs, assessed by consultant rheumatologists. The classification of an erosive disease was the presence of at least one erosion.

3.1.4 Haemanalysis and co-morbid condition

At recruitment, all patients underwent a physical and medical examination, in which pulse, blood pressure, blood tests, etc were assessed. Blood test parameters included haemoglobin, leukocyte count, platelet count, cholesterol and creatinine. Blood samples were collected for DNA isolation and serum separation.

Evidence of CVD (IHD, previous MI, heart failure, etc) was obtained from a structured interview, review of the medical notes and current and cumulative medication. Furthermore, all patients also underwent resting 12-lead electrocardiography (ECG). A diagnosis of IHD was based on presence of angina pectoris, previous MI (physician diagnosed) or evidence of coronary artery disease based on angiography, functional testing or previous revascularization procedures such as coronary artery bypass grafting. ECG was

further used to identify possible unrecognized previous MI (Q-wave development in absence of any conduction defect, suggesting full thickness MI). Hypertension was considered to be present if there was a physicianôs diagnosis in the medical notes, the patient was on anti-hypertensive medication, or high blood pressure (Ó140 mmHg systolic or Ó 90 mmHg diastolic) recorded during the medical examination at recruitment. Hypercholesterolemia was considered to be present if a diagnosis had been recorded in the medical notes, or if the patient had ever been prescribed lipid-lowering medication. Evidence of diabetes (type 1 and 2) was based on a physicianôs diagnosis in the medical notes, or use of anti-diabetic medications. Similarly, information of other co-morbid conditions (e.g. cancer, renal disease, liver disease, respiratory disease, cerebrovascular disease, etc) was obtained based on the medical notes, interviews and prescribed medications. New occurrence of any co-morbid condition was/would be recorded in the years of follow up.

3.1.5 Drug therapy regimen

All patients were receiving anti-inflammatory and/or anti-rheumatic drugs. Most patients (93%) had been treated with one or more DMARDs. The majority were being treated with MTX, SSZ or hydroxychloroquine. The commonest combination therapy was MTX and SSZ. A small number of patients were being treated with steroids (10%) or cytotoxic drugs such as azathioprine or cyclophospamide (< 5%). A number of patients were on a biologic agent (mainly etanercept and infliximab) at the time of recruitment (14%).

3.2 STUDY DESIGN

3.2.1 Study design

The initial design of this study was a prospective cohort that would be followed up annually for a 5-year period, to investigate the prevalence of co-morbid diseases in a typical RA population and the mortality rate attributable to certain co-morbid conditions, and to assess the relationship with specific genetic and environmental factors. Development of a co-morbidity index in this RA population was also planned.

For this particular study, we used this group of RA patients to investigate the association of genetic variations and smoking with the disease activity/severity and co-morbidities. It was cross-sectional in nature. All the measures used in the present thesis were the baseline data (recorded at the time of recruitment), except for the mean time-averaged DAS over 24 months (DAS28 at baseline, 12^{th} month and 24^{th} month). However, in future, it will be possible to assess the influence of genetic variations and smoking on further development of co-morbidities, RA progression (measured by the change of clinical parameters) and any fatal events, during a certain period.

3.3 GENOTYPING

3.3.1 Isolation of genomic DNA from peripheral blood

In order to perform genotyping, the first step was to isolate genomic DNA from each patient. To achieve this, a Nucleon® genomic DNA extraction kit (GE Healthcare, Buckinghamshire, UK) was used and performed according to manufacturerôs instructions. All reagents and buffers used were integral components of the kit, except for ethanol and chloroform. It is an ethanol precipitation based procedure that isolates genomic DNA from fresh or frozen blood samples. The method utilises salts to neutralise the positive charge of the sugar-phosphate DNA backbone and ethanol to precipitate the DNA by promoting ion pair formation between the DNA and salt ions. The kit is able to isolate genomic DNA ranging from 250 εl up to 10 ml of whole blood.

Cell preparation from whole blood

The blood samples were removed from storage at -80°C and were thawed at room temperature. After a 10 seconds vortex, 1 ml of sample was transferred to fresh 15 ml centrifuge tubes. Approximately 4 ml of Reagent A (4 times the volume) was added to the tubes. Then, rotary shaking for 4 minutes at room temperature was performed to mix the blood and Reagent A completely. After that, samples were centrifuged at 4,000 rpm for 5 minutes. The supernatant was then discarded without disturbing the cell pellet.

Cell lysis

For cell lysis, 350 εl of Reagent B (ensuring that the detergent is fully dissolved) was added to the pellet, briefly vortexed to resuspend the pellet, and incubated at 37°C for 10 minutes. After that, the suspension was transferred to a fresh 1.5 ml microtube.

Deproteinisation

A hundred εl of $NaClO_4$ solution was added to the suspension. This was mixed by inverting at least 7 times by hand. After that, 600 εl of $CHCl_3$ was added. To emulsify the phases, tubes were inverted at least 7 times for the second time. After adding 75 εl of Nucleon Resin (without remixing the phases), samples were centrifuged at 1,500 rpm for 1 minute.

DNA precipitation

The upper phase (~ 450 εl) was transferred to a new 1.5ml tube without disturbing the Nucleon Resin layer (brown in colour). To the upper phase, approximately 2 volumes (~ 900 εl) of cold absolute ethanol (stored at 4°C) were added. Tubes were inverted to precipitate the DNA.

DNA washing

The DNA was pelleted by centrifuging at 12,000 rpm for 5 minutes. After discarding the supernatant, 1 ml cold 70% ethanol was added to the pellet. The tubes were inverted

several times before the samples were centrifuged and the supernatant was discarded again. Pellets were then allowed to air dry (ensuring the complete removal of all ethanol trace). After that, 50 ï 200 εl (depend on the size of the pellet) of sterile water was added to each sample to elute the isolated DNA. The DNA samples were placed at room temperature for 3 days to dissolve before being stored at 4°C.

3.3.2 Polymerase chain reaction amplification

Following genomic DNA isolation, the next step was to use polymerase chain reaction (PCR) technology to amplify the specific sequences of DNA containing each of the polymorphism studied in this project. The result of such amplification is the generation of a DNA solution that contains upwards of 1 billion copies of the desired sequence. These enhanced amounts of identical DNA fragment can then be visualised and analysed using electrophoretic methodology. PCR involves the following three steps, denaturation, annealing and extension. First, the genetic material is denatured, converting the double stranded DNA molecules to single strand. The primers are then annealed to the complementary regions of the single stranded molecules. In the third step, they are extended by the action of the DNA polymerase. All these steps are temperature sensitive and the generic choice of temperature is 94 - 95°C, 55 - 60°C and 70 - 72°C for denaturation, annealing and extension respectively.

PCR primers

To be able to amplify the desired DNA sequence, it is necessary to use sequence-specific oligonucleotide primers. For each polymorphism, a pair of primers is used which flanks the

polymorphism site, one primer binding upstream and the other binding downstream. When bound they act both as priming molecules for DNA synthesis to occur and also as boundaries within which the synthesis proceeds. This results in the generation of a population of pure identical DNA fragments where the targeted polymorphism is located.

To design such primers, the DNA sequence of the targeted region needs to be known. For the SNP studies particularly, this information is available in the National Centre for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp). There are several parameters for primer design such as primer length (optimal, 18 ï 22 bp), primer melting temperature (optimal, 52 - 58°C), primer GC content (optimal, 40 ï 60%), product length (optimal, Ò500 bp in standard PCR) and secondary structure of primer and template (to avoid). A number of primer design tools are available that can assist in PCR primer design, such as the online software Primer3 (version 0.4.0, http://frodo.wi.mit.edu/primer3) which was used in the present study.

PCR optimization

The PCR reaction needed to be very specific so as to make sure that the DNA was amplified well, and that only the desired fragment was selectively amplified. To achieve this, the amplification conditions were carefully optimised. This required numerous preliminary PCR reactions where the condition variables could be individually assessed. This included optimising the concentration of each of the PCR reaction mixture constituents (in particular, $MgCl_2$, deoxynucleotide triphosphates (dNTPs), Taq polymerase and primers), adjusting the annealing temperature and determining the optimum incubation periods for each step and the number of PCR cycles. Once established, the optimised

reaction conditions determined for each genetic variant were used in all subsequent PCR amplification reactions.

PCR primers and conditions for each genetic variant

Each PCR amplification was performed in 20 εl volume contained approximately 20 ng genomic DNA (2 εl of diluted genomic DNA) in a 0.2 ml PCR tube (NUNC, Roskilde, Denmark). All amplification reactions were performed in a Flexigene thermal cycler (Techne Limited, Cambridge, UK) using a 96 well heating block. Table 3.3.2a lists the primers and PCR conditions for each polymorphism studied in this project.

Table 3.3.2a The primers and conditions of PCR amplifications for studied genetic polymorphisms

GSTM1 and T1 combined*

Primers for GSTM1: Forward: 5ô-GAACTCCCTGAAAAGCTAAAGC-3ô,

Reverse: 5ô-GTTGGGCTCAAATATACGGTGG-3ô

Primers for GSTT1: Forward: 5ô-TTCCTTACTGGTCCTCACATCTC-3ô

Reverse: 5ô-TCACCGGATCATGGCCAGCA-3ô

Solution mixture: 1 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1 εl of 50 mM $MgCl_2$, 4.8 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 0.5 εl of each 10 εM primer, 0.1 εl of 5 U/εl Taq polymerase and 7.5 εl of double-distilled water (ddH_2O).

Cycling setting: 2.5 minutes at 94°C, followed by 39 cycles of 1 minute at 94°C, 1 minute at 58°C, 1 minute at 72°C, with final extension at 72°C for 7.5 minutes.

VEGFA-2578 SNP (rs699947)

Primers: Forward: 5ô-GGCCTTAGGACACCATACC-3ô,

Reverse: 5ô-CACAGCTTCTCCCCTATCC-3ô(Han et al, 2004).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 4.8 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.12 εl of 5 U/εl Taq polymerase and 6.28 εl of ddH_2O.

Cycling setting: 8 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C, with final extension at 72°C for 10 minutes.

VEGFA-460 SNP (rs833061)

Primers: Forward: 5ô-TGTGCGTGTGGGGTTGAGCG-3ô,

Reverse: 5ô-TACGTGCGGACAGGGCCTGA-3ô(Watson et al, 2000).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 4.8 εl of 2 × polymate additive, 1.3 εl of 2 mM dNTPs mix, 0.6 εl of each 10 εM primer, 0.16 εl of 5 U/εl Taq polymerase and 7.34 εl of ddH_2O.

Cycling setting: 8 minutes at 94°C, followed by 35 cycles of 30 secnds at 94°C, 30 seconds at 62°C, 1 minute at 72°C, with final extension at 72°C for 10 minutes.

VEGFA+405 SNP (rs2010963)

Primers: Forward: 5ô-ATTTATTTTTGCTTGCCATT-3ô,

Reverse: 5ô-GTCTGTCTGTCTGTCCGTCA-3ô(Watson et al, 2000).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.6 εl of 50 mM $MgCl_2$, 3.2 εl of 2 × polymate additive, 2 εl of 2 mM dNTPs mix, 0.8 εl of each 10 εM primer, 0.12 εl of 5 U/εl Taq polymerase and 7.48 εl of ddH_2O.

Cycling setting: 8 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, with final extension at 72°C for 10 minutes.

VEGFA+936 SNP (rs3025039)

Primers: Forward: 5ô-ATTTATTTTTGCTTGCCATT-3ô,

Reverse: 5ô-GTCTGTCTGTCTGTCCGTCA-3ô(Han et al, 2004).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 0.6 εl of 50 mM $MgCl_2$, 3.2 εl of 2 × polymate additive, 2 εl of 2 mM dNTPs mix, 0.8 εl of each 10 εM primer, 0.12 εl of 5 U/εl Taq polymerase and 8.48 εl of ddH_2O.

Cycling setting: 8 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, 1 minute at 72°C, with final extension at 72°C for 10 minutes.

ENOS+894 SNP (rs1799983)

Primers: Forward: 5ô-AAGGCAGGAGACAGTGGATGGA-3ô,

Reverse: 5ô-CCCAGTCAATCCCTTTGGTGCTCA-3ô (Isordia-Salas et al, 2010).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 3 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.2 εl of 5 U/εl Taq polymerase and 8 εl of ddH_2O.

Cycling setting: 4 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 72°C, with final extension at 72°C for 10 minutes.

MMP3 5A6A (rs3025058)

Primers: Forward: 5ô-GGTTCTCCATTCCTTTGATGGGGGGAAAgA-3ô,

Reverse: 5-CTTCCTGGAATTCACATCACTGCCACCACT-3ô (Gnasso et al, 2000).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 0.6 εl of 50 mM $MgCl_2$, 2 εl of 2 mM dNTPs mix, 1.6 εl of each 10 εM primer, 0.16 εl of 5 U/εl Taq polymerase and 10.04 εl of ddH_2O.

Cycling setting: 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C, with final extension at 72°C for 5 minutes.

MMP3 Glu45Lys (rs679620)

Primers: Forward: 5ô-TCCCCGTCACCTCCAATCCAA-3ô,

Reverse: 5ô-TGAGTAACAAGTGGAGAGTGG-3ô(Shibata et al, 2005).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1 εl of 50 mM $MgCl_2$, 3 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.2 εl of 5 U/εl Taq polymerase and 8.2 εl of ddH_2O.

Cycling setting: 4 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, with final extension at 72°C for 5 minutes.

Rs495366 (intergenic SNP between MMP1 and 3 genes)

Primers: Forward: 5ô-TGAGCTCCCGAAAGGTAGAA-3ô,

Reverse: 5ô-TGAGACCACATCAGTCTT-3ô.

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 3 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.2 εl of 5 U/εl Taq polymerase and 8 εl of ddH_2O.

Cycling setting: 4 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 72°C, with final extension at 72°C for 5 minutes.

MMP1 1G2G (rs1799750)

Primers: Forward: 5ô-TGACTTTTAAAACATAGTCTATGTTCA-3ô,

Reverse: 5ô-TCTTGGATTGATTTGAGATAAGTCATAgC-3ôÿ.

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1 εl of 50 mM $MgCl_2$, 4.8 εl of 2 × polymate additive, 2 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.16 εl of 5 U/εl Taq polymerase and 6.04 εl of ddH_2O.

Cycling setting: 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 30 seconds at 72°C, with final extension at 72°C for 5 minutes.

MMP2-1306 (rs243865)

Primers: Forward: 5ô-CTTCCTAGGGCTGGTCCTTACTGA-3ô,

Reverse: 5ô-CTGAGACCTGAAGAGCTAAAGAGCT-3ô.

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 3 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.2 εl of 5 U/εl Taq polymerase and 8 εl of ddH_2O.

Cycling setting: 4 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 59°C, 1 minute at 72°C, with final extension at 72°C for 5 minutes.

TGFB1-509 (rs1800469)

Primers: Forward: 5ô-CAGTAAATGTATGGGGTCGCAG-3ô,

Reverse: 5ô-GGTGTCAGTGGGAGGAGGG-3ô(Amirghofran et al, 2009).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 3 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.2 εl of 5 U/εl Taq polymerase and 8 εl of ddH_2O.

Cycling setting: 3 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 61°C, 1 minute at 72°C, with final extension at 72°C for 5 minutes.

TGFB1+868 (rs1800470) ¶

Primers: Forward (generic primer): 5ô-TCCGTGGGATACTGAGACAC-3ô,

Reverse (for the C allele): 5ô-GCAGCGGTAGCAGCAGCG-3ô,

Reverse (for the T allele): 5ô-AGCAGCGGTAGCAGCAGCA-3ô (Perrey et al, 1999).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 0.6 εl of 50 mM $MgCl_2$, 5 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer (internal control primers, generic primer and the primer for the C allele/internal control primers, generic primer and the primer for the T allele), 0.2 εl of 5 U/εl Taq polymerase and 4.6 εl of ddH_2O.

Cycling setting: 1 minute at 95°C, followed by 10 cycles of 15 seconds at 95°C, 50 seconds at 65°C and 40 seconds at 72°C, and 20 cycles of 20 seconds at 95°C, 50 seconds at 59°C and 50 seconds at 72°C.

TGFB1+913 (rs1800471) ¶

Primers: Forward (for the G allele): 5ô-GTGCTGACGCCTGGCCG-3ô,

Forward (for the C allele): 5ô-GTGCTGACGCCTGGCCC-3ô,

Reverse (generic primer): 5ô-GGCTCCGGTTCTGCACTC-3ô (Perrey et al, 1999).

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1.2 εl of 50 mM $MgCl_2$, 3 εl of 2 × polymate additive, 1.6 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer (internal control primers, generic primer and the primer for the G allele/internal control primers, generic primer and the primer for the C allele), 0.2 εl of 5 U/εl Taq polymerase and 6 εl of ddH_2O.

Cycling setting: 1 minute at 95°C, followed by 10 cycles of 15 seconds at 95°C, 50 seconds at 65°C and 40 seconds at 72°C, and 20 cycles of 20 seconds at 95°C, 50 seconds at 59°C and 50 seconds at 72°C.

PTPN22 R620W (rs2476601)

Primers: Forward: 5ô-TCACCAGCTTCCTCAACCACA-3ô,

Reverse: 5ô-GATAATGTTGCTTCAACGGAATTT-3ô.

Solution mixture: 2 εl (~ 20 ng) of diluted genomic DNA, 2 εl of 10 × reaction buffer, 1 εl of 50 mM $MgCl_2$, 2 εl of 2 mM dNTPs mix, 1 εl of each 10 εM primer, 0.16 εl of 5 U/εl Taq polymerase and 10.84 εl of ddH_2O.

Cycling setting: 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 72°C, with final extension at 72°C for 2 minutes.

* This PCR was able to distinguish between homozygous GSTM1*0/GSTT1*0 and genotypes with least 1 copy of expressible allele. Since the amplified GSTM1 and GSTT1 have distinguishable difference in length, 2 PCRs were combined as one and the genotypes of both genes can be identified in a single agarose gel using gel electrophoresis. Positive control tests, using ß-globin (primers forward: 5ô-ACACAACTGTGTTCACTAGC-3ôand reverse: 5ô-CAACTTCATCCACGTTCACC-3ô, product size 325 bp), were carried out for samples with no PCR products in order to determine whether these patients carrying both homozygous GSTM1*0 and GSTT1*0 or the failure of amplification was due to insufficient genomic DNA; [À] A mismatch was made at the nucleotide written in lower case in order to create a recognition site for the enzyme Tth111I in the case of a 5A allele; [ÿ] A mismatch was made at the nucleotide written in lower case in order to create a recognition site for the enzyme AluI in the case of a 1G allele; [¶] Allele-specific PCR systems were used (Dennis Lo, 1998). Internal control amplifies a product of 429 bp (primers forward: 5ô-GCCTTCCCAACCATTCCCTTA-3ôand reverse: 5ô-TCACGGATTTCTGTTGTGTTTC-3ô).

3.3.3 Restriction fragment length polymorphism analysis

Following amplification of the desired DNA sequences, the final step was to use restriction fragment length polymorphism (RFLP) analysis to determine which allele each patient carried at a particular SNP site. The RFLP analysis involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. Selected restriction enzymes are used to cut DNA at a recognition site in the case of one allele, but not the other one. The resulting DNA fragments are then separated by length using gel electrophoresis. The alleles that a patient carried are therefore able to be distinguished according to the digest outcome on gels.

Restriction enzyme and digestion conditions for each genetic variant

Initial optimisation digestion reactions were performed first to produce an efficient system that generated reproducible results. Generally, each digestion reaction was performed in a 20 εl volume made up of 10 εl of PCR product (or more in case there was not enough amplified DNA), 1 × appropriate (numbered 1 - 4) reaction buffer, 1 × bovine serum albumin (BSA) when necessary, ddH_2O, and appropriate restriction enzyme. The incubation temperature and period varied between assays, usually ranging from 25°C (as room temperature) to 65°C and ranging from 1 hour to 20 hours respectively. Table 3.3.3a lists the restriction enzyme and digestion conditions for each polymorphism studied.

Table 3.3.3a The restriction enzymes and digestion conditions for studied genetic polymorphisms

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BstYI (for VEGFA-2578 A/C SNP)

Recognition site: 5ôé R$^{\xi}$ GATCYé 3ô

3ôé YCTAG$_{z}$ Ré 5ô(New England Biolabs, Inc.).

Cuttable allele: A.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 2, 2 units of BstYI (0.2 εl) and 7.8 εl of ddH_2O. Incubation: at 60°C for 3 hours.

BstU I (for VEGFA-460 T/C SNP)

Recognition site: 5ôé CG$^{\xi}$ CGé 3ô

3ôé GC$_{z}$ GCé 5ô(New England Biolabs, Inc.).

Cuttable allele: C.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 3 units of BstUI (0.3 εl) and 7.7 εl of ddH_2O. Incubation: at 60°C for 3 hours.

BsmF I (for VEGFA+405 C/G SNP)

Recognition site: 5ôé GGGAC(N)$_{10}{}^{\xi}$ é 3ô

3ôé CCCTG(N)$_{14z}$ é 3ô(New England Biolabs, Inc.).

Cuttable allele: G.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 2 units of BsmFI (1 εl), 0.2 εl of BSA and 6.8 εl of ddH_2O. Incubation: at 65°C for 3 hours.

NlaIII (for VEGFA+936 C/T SNP)

Recognition site: 5ôé CATG$^{\xi}$ é 3ô

3ôé $_{z}$ GTAGé 5ô(New England Biolabs, Inc.).

Cuttable allele: T.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 1 unit of NlaIII (0.1 εl), 0.2 εl of BSA and 7.7 εl of ddH_2O. Incubation: at 37°C for 3 hours.

MboI (for eNOS+894 G/T SNP)

Recognition site: 5ôé $^{\delta}$ GATCé 3ô

3ôé CTAG$_{z}$ é 5ô(New England Biolabs, Inc.).

Cuttable allele: T.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 2 units of MboI (0.4 εl) and 7.6 εl of ddH_2O. Incubation: at 37°C for 20 hours.

Tth111I (for MMP3 5A6A polymorphism)

Recognition site 5ôé GACN$^{\delta}$ NNGTCé 3ô

3ôé CTGNN$_{z}$ NCAGé 5ô(New England Biolabs, Inc.).

Cuttable allele: 5A.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 4 units of Tth111I (1 εl), 0.2 εl of BSA and 6.8 εl of ddH_2O. Incubation: at 65°C for 3 hours.

Taq$^{\alpha}$I (for MMP3 Glu45Lys A/G SNP)

Recognition site: 5ôé T$^{\delta}$ CGAé 3ô

3ôé AGC$_{z}$ Té 5ô(New England Biolabs, Inc.).

Cuttable allele: G.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 10 units of Taq$^{\breve{}}$I (0.5 εl), 0.2 εl of BSA and 7.3 εl of ddH_2O. Incubation: at 65°C for 2 hours.

SfcI (for rs495366 A/G SNP)

Recognition site: 5ôé C$^{\delta}$ TRYAGé 3ô

3ôé GAYRT$_{z}$ Cé 5ô(New England Biolabs, Inc.).

Cuttable allele: G.

Digestion condition: 10 εl of PCR product, 2 εl of reaction buffer 4, 2 units of SfcI (0.2 εl), 0.2 εl of BSA and 7.6 εl of ddH_2O. Incubation: at 25°C for 16 hours.

AluI (for MMP1 1G2G polymorphism)

Recognition site: 5ôé AG$^{\delta}$ CTé 3ô

3ôé TC$_{z}$ GAé 5ô(New England Biolabs, Inc.).

Cuttable allele: 1G.

Digestion condition: 15 εl of PCR product, 2 εl of reaction buffer 4, 10 units of AluI (1 εl) and 2 εl of ddH_2O. Incubation: at 37°C for 16 hours.

BfaI (for MMP2-1306 C/T SNP)

Recognition site: 5ôé C$^{\delta}$ TAGé 3ô

3ôé GAT$_{z}$ Cé 5ô(New England Biolabs, Inc.).

Cuttable allele: T.

Digestion condition: 10 ε l of PCR product, 2 ε l of reaction buffer 4, 2.5 units of BfaI (0.5 ε l) and 7.5 ε l of ddH_2O. Incubation: at 37°C for 16 hours.

Bsu36I (for TGFB1-509 C/T SNP)

Recognition site: 5ôé CC$^{\delta}$ TNAGGé 3ô

3ôé GGANT$_{z}$ CCé 5ô(New England Biolabs, Inc.).

Cuttable allele: C.

Digestion condition: 10 ε l of PCR product, 2 ε l of reaction buffer 3, 5 units of Bsu36I (0.5 ε l), 0.2 ε l of BSA and 7.3 ε l of ddH_2O. Incubation: at 37°C for 16 hours.

XcmI (for PTPN22 R620W C/T SNP)

Recognition site: 5ôé CCANNNNN$^{\delta}$ NNNNTGGé 3ô

3ôé GGTNNNN$_{z}$ NNNNNACCé 5ô(New England Biolabs, Inc.).

Cuttable allele: T.

Digestion condition: 12 ε l of PCR product, 2 ε l of reaction buffer 2, 5 units of XcmI (1 ε l) and 5 ε l of ddH_2O. Incubation: at 37°C for 4 hours.

R = A or G; Y = C or T; N = A, C, G or T.

Agarose gel electrophoresis of digestion fragments for each polymorphism

To identify which alleles each patient carried, the digested products were analysed using agarose gel electrophoresis on (1 ï 4%) agarose gels. Better separation and resolution are provided by increasing the percentage of agarose. However, it increases running time and costs more in materials. Each gel was prepared by dissolving the appropriate amount of agarose in 30 ml of running buffer (3 ml 5 × Tris/Borate/EDTA (TBE) buffer plus 27 ml ddH_2O), with the addition of 3 ε l ethidium bromide (500 ε g/ml, Sigma-Aldrich Inc.). The solution was then boiled and poured into a pre-assembled gel eletrophoresis unit. Each gel

was allowed to set at room temperature or at 4℃. Concurrently, restriction fragments generated from digestion were prepared by the addition of the appropriate volume (~ 3 εl) of 6 × loading buffer (Novagen, Darmstadt, Germany). Gels were then loaded with 10 εl of each digest sample, 10 εl of negative control and 5 εl of DNA marker (1.5 kb DNA ladder, Bioline Ltd.). The gel tanks were subsequently filled with running buffer to submerse the gels and the samples eletrophoretically separated at 120 volts until sufficient separation of the bands had been seen. To analyse the banding pattern, gels were exposed to UV illumination on a transilluminator. A photograph of the banding pattern was taken as a permanent record using film or digital camera specifically designed for gel photography. A diagram of digestion outcome for each polymorphism is shown in Appendix 8.4.

3.4 QUANTIFICATION OF SERUM PROTEINS

3.4.1 Serum collection

Serum was collected from the blood by the use of a serum separator tube (SST) and allowed to clot for 30 minutes prior to centrifugation at 3,000 rpm. When collected, samples were stored at -80°C before assay.

3.4.2 Types of assay used in the present study

Luminex suspension array system (Bio-Rad) was used in this project to measure the MMPs (including MMP-1, -2, -3, -8 and -9), TIMPs (including TIMP-1, -2, -3 and -4) and the angiogenesis associated proteins (including Angiopoietin-2, Follistatin, granulocyte

colony-stimulating factor (G-CSF), hepatocyte growth factor (HGF), IL-8, Leptin, platelet-derived growth factor (PDGF-BB), platelet endothelial cell adhesion molecule (PECAM-1) and VEGF-A). The system is based on a bead-based ELISA and a flow cytometer with 2 lasers, which allows measurement of numerous analytes (up to 100 analytes) in parallel. The microsphere beads used contain a 2-dye fluorescent variable intensity to create a 100-distinct bead set. Molecules bind to the specific probes attached to the bead surface which are then tagged by fluorescent reporter labels. The beads pass individually through a 2 laser column in the machine and their fluorescent biochemical reactions are measured and reported (see http://www1.imperial.ac.uk/resources/6DB4DBCF-7A48-4655-85ED-21C3E2C63932/).

A standard ELISA was used in the present study to determine the level of TGF-ß1 in each patient.

3.4.3 Luminex assay for angiogenesis associated proteins

Measurement of circulating angiogenesis associated proteins was performed on the serum samples using Bio-Plex Pro™ Human Angiogenesis 9-plex immunoassay kits (Bio-Rad) and was read on a Bio-Plex 200™ system (Bio-Rad). Sample preparation and assay procedure were followed according to the manufacturerôs recommendation. In addition, 3 εg/ml of Heteroblock (Omega Biologicals, Bozeman, USA) was added into the sample diluent prior to assay to block any non-specific binding to RF (Hueber et al, 2007).

Preparation of samples

The serum samples were removed from storage at -80°C and were thawed at 4°C. Samples were centrifuged at 13,200 rpm for 10 minutes at 4°C to clear the precipitate. Immediately, 50 εl of serum was diluted with 150 εl of Sample Diluent containing 4 εg/ml of Heteroblock (dilution at 1:4). The prepared samples were kept on ice until ready for use.

Preparation of standards

One vial of lyophilized standard was reconstituted with 500 εl of the Standard Diluent. After a gentle vortex (1 ï 3 seconds), the standard solution was incubated on ice for 30 minutes.

A set of tubes was labelled as S1 (standard 1), S2, S3, S4, S5, S6, S7 and S8. Four hundred εl of reconstituted standard was added to the S1 tube. Four hundred and fifty εl of Serum Standard Diluents were added into the remaining tubes. A 4-fold dilution series were produced by transferring 150 εl of solutions from the tube ahead to the next one (S1Ÿ S2Ÿ S3Ÿ S4Ÿ S5Ÿ S6Ÿ S7Ÿ S8). The reconstituted standard served as the high standard, whereas the Serum Standard Diluent served as the blank. Vortexing and change of the pipet tips were performed during every transfer. The serial standards were kept on ice until ready for use.

Preparation of controls

One vial of lyophilized control was reconstituted with 800 εl Serum Standard Diluent. After a gentle vortex (1 ï 3 seconds), the control solution was incubated on ice for 30 minutes.

The reconstituted control was further diluted to create the desired quality control samples (3-level controls). A set of tubes was labelled as C1 (control 1), C2 and C3. Three hundred εl of Serum Standard Diluents were added into these three tubes. After that, 150 εl of the reconstituted control solution was added into the C1 tube. A 3-fold dilution series were made by transferring 150 εl of solutions from the tube ahead to the next one (C1Ÿ C2Ÿ C3). Vortexing and change of the pipet tips were performed during every transfer. Control samples were kept on ice until ready for use.

Preparation of magnetic beads, detection antibody and streptavidin-PE

The coupled magnetic beads were protected from light by covering the tubes with aluminium foil. The beads (25×) were vortexed at medium speed for 15 ï 20 seconds before diluting. Assay Buffer was used to dilute beads from 25× to 1×. Each well required 50 εl of beads (1×) (2 εl of beads (25×) + 48 εl of Assay Buffer). Prepared beads were kept on ice until ready to use.

Detection Antibody Diluent was used to dilute detection antibody from 10× to 1×. Each well required 25 εl of detection antibody (1×) (2.5 εl of detection antibody (10×) + 22.5 εl of Detection Antibody Diluent).

The streptavidin-PE (100×) was diluted with Assay Buffer to the concentration of 1×. Each well required 50 εl of streptavidin-PE (1×) (0.5 εl of streptavidin-PF (100×) + 49.5 εl of Assay Buffer).

Assay procedure

The diluted standards, samples and controls, and all buffer solutions were brought to room temperature 20 minutes prior to use. The wells of the 96-well filter plate were pre-wetted with 100 εl of Assay Buffer and then removed by a vacuum manifold designed to accommodate a 96-well plate. Coupled magnetic beads (1×) were vortexed for 15 ï 20 seconds. After that, 50 εl of the beads was added to each well and vacuum-filtration was performed immediately. Washing was performed by adding 100 εl of Wash Buffer to each well and performing vacuum-filtration to remove the liquid. Washing was carried out twice. After washing, 50 εl of standard, control or sample (gently vortexed, 1 ï 3 seconds) was added into each well, followed by incubation at room temperature on a horizontal orbital shaker for 30 minutes (plate covered with a foil plate sealer during incubation). During this incubation period, the detection antibody (1×) was prepared (as described). After incubation, the plate was filtered through the vacuum manifold, and washed 3 times. Then, 25 εl of detection antibody (gently vortexed, 1 ï 3 seconds) was added into each well, followed by the second incubation at room temperature on an orbital shaker for 30 minutes. In this period, streptavidin-PE (1×) was prepared (as described). After the detection antibody incubation, vacuum-filtration and washing (3×) were performed. Addition of 50 εl of streptavidin-PE (gently vortexed, 1 ï 3 seconds) into each well was carried out before the third incubation at room temperature on an orbital shaker for 10 minutes. When the streptavidin-PE incubation was completed, further vacuum-filtration

and washing (3×) were carried out. After the final wash, 125 εl of Assay Buffer was added into each well, followed by a shaking at room temperature on an orbital shaker for 30 seconds. The plate was then ready to be read on the Luminex Bio-Rad Analyzer (setting for data acquisition not shown).

3.4.4 Luminex assay for MMPs

Quantification of circulating MMPs was performed on the serum samples using Human MMP Fluorokine MAP multiplex kits (R&D Systems, Minneapolis, USA) and was read on a Bio-Plex 200^{TM} system (Bio-Rad). Sample preparation and assay procedure were followed according to the manufacturerôs recommendation. Three εg/ml of Heteroblock (Omega Biologicals, Bozeman, USA) was added into the sample diluent prior to assay to block any non-specific binding to RF (Hueber et al, 2007).

The procedure of this assay was similar to that for the angiogenic proteins. This assay measured the pro-form of MMPs.

3.4.5 Luminex assay for TIMPs

Measurement of circulating TIMPs was performed on the serum samples using Human TIMP Fluorokine MAP multiplex kits (R&D Systems, Minneapolis, USA) and was read on a Bio-Plex 200^{TM} system (Bio-Rad). Sample preparation and assay procedure were followed according to the manufacturerôs recommendation. Three εg/ml of Heteroblock (Omega Biologicals, Bozeman, USA) was added into the sample diluent prior to assay to block any non-specific binding to RF (Hueber et al, 2007).

The procedure of the TIMP assay was similar to that of the assay for the angiogenic proteins.

3.4.6 ELISA assay for TGF-β1

Measurement of circulating TGF-ß1 was performed on the serum samples using a Duoset Human TGF-ß1 ELISA kit (R&D Systems, Minneapolis, USA) and was read on a TiterTek Multiskan Plus MKII microplate reader (Flow Laboratories Ltd., Richmansworth, UK). Sample and solution preparations and assay procedure were followed according to the manufacturerôs recommendation.

Preparation of plate

Capture antibody (mouse anti-TGF-ß1) was reconstituted with 1 ml of phosphate buffered saline (PBS). The reconstituted capture antibody (360 εl/ml) was stored at 4°C. To dilute the capture antibody to the working concentration (2 εl/ml), 66.6 εl of reconstituted capture antibody was added into 11,933.4 εl of PBS. For coating a 96-well microplate (Costar 3590, Corning Incorporated, NY, USA), 100 εl of the diluted capture antibody was added into each well. After that, the plate was sealed with foil and was incubated overnight at room temperature. By aspirating each well, and inverting the plate and blotting it against clean paper towels, liquid was removed from the wells. Washing was carried out 4 times. Each wash step was performed by adding 300 εl of Wash Buffer (0.05% Tween® 20 in PBS) into each well and removing the liquid as described above. For blocking the plate, 300 εl of Block Buffer (5% Tween® 20 in PBS with 0.05% NaN_3) was added into each

well and the plate was incubated at room temperature for 1.5 hours. After that, another washing (4×) was required before the plate was ready for sample addition.

TGF-β1 activation and dilution for samples

To activate latent TGF-ϐ1 to immunoreactive protein, an acid activation was required, followed by a neutralization. To do this, firstly, 20 εl of 1 N HCl was mixed with 40 εl of serum and the mixture was incubated at room temperature for 10 minutes. Then, the acidified sample was neutralized by adding 20 εl of 1.2 N NaOH with 0.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). During this, samples were diluted 1:2.

Prior to the assay, a further 20-fold dilution was performed by adding 20 εl of activated sample into 380 εl of Reagent Diluent (Catalog # DY997, R&D Systems, Minneapolis, USA). Thus, the final dilution was at 1:40.

Preparation of standards

One vial of lyophilized standard was reconstituted with 500 εl of Reagent Diluent. The reconstituted standard (120 ng/ml) was allowed to sit for 20 minutes with gentle agitation prior to making dilutions for serial standards.

A set of tubes was labelled as S1, S2, S3, S4, S5, S6 and S7. Into the S1 tube, 16.7 εl of reconstituted standard and 983.3 εl of Reagent Diluent were added to make the high standard (2,000 pg/ml). Five hundred εl of Reagent Diluents were added into the

remaining tubes. A 2-fold dilution series were produced by transforming 500 εl of solutions from the tube ahead to the next one (S1Ÿ S2Ÿ S3Ÿ S4Ÿ S5Ÿ S6Ÿ S7). A 7-point standard curve was then made and the Reagent Diluent served as the blank. Vortexing and change of pipet tips were performed during every transfer.

Preparation of detection antibody and streptavidin-HRP

Detection antibody (biotinylated chicken anti-TGF-ƃ1) was reconstituted with 1 ml of Reagent Diluent. The reconstituted detection antibody (54 εg/ml) was stored at 4°C. To dilute the detection antibody to the working concentration (300 ng/ml), 66.6 εl of reconstituted detection antibody was mixed with 11,933.4 εl of Reagent Diluent.

To dilute the streptavidin-HRP to the working concentration, a 1:200 dilution was required. Addition of 60 εl of streptavidin-HRP into 11,940 εl of Reagent Diluent was carried out for each assay.

Assay procedure

One hundred εl of sample or standard was added to each well, followed by incubation at room temperature for 2 hours (plate covered with an adhesive strip when incubating). After removing the liquid from wells (as described), washing was carried out 3 times (as described). Then, 100 εl of diluted detection antibody was added into each well, followed by incubation at room temperature for 2 hours. Washes were carried out 3 times. After that, 100 εl of diluted streptavidin-HRP was added into each well. The plate was protected from light and was incubated at room temperature for 20 minutes. Washes (3×) were performed

after this incubation. Addition of 100 εl of Substrate Solution (1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (tetramethylbenzidine), Catalog # DY999, R&D Systems, Minneapolis, USA) to each well was performed, followed by incubation at room temperature for 20 minutes (avoiding the light). Before reading, 50 εl of Stop Solution (2 N H_2SO_4, Catalog # DY994, R&D Systems, Minneapolis, USA) was added into each well and the plate was gently tapped to ensure thorough mixing. Determination of the optical density of each well was carried out immediately, using a reader set to 550 nm for wavelength correction and 450 nm for result-obtaining reading.

3.5 STATISTICAL ANALYSIS

3.5.1 Significance level

In the present study, univariate statistics and multivariate regression analysis were applied to assess relationships. The significance level was set at a p value of 0.05. All p values were based on 2-tailed tests.

3.5.2 Analysis for genotypic distribution, linkage disequilibrium and haplotype

Hardy-Weinberg equilibrium (HWE) for the genotypic distributions of each polymorphism was tested with the Chi-Square goodness-of-fit test. LD indexes, *D'* (Lewontin, 1964) and r^2 were used to indicate the strength of LD and the correlation of linked alleles between each pair of markers respectively. The LD block was defined by the four gamete rule. The haplotype frequencies were estimated using an expectation-maximization algorithm to

determine the maximum-likelihood frequencies of multi-locus haplotypes (Excoffier and Slatkin, 1995). Haploview (version 4.2) (Barrett et al, 2005) was applied to perform these analyses and to export related figures.

3.5.3 Analysis for relationship between categorical variables

The relationship between categorical variables was initially assessed using contingency tables. In the present study, categorical variables for outcome measures were all binary variables. The p value obtained from Chi-Square tests was used to determine whether there was significant difference between at least 2 groups. Odds ratio (OR) with 95% confidence interval (CI) was calculated based on proportions (Fisherôs Exact Test was applied where samples sizes were small (Ò5)). If a trend was seen, the p value for trend was determined by extended Mantel-Haenszel Test. Thereafter, multivariate logistic regression was used to test the association when adjusting for confounding factors (e.g. sex, age, DD, etc) and to investigate whether the risk factor was independent of other established risk factors (outcome variable as dependent variable, and risk variables and confounders as independent variables). The Number Cruncher Statistical System for Windows (version NCSS 2000) was used to perform all analysis, except for the assessment of p for trend. P for trend was obtained using WinPEPI (version 9.4).

3.5.4 Analysis for relationship between categorical and quantitative variables

The relationship between categorical variables and quantitative variables was assessed using T-test (categories = 2) or using one-way ANOVA (categories Ó3) initially. For T-test, if 2 populations being compared were both in normal distribution and had equal variance,

Studentôs T-test was applied; if they were both in normal distribution but had the unequal variance, Aspin-Welch Test was applied; and if they were neither in normal distribution nor had the unequal variance, the Mann-Whitney Test was used. For one-way ANOVA, if compared groups were all in normal distribution and had equal variance, the standard test were applied; if they were not all in normal distribution but had equal variance, Kruskal-Wallis Test was used. When there was a trend, the p value for trend was assessed. In the present study, the p value for trend was determined by the Jonckheere Terpstra Test (assessment of trend among three non-parametric distributed quantitative trait groups). Multivariate multiple regression analysis was carried out to test the independence of associations and to adjust for potential confounding factors (outcome variable as dependent variable, and risk variables and confounders as independent variables). NCSS performed most analyses, while WinPEPI was used to calculate the p for trend.

3.5.5 Analysis for relationship between quantitative variables

Correlation testing was used to assess the relationship between quantitative variables. Since the majority of these variables were not in normal distribution, Spearmanôs Correlation was mostly applied. Multiple regression analysis was also used to assess the independence of association and to adjust for potential confounding factors. When using multiple regression analysis, a normal distribution of the dependent variable was required and where possible a normal distribution of the independent variables. Thus, some transformations to normality were applied (e.g. log, square root, etc). These analyses were carried out using NCSS.

3.5.6 Analysis for relationship involving haplotype

The relationship between haplotypes and binary variables was analyzed in Haploview. The analysis was based on the estimated frequencies of haplotypes and the contingency tables. The relationship between haplotypes and quantitative variables was assessed under regression-based models in HAPSTAT (version 3.0, Department of Biostatistics, University of North Carolina at Chapel Hill, USA) (Lin et al, 2005).

3.5.7 Assessment of biological interaction

Evidence of interaction between categorical variables in relation to a binary variable was assessed by examining for evidence of departure from additivity using the methods of Rothman and Greenland (Rothman and Greenland, 1998). Using this approach the attributable proportion due to interaction (AP) was calculated, together with 95% CI, as detailed by Andersson et al (Andersson et al, 2005). The AP refers to the attributable proportion of disease that is due to interaction among persons with both exposures. In the case of no biological interaction, AP = 0, while an AP = 1 corresponds to complete additive interaction.

CHAPTER 4 RESULTS

PART A: SMOKING, AUTOANTIBODIES, SEVERITY AND CO-MORBIDITY IN RA (4.1 – 4.4)

4.1 SMOKING AND AUTOANTIBODIES

4.1.1 Brief introduction

Cigarette smoking has been identified as a risk factor for seropositive RA regardless of gender. However, with which marker (RF vs. anti-CCP) it is primarily associated remains to be fully evaluated. With the interaction involving HLA-DRB1 SE alleles smoking seems to be responsible for the presence of anti-CCP (Vittecoq et al, 2008), while other observations have shown that smoking is more significantly associated with the RF production in patients carrying certain genotypes such as GSTM1-null (Mattey et al, 2002). It was therefore of interest to evaluate the relationship between smoking and the presence of autoantibodies (RF and anti-CCP) in our RA population.

4.1.2 Smoking characteristics

Smoking status was successfully recorded on 428 patients. The demographic and clinical characteristics of the total RA population, and patients stratified by ever-smoking status are shown in Table 4.1.2a. The majority of smokers had started smoking as teenagers, with median age = 17 (interquartile range (IQR) 15 - 19). Table 4.1.2b shows the smoking history of patients stratified by gender. A higher frequency of smokers (ever-smoked) was found in males ($p = 0.037$). Male smokers consumed more cigarettes per day than female

smokers did ($p = 0.0013$). However, between genders there was no significant difference in smoking duration. In terms of total amount of smoking, males were more likely to be heavier smokers compared to females ($p = 0.024$).

Preliminary analysis showed that there was no significant difference in outcome variables between past and current smokers (data not shown). In the present section (Part A, Results Chapter), we therefore combined past and current smokers and thus present the data only on patients who had ever smoked.

Table 4.1.2a Demographic and clinical characteristics of RA patients stratified by ever-smoking status

Variable	All patients	Non-smoking	Ever-smoking	p[ÿ]
Demographics				
Age, years	62.0 (54.0 ï 69.0)	60.0 (53.0 ï 67.0)	63.0 (55.0 ï 70.0)	NS
Male: Female	137:291	24:121	113:170	< 0.0001
BMI, kg/m^2	27.3 (24.5 ï 30.5)	27.3 (24.6 ï 30.6)	27.4 (24.5 ï 30.4)	NS
CSUM (Ómedian)	196/400 (49.0)	57/134 (42.5)	138/266 (51.9)	NS
RA characteristics				
Age of onset	50.0 (41.0 ï 58.0)	48.8 (38.0 ï 56.0)	50.0 (42.0 ï 59.0)	NS
Duration, years	9.0 (3.0 ï 18.0)	9.0 (3.3 ï 17.8)	9.0 (3.0 ï 18.0)	NS
RF	243/426 (57.0)	64/143 (44.8)	179/283 (63.3)	0.05
Anti-CCP	314/415 (75.7)	96/138 (69.6)	218/277 (78.7)	NS
ESR, mm/hr	19.0 (10.0 ï 35.0)	18.0 (10.0 ï 33.0)	20.0 (10.5 ï 38.0)	NS
CRP (Ó10 mg/l)	229/428 (53.5)	72/143 (50.3)	157/282 (55.7)	NS
*DAS28	4.19 (1.38)	4.07 (1.34)	4.25 (1.40)	NS
Erosive status	312/423 (73.8)	99/144 (68.8)	212/278 (76.3)	NS
MJS	7.0 (3.0 ï 15.0)	7.0 (3.0 ï 15.0)	7.0 (3.0 ï 15.0)	NS
HAQ score	1.63 (1.0 ï 2.0)	1.44 (0.78 ï 2.0)	1.63 (1.13 ï 2.0)	NS
Nodular disease	54/399 (13.5)	15/137 (10.9)	39/261 (14.9)	NS
Co-morbidities				
IHD	78/424 (18.4)	11/143 (7.7)	67/281 (23.8)	< 0.0001
MI	46/424 (10.8)	4/143 (2.8)	42/281 (14.9)	0.0001
Diabetes type 1	11/424 (2.6)	3/143 (2.1)	8/281 (2.8)	NS
Diabetes type 2	22/424 (5.2)	6/143 (4.2)	15/281 (5.3)	NS
Asthma	44/423 (10.4)	13/143 (9.1)	31/280 (11.1)	NS
CVA	15/424 (3.5)	4/143 (2.8)	11/281 (3.9)	NS
Solid cancers	31/398 (7.8)	10/137 (7.3)	21/261 (8.0)	NS
Hypertension	167/424 (39.4)	48/143 (33.6)	119/281 (42.3)	NS
Hypercholesterolemia	70/424 (16.5)	19/143 (13.3)	51/281 (18.1)	NS
Haemanalysis				
Hemoglobin, g/dl	13.0 (12.1 ï 13.9)	13.0 (12.0 ï 13.7)	13.1 (12.2 ï 13.9)	NS
Leukocyte, × 10^9/l	7.5 (6.2 ï 9.3)	7.3 (6.1 ï 8.9)	7.7 (6.2 ï 9.5)	NS

Platelet, × 10^9/l	293 (240 ï 366)	291 (252 ï 373)	297 (231 - 365)	NS

Values are n (%) or median (IQR); * Values are mean (SD); ÿ p values (from Chi-square or two samplesôT-test) show significant differences between patients who never smoked and who ever smoked, without adjustment for potential confounding factors; NS, not significant.

Table 4.1.2b Characteristics of cigarette smoking in all RA patients and patients stratified by gender

	All (n = 428)	Male (n = 137)	Female (n = 291)
Ever-smoked	285/428 (66.6)	113/137 (82.5)	170/291 (58.4)
Current smokers, n (%)	75/428 (17.5)	25/137 (18.2)	50/291 (17.2)
Past smokers, n (%)	210/428 (49.1)	88/137 (64.2)	120/291 (41.2)
*Cigarettes per day, n	15 (8 - 20)	20 (10 - 20)	10 (7 - 20)
*Years of smoking, year	31 (18 - 41)	33 (18.8 - 44)	29 (18 - 40)
*Total amount, pack-years	18.8 (8.5 - 33)	22 (9 - 37)	16.5 (7.75 - 30)

* Excluded the non-smoking samples; Values are n of positive case (%) or median (IQR); pack-years, packs (20 cigarettes) per day × years of smoking.

4.1.3 Smoking, RF and anti-CCP

Complete data of RF, anti-CCP and ever-smoked status was available in 413 patients. RF and anti-CCP status were strongly associated with each other ($p < 0.0001$). Among 413 patients, 221 (53.5%) were positive for both RF and anti-CCP, while 84 (20.3%) were negative for both markers. There were 91 (22.0%) patients recorded as RF-/anti-CCP+, and 17 (4.1%) as RF+/anti-CCP-. A trend in terms of frequencies of ever smokers in each subset (Table 4.1.3a) implied that smoking may be more closely associated with RF in this

RA population. This was a general indication without consideration of any genetic interaction with smoking.

Table 4.1.3a Combination of RF and anti-CCP status stratified by smoking

	Never-smoked (n = 137)	Ever-smoked (n = 276)	OR (95% CI)
RF-/anti-CCP-	38 (45.2%)	46 (54.8%)	Referent
RF-/anti-CCP+	36 (39.6%)	55 (60.4%)	1.26 (0.69 ï 2.29)
RF+/anti-CCP-	4 (23.5%)	13 (76.5%)	2.48 (0.79 ï 7.84)
RF+/anti-CCP+	59 (26.7%)	162 (73.3%)	2.26 (1.34 ï 3.80)

Values are n (%).

We tested the effects of smoking (ever-smoked) and anti-CCP status on the presence of RF using multivariate logistic regression analysis. In a model that contained both smoking and anti-CCP as independent variables and RF as dependent variable, it was found that smoking and anti-CCP were both independently associated with RF (OR 1.92, 95% CI 1.18 ï 3.13 and OR 11.75, 95% CI 6.50 ï 21.23 respectively, corrected for sex, age and RA duration). The effect of smoking (independent variable) on the presence of anti-CCP (dependent variable) also achieved significance (OR 1.92, 95% CI 1.17 ï 3.15, corrected for sex, age and RA duration) in a model without inclusion of RF. However, addition of RF (independent variable) as well as smoking in the same model revealed a strong association with RF, while the association with smoking became insignificant (OR 1.32, 95% CI 0.75 ï 2.29, corrected for sex, age and RA duration).

The level of RF and anti-CCP were strongly correlated with each other ($r = 0.44$, $p < 0.0001$). In smokers (ever-smoked), the RF level was associated with the number of years

of smoking ($p < 0.0001$, corrected for sex, age and RA duration), but not with the cigarettes per day. It was also associated with the pack-years ($p = 0.007$, corrected for sex, age and RA duration), although this association disappeared when included in a model with the number of years of smoking. The latter appeared to be the primary association. Anti-CCP level was also associated with the number of years of smoking ($p = 0.0012$, corrected for age, sex and RA duration), but not with the cigarettes per day. Once adjusted for RF level, the significance of this association reduced ($p = 0.074$, corrected for age, sex and RA duration).

In both genders, smoking was associated with the presence of RF. It showed a more significant effect in males with OR = 4.35 (95% CI 1.61 ï 11.74, corrected for age and RA duration), compared to that in females (OR 1.95, 95% CI 1.20 ï 3.14, corrected for age and RA duration). In either males or females only, no significant association was achieved between smoking and anti-CCP status (adjusted for age and RA duration).

4.1.4 Smoking and RF in GSTM1-null genotype

A previous report from our laboratory demonstrated that RF production was associated with cigarette smoking only in GSTM1-null patients (Mattey et al, 2002). In the present study we have confirmed this association in a larger group of patients. GSTM1 genotyping, RF and smoking status were available in 423 patients. In GSTM1-null patients (n = 236), ever-smoked status was associated with higher rate of RF positivity compared with never-smoking (never-smoking vs. ever-smoking, 32/80 (40.0%) vs. 101/155 (65.2%), OR 95% CI 3.32 (1.84 ï 5.98), $p = 0.046$, corrected for age, sex and RA duration). In contrast, in GSTM1-expressing patients (n = 187) there was no such significant difference (never-

smoking vs. ever-smoking, 31/61 (50.8%) vs. 75/124 (60.5%), OR 95% CI 1.50 (0.79 ï 2.86), p = 0.51, adjusted for age, sex and RA duration).

4.2 SMOKING AND DISEASE SEVERITY

4.2.1 Brief introduction

The presence of RF and anti-CCP is known to be associated with a more significant disease activity and worse functional outcome. Whether there are effects of smoking on disease severity independent of its influence on seropositive status remains to be clarified.

4.2.2 RF, anti-CCP and RA clinical measures

In order to assess whether the effects of smoking on RA outcome were independent of RF and/or anti-CCP, the effects from these autoantibodies were first tested. Activity/severity of RA were estimated by several clinical measures including DAS28, MJS, HAQ, and erosive and nodular diseases. Since disease activity will largely be determined by the levels of inflammation over time we also investigated associations with inflammatory markers including ESR and CRP in these patients.

RF positive patients displayed higher levels of disease activity and worse outcome for all clinical measures, compared to that in RF negative patients (Table 4.2.2a). Significant differences were achieved in ESR, CRP, DAS28, nodular disease, MJS and HAQ. When stratified by anti-CCP status, it was found that ESR, CRP, DAS28, MJS, and erosive and

nodular diseases showed significant differences between positive and negative patients (Table 4.2.2a).

As shown, RF and anti-CCP were highly correlated with each other. In order to test their individual impact on disease measures, the confounding effect between these 2 factors needed to be considered. Multivariate multiple or logistic regression models that contained both RF and anti-CCP as independent variables thus were used. In these models, the association of RF with disease measures remained significant, whereas the association of anti-CCP with most parameters was lost except for that with ESR and erosive disease (Table 4.2.2b). Based on these adjusted results, RF was strongly associated with most measures of disease activity and severity, although it was not predictive of erosive disease. On the other hand, anti-CCP was a marker that specifically associated with erosive disease in RA.

Table 4.2.2a Difference of disease process and outcome measures between seropositive and negative RA

	Process (activity) measures			Outcome (severity) measures			
	ESR	CRP	DAS28	Nodules	Erosion	MJS	HAQ
Stratified by RF							
+	26.0 (13.0 ï 42.0)	12.3 (5.0 ï 23.1)	4.49 ± 1.41	42/245 (17.1%)	186/231 (77.2%)	8.0 (4.0 ï 18.0)	1.8 (1.1 ï 2.1)
ï	14.5 (8.3 ï 25.0)	8.0 (3.8 ï 16.1)	3.79 ± 1.24	10/184 (5.4%)	124/180 (68.9%)	6.0 (2.0 ï 13.0)	1.4 (0.8 ï 2.0)
Positive vs. negative	p < 0.0001	p = 0.003	p < 0.0001	p = 0.0002	NS	p = 0.011	p < 0.0001
Stratified by anti-CCP							
+	22.0 (11.0 ï 39.0)	11.5 (4.5 ï 20.8)	4.27 ± 1.38	46/315 (14.6%)	245/312 (78.3%)	8.0 (4.0 ï 17.0)	1.6 (1.0 ï 2.1)
ï	14.0 (7.0 ï 25.3)	8.1 (3.8 ï 15.8)	3.89 ± 1.39	6/103 (5.8%)	57/97 (58.8%)	4.0 (2.0 ï 11.0)	1.5 (0.8 ï 2.0)
Positive vs. negative	p < 0.0001	p = 0.038	p = 0.008	p = 0.019	p = 0.0001	p = 0.0008	NS

Values are median (IQR) or mean ± standard deviation (SD); NS, not significant.

Table 4.2.2b Relationship between disease measures and RF/anti-CCP status in multivariate multiple/logistic regression models

	Dependent variable						
	Model 1: ESR*	Model 2: CRP*	Model 3: DAS28	Model 4: Nodules	Model 5: Erosion	Model 6: MJS[À]	Model 7: HAQ
Independent variable							
RF +/-	p < 0.0001	p = 0.016	p < 0.0001	p = 0.003 OR (95% CI): 3.55 (1.52 ï 8.31)	NS	p = 0.020	p < 0.0001
Anti-CCP +/-	p = 0.041	NS	NS	NS	p = 0.017 OR (95% CI): 2.06 (1.14 ï 3.72)	NS	NS

OR, odds ratio; NS, not significant; * and [À], log and square root transformation respectively; Multiple or logistic regression models (1 - 7) contained both RF and anti-CCP status as independent variables at the same time, and adjusted for age, sex and RA duration.

4.2.3 Smoking and RA clinical measures

Initial analysis indicated that there were relationships between smoking and disease outcome measures including HAQ ($p = 0.023$) and erosive disease ($p = 0.050$), adjusted for sex, age and RA duration. However, these may be due to the confounding associations of RF with HAQ and anti-CCP with erosive disease. Adjustment of these confounders in multivariate regression (multiple or logistic) models revealed an insignificant effect of smoking on disease outcome measures (HAQ and erosive disease) in the whole RA cohort (Table 4.2.3a and 4.2.3b).

Interestingly, when we analysed the relationship in seropositive and negative patients separately, we found that smoking was associated with HAQ in RF- and anti-CCP- patients. Also, smoking was associated with erosive disease in anti-CCP- patients. It was associated with an increase in the HAQ score and the occurrence of erosive disease in seronegative patients who displayed less disease severity compared to seropositive patients (Table 4.2.3a and 4.2.3b). Similar trends were also seen with regard to inflammatory and disease activity measures (ESR, CRP and DAS28), but only the association with ESR achieved significance in anti-CCP negative patients (data not shown). Effects on MJS and nodular disease were not seen.

To completely remove any effect from RF and anti-CCP, we also carried out analyses restricted to patients who were negative for both RF and anti-CCP. Among these samples, 46 (54.8%) were classified as ever-smokers, while 38 (45.2%) were non-smokers. Differences were found in regard to HAQ and erosive disease between patients who had ever or never smoked (Table 4.2.3a and 4.2.3b).

Table 4.2.3a Relationship between smoking and HAQ in patients stratified by RF or anti-CCP

Patient groups	HAQ
In all patients	
Ever-smoked (n = 283)	1.63 (1.13 ï 2.0)
Never-smoked (n = 145)	1.44 (0.78 ï 2.0)
Ever-smoked vs. never-smoked	NS
In RF+ patients	
Ever-smoked (n = 179)	1.75 (1.25 ï 2.13)
Never-smoked (n = 65)	1.75 (1.13 ï 2.25)
Ever-smoked vs. never-smoked	NS
In RF- patients	
Ever-smoked (n = 103)	1.50 (0.88 ï 2.0)
Never-smoked (n = 79)	1.25 (0.63 ï 1.88)
Ever-smoked vs. never-smoked	p = 0.038 (age, sex and RA duration adjusted)
In anti-CCP+ patients	
Ever-smoked (n = 218)	1.63 (1.10 ï 2.13)
Never-smoked (n = 95)	1.63 (1.0 ï 2.10)
Ever-smoked vs. never-smoked	NS
In anti-CCP- patients	
Ever-smoked (n = 58)	1.63 (1.0 ï 2.0)
Never-smoked (n = 42)	1.25 (0.50 ï 1.91)
Ever-smoked vs. never-smoked	p = 0.037 (age, sex, RA duration and RF adjusted)
In RF- and anti-CCP- patients	
Ever-smoked (n = 46)	1.50 (0.88 ï 2.0)
Never-smoked (n = 38)	1.19 (0.44 ï 1.78)
Ever-smoked vs. never-smoked	p = 0.027 (age, sex and RA duration adjusted)

Values are median (IQR); NS, not significant; P values are obtained from multivariate multiple regression models and are adjusted for confounding factors where appropriate.

Table 4.2.3b Relationship between smoking and erosive status in patients stratified by RF or anti-CCP

Patient groups	Erosive status
In all patients	
Ever-smoked	212/278 (76.3)
Never-smoked	45/144 (68.8)
Ever-smoked vs. never-smoked	NS
In RF+ patients	
Ever-smoked	137/176 (77.8)
Never-smoked	48/64 (75.0)
Ever-smoked vs. never-smoked	NS
In RF- patients	
Ever-smoked	74/101 (73.3)
Never-smoked	50/29 (63.3)
Ever-smoked vs. never-smoked	NS
In anti-CCP+ patients	
Ever-smoked	171/216 (79.2)
Never-smoked	72/95 (75.8)
Ever-smoked vs. never-smoked	NS
In anti-CCP- patients	
Ever-smoked	35/55 (63.6)
Never-smoked	22/42 (52.4)
Ever-smoked vs. never-smoked	p = 0.030 (age, sex, RA duration adjusted)
In RF- and anti-CCP- patients	
Ever-smoked	27/44 (61.4)
Never-smoked	19/38 (50.0)
Ever-smoked vs. never-smoked	p = 0.047 (age, sex and RA duration adjusted)

Values are n (%); NS, not significant; P values are obtained from multivariate logistic regression models and are adjusted for confounding factors where appropriate.

4.3 SMOKING AND CO-MORBIDITY

4.3.1 Brief introduction

Cigarette smoking affects multiple organ systems and results in numerous smoking-induced diseases, notably CVD. The most common CVD is IHD. In RA, the clinical presentation of IHD appears to be different to that in the normal population. It was therefore of a great interest to investigate the relationship between smoking and IHD as well as other disease conditions in this specific subset of the RA population.

4.3.2 Smoking, IHD and MI

Patients who had ever smoked had an increased likelihood of IHD and MI, compared to those who had never smoked (OR 95% CI, 2.52 (1.28 ï 4.95) and OR 95% CI, 4.24 (1.45 ï 12.37) respectively, adjusted for age, sex and RA duration). Amongst smokers, strong associations were found between pack-years and IHD/MI ($p = 0.009$ and $p = 0.006$ respectively, corrected for age, sex and RA duration). Further details, including associations with VEGFA-smoking interaction and TGFB1-smoking interaction are presented in Part C and Part F of the Results respectively.

4.3.3 Smoking and the number of presented co-morbidities

Although no significant associations were found between smoking and other co-morbid conditions (including diabetes type 1 and 2, cerebrovascular accident (CVA), asthma, cancers, hypertension and hypercholesterolaemia), patients who had ever smoked had an

increased likelihood of presenting with more co-morbidities (Table 4.3.3a, n < 3 vs. n Ó3 (n: any recorded co-morbid condition), OR 2.02, 95% CI 1.10 ï 3.73, corrected for age, sex and RA duration). This suggests that smoking may be a risk factor for several co-morbid conditions in RA. The lack of association of smoking with individual co-morbidities (apart from CVD) may be a reflection of the relatively low numbers of some conditions in this group of patients.

Table 4.3.3a Relationship between ever-smoked status and the number of presentation of co-morbid conditions (n < 3 vs. n Ó3)

	Number of co-morbid conditions Ó3	
Ever-smoked status	ï	+
ï	118	20 (14.5)
+	194	75 (27.9)

4.4 SUMMARY OF FINDINGS FOR 4.1 – 4.3

- Smoking was more closely associated with the presence of RF than anti-CCP in this RA population. The release of RF was associated with the number of years of smoking rather than cigarettes per day or pack-years.

- This study provided support for a previous report (Mattey et al, 2002) that smoking was only significantly associated with RF in GSTM1-null patients.

- Seropositive RA was more likely to have high disease activity, leading to more severe disease. In particular, RF appeared to be more closely associated with markers of disease activity (ESR, CRP and DAS28), nodular status and those disease severity measures that included an element of functional outcome (MJS and HAQ). On the other hand, there was superiority of anti-CCP over RF in predicting an erosive course.

- Smoking was associated with RA outcome measures (HAQ and erosive disease) particularly in seronegative patients.

- There were associations of smoking with the presence of IHD and previous MI in patients with RA.

- Smoking was associated with increased number of co-morbid conditions in patients with RA.

PART B: GSTM1, T1 POLYMORPHISM, SEVERITY AND CO-MORBIDITY IN RA (4.5 – 4.8)

4.5 GSTM1, T1 POLYMORPHISM

4.5.1 Brief introduction

The GST enzymes are believed to be important in detoxifying products generated by the activity of ROS. Homozygous deletions of the GSTM1 and GSTT1 genes are common and result in a complete loss of enzyme activity (Rebbeck 1997; Bell et al, 1993; Pemble et al, 1994). It has been shown that GSTM1-null and GSTT1-null genotypes are associated with increased risk of smoking-related diseases, such as lung cancer (Seidergard et al, 1990; Taioli et al, 2003). Thus, it was of interest to investigate whether the GSTM1/T1 polymorphism was associated with disease severity and/or co-morbid conditions in RA population.

4.5.2 Non-functional null polymorphism

Both GSTM1 and T1 genes have expressible alleles and a non-functional null allele (GSTM1*0/GSTT1*0) caused by deletion of the GSTM1/GSTT1 gene sequence. By amplifying the gene sequence (using PCR), it is possible to distinguish between homozygous GSTM1*0/GSTT1*0 and genotypes with least 1 copy of the expressible allele.

Genotypic distribution

GSTM1 and GSTT1 genotypes were both determined in 430 RA patients. Two hundred and thirty eight (55.3%) patients did not express GSTM1 enzyme (homozygous GSTM1*0), while 67 (15.5%) patients did not express GSTT1 enzyme (homozygous GSTT1*0). The frequencies of homozygosity for GSTM1*0 and GSTT1*0 in this group of RA patients were similar to those in the general population. Homozygosity for GSTM1*0 and GSTT1*0 occurred in ~ 50% and ~ 20% of Caucasians respectively (Hatagima et al, 2004). Table 4.5.2a shows the combination of GSTM1 and T1 genotypes in this group of RA patients.

Table 4.5.2a Combination of GSTM1 and T1 genotypes (expressing vs. null) in RA

Combination	Number (%)
M1-expressing, T1-expressing	157 (36.5%)
M1-null, T1-expressing	206 (47.9%)
M1-expressing, T1-null	35 (8.1%)
M1-null, T1-null	32 (7.4%)

4.6 GSTM1, T1 POLYMORPHISM AND DISEASE SEVERITY

4.6.1 Brief introduction

It has been well established that smoking plays a significant role in RA. Previous work from our laboratory indicated that the non-functional null polymorphism of the GSTM1 gene was associated with worse radiographic damage (Larsen score) and functional

outcome (HAQ) in a group of 164 female RA patients. This association primarily occurred in ever smokers, which may suggest the importance of this enzyme in detoxifying products generated by cigarette smoking. Furthermore, it was also found that the production of RF was only linked to smoking in the absence of functional GSTM1 gene, implying this gene may be associated with the prevention of RF release (Mattey et al, 2002). This study was to confirm the previous data and to evaluate the GSTT1 function in a larger population of RA.

4.6.2 GSTM1, T1 genotype, smoking and RF

In patients carrying homozygosity for GSTM1*0, there was an association of ever-smoked status with RF production, whereas in patients with functional GSTM1 gene, this association did not exist. This observation was described in Section 4.1.4. Stratified by GSTT1 genotypes (T1-expressing vs. T1-null) ever-smoked status was associated with RF in both subsets of patients (data not shown), suggesting that the GSTT1 gene was not involved in this association.

4.6.3 GSTM1 genotype and erosive status

In all patients, no association of GSTM1 and T1 genotypes with disease measures including DAS28, MJS, HAS and nodular and erosive status was found. A significant association of GSTM1-null genotype with erosive disease occurred in patients with RF positive status (Table 4.6.3a). A similar relationship was also found when patients were stratified by anti-CCP or ever-smoked status, although the result was not significant (Table

4.6.3b and c). Combination of GSTM1 and T1 genotypes revealed no additional associations.

To test which factors (RF, anti-CCP, ever-smoked) were the primary ones that interacted with GSTM1-null in terms of the development of erosive disease, we carried out a multivariate logistic regression analysis, which contained RF, anti-CCP and ever-smoked status together as independent variables and erosive status as dependent variable, within patients carrying GSTM1-null genotype. Interestingly, the model indicated that ever-smoking and anti-CCP+ were independently associated with erosive disease in GSTM1-null patients, while RF+ status was not significantly involved in the same patients (Table 4.6.3d). Analysis within the patients carrying GSTM1-expressing genotype revealed that none of these factors were associated with erosive disease (Table 4.6.3d). This suggested that the association of ever-smoking and anti-CCP+ with erosive disease depended on their interactions with the GSTM1-null genotype.

Table 4.6.3a Relationship between GSTM1 genotype (expressing vs. null) and erosive disease in all patients and in patients stratified by RF status

	All patients		RF-patients		RF+ patients	
GSTM1	Erosive-	Erosive+	Erosive-	Erosive+	Erosive-	Erosive+
expressing	56	133 (70.4)	23	56 (70.9)	33	75 (69.4)
null	55	176 (76.2)	33	67 (67.0)	22	108 (83.1)
		NS		NS		*p = 0.028

Values are n (%); * Adjustment for age, sex and RA duration; NS, not significant.

Table 4.6.3b Relationship between GSTM1 genotype (expressing vs. null) and erosive disease in all patients and in patients stratified by anti-CCP status

	All patients		Anti-CCP- patients		Anti-CCP+ patients	
GSTM1	Erosive-	Erosive+	Erosive-	Erosive+	Erosive-	Erosive+
expressing	56	133 (70.4)	17	27 (61.4)	37	103 (73.6)
null	55	176 (76.2)	23	30 (56.6)	31	139 (81.8)
		NS		NS		NS (*p = 0.095)

Values are n (%); * Adjustment for age, sex and DD; NS, not significant.

Table 4.6.3c Relationship between GSTM1 genotype (expressing vs. null) and erosive disease in all patients and in patients stratified by ever-smoked status

	All patients		Never-smoked patients		Ever-smoked patients	
GSTM1	Erosive-	Erosive+	Erosive-	Erosive+	Erosive-	Erosive+
expressing	56	133 (70.4)	19	43 (69.4)	35	89 (71.8)
null	55	176 (76.2)	25	54 (68.4)	29	121 (80.7)
		NS		NS		NS (*p = 0.074)

Values are n (%); * Adjustment for age, sex and DD; NS, not significant.

Table 4.6.3d Relationship between ever-smoking, anti-CCP and RF status, and erosive disease in multivariate logistic regression, analysed separately in GSTM1-null and GSTM1-expressing patients

	Erosive status (GSTM1-null patients)			Erosive status (GSTM1-expressing patients)		
Independent variable	Regression coefficient	p value	OR 95% CI	Regression coefficient	p value	OR 95% CI
Ever-smoked	0.77	0.033	2.14 (1.06 ï 4.33)	-0.029	0.94	0.97 (0.46 ï 2.04)
Anti-CCP+	0.81	0.010	2.61 (1.26 ï 5.38)	0.66	0.16	1.94 (0.77 ï 4.88)
RF+	0.32	0.43	1.37 (0.62 ï 3.02)	-0.45	0.29	0.63 (0.28 ï 1.47)
Age	-0.047	0.011	0.95 (0.92 ï 0.99)	0.019	0.23	1.02 (0.99 ï 1.05)
Female	0.49	0.19	1.63 (0.78 ï 3.38)	-0.24	0.56	0.79 (0.36 ï 1.74)
RA duration	0.085	0.0002	1.09 (1.04 ï 1.14)	0.064	0.0037	1.06 (1.02 ï 1.12)

4.7 GSTM1, T1 POLYMORPHISM AND CO-MORBIDITY

4.7.1 Brief introduction

Associations of GSTM1-null and T1-null genotypes with the development of atherosclerosis and coronary artery disease have been observed in the general population (Abu-Amero et al, 2006; Wang et al, 2010; Girisha et al, 2004; Wilson et al, 2003). In particular, there were numerous studies suggested that interaction of these genotypes with smoking may be responsible for such associations (Wang et al, 2008; Kim et al, 2008; Manfredi et al, 2007; Tamer et al, 2004; Miller et al, 2003; Olshan et al, 2003; Masetti et al, 2003; Wang et al, 2002; Li et al, 2000). Furthermore, both M1 and T1 have been

associated with the risk of MI (Wilson et al, 2000; Cornelis et al, 2007). Recently, studies focusing on these 2 polymorphisms also indicated that they were associated with hypertension in the general population (Cruz-Gonzalez et al, 2009; Capoluongo et al, 2009; Delles et al, 2008; Oniki et al, 2008; Marinho et al, 2007). It was therefore interesting to see if there were such relationships in this RA population.

4.7.2 GSTM1, T1 genotype and co-morbid condition

No association of GSTM1 or T1 genotype with any co-morbid condition recorded in the studied population was found. No evidence of interaction with smoking was found.

4.8 SUMMARY OF FINDINGS FOR 4.5 – 4.7

- The frequencies of homozygosity for GSTM1*0 and GSTT*0 in RA population were similar to those in the general population.

- We confirmed that the association of smoking with RF only occurred in patients with non-functional GSTM1 genotype.

- In univariate analyses, RF+ (significantly), anti-CCP+ (insignificantly) and ever-smoked status (insignificantly) were only associated with erosive disease in GSTM1-null patients. Multivariate logistic regression containing these 3 factors together as independent variables indicated that anti-CCP+ and ever-smoked status were independent risks for erosive disease in patients with GSTM1-null genotype.

PART C: VEGFA POLYMORPHISM, SERUM LEVEL OF VEGF-A, SEVERITY AND CO-MORBIDITY IN RA (4.9 – 4.12)

4.9 VEGFA POLYMORPHISM AND SERUM VEGF-A LEVEL

4.9.1 Brief introduction

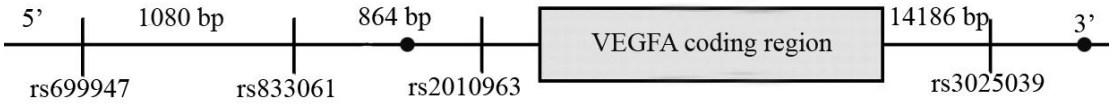
Polymorphisms in the VEGFA gene have been reported to be associated with many disorders where angiogenesis may play a critical role in disease development. It has been suggested that these associations may be explained by the alteration of VEGF-A expression. SNPs in the promoter region (rs699947 and rs833061), 5ô-untranslated region (UTR) (rs2010963) and 3ô-UTR (rs3025039) have been associated with differential expression of VEGF-A protein, although there is inconsistency between studies (Watson et al, 2000; Awata et al, 2002; Stevens et al, 2003; Renner et al, 2000; Koukourakis et al, 2004; Prior et al, 2006). This relationship has not yet been investigated in subjects with RA. As a key promoter of angiogenesis, VEGF-A is thought to play an important role in the pathogenesis and progression of RA. Thus, it was interesting to investigate whether polymorphism in the VEGFA gene was associated with the circulating level of VEGF-A protein in patients with RA.

4.9.2 VEGFA polymorphism

Studied SNP

Polymorphism rs699947 is normally known as VEGFA-2578(A/C), named after its position relative to the translation start site of the gene (-1540 relative to the transcription start site). SNPs rs833061 and rs2010963 are known as VEGFA-460(C/T) and +405(C/G) respectively. They are identified by their positions relative to the transcription start site. Alternatively, rs2010936 is also referred to as VEGFA-634 based on its location according to the translation start site. These 3 polymorphisms are upstream of the VEGFA coding region, with -2578 and -460 in the promoter region, and +406 in the 5ô-UTR. SNP rs3025039, known as VEGFA+936(C/T), is named after its position relative to the transcription end site. It is located in the 3ô-UTR, downstream of the VEGFA coding region. These 4 SNPs are the most common and studied polymorphisms in the VEGFA gene. Figure 4.9.2a shows the location of each SNP and their physical distance between each other.

Figure 4.9.2a Diagram of human VEGFA gene highlighting the location of its common polymorphisms



The positions of rs699947, rs833061, rs2010963 and rs3025039 at chromosome 6 are 43736389, 43737486, 43738350 and 43752536 respectively. Physical distances in bp between SNPs are shown. Left and right dots indicate the transcription start site and end site respectively.

Allelic and genotypic distribution

Genotypes were determined in 428 RA subjects for VEGFA-2578 SNP, 430 subjects for -460 and 429 subjects for +405 and +936. The allelic and genotypic frequencies of the 4 VEGFA SNPs in our RA patients and in healthy subjects of UK Caucasoid origin are shown in Table 4.9.2a. Genotypes of -2578, -460, +405 and +936 were all distributed in accordance with a close fit to HWE. No significant difference of allelic distribution between our RA patients and healthy UK Caucasians was observed.

Table 4.9.2a Distribution of allelic and genotypic frequencies of VEGFA-2578(A/C), -460(C/T), +405(C/G) and +936(C/T) polymorphisms in RA patients and in the normal healthy UK Caucasians

	Patients with RA n (%)	Healthy subjects* n (%)		Patients with RA n (%)	Healthy subjects* n (%)		Patients with RA n (%)	Healthy subjects* n (%)		Patients with RA n (%)	Healthy subjects* n (%)
-2578			-460			+405			+936		
Genotype			Genotype			Genotype			Genotype		
AA	112(26.2)	16(21.6)	CC	106(24.7)	NA	CC	40(9.3)	NA	CC	308(71.8)	NA
AC	207(48.4)	34(45.9)	CT	218(50.7)	NA	CG	186(43.4)	NA	CT	107(24.9)	NA
CC	109(25.5)	24(32.4)	TT	106(24.7)	NA	GG	203(47.3)	NA	TT	14(3.3)	NA
Allele			Allele			Allele			Allele		
A	431(50.4)	66(44.6)	C	430(50.0)	121(52.6)	C	266(31.0)	67 (29.1)	C	723(84.3)	NA
C	425(49.6)	82(55.4)	T	430(50.0)	109(47.4)	G	592(69.0)	163(70.9)	T	135(15.7)	NA
		$p > 0.05$			$p > 0.05$			$p > 0.05$			

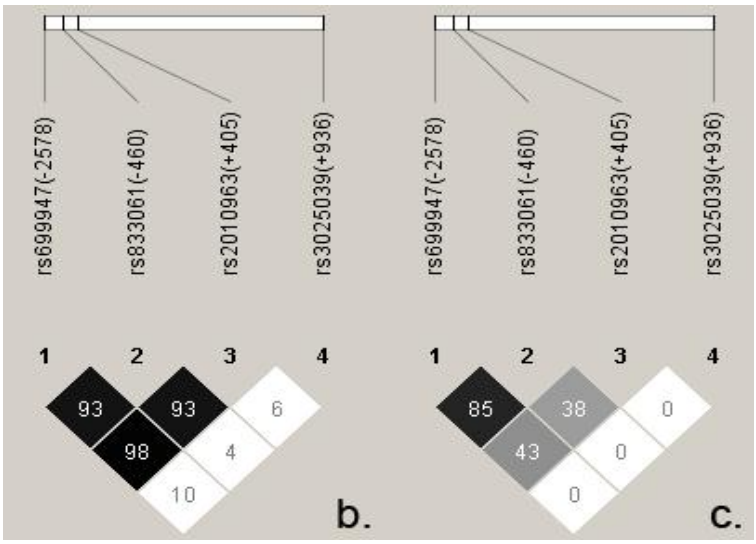
* Adapted data from Brogan et al, 1999 (VEGFA-2578) and Watson et al, 2000 (VEGFA-460 and +405); NA, not available.

Linkage disequilibrium and haplotype

Pair-wise LD coefficients (*D'*) and correlations (r^2) between the 4 SNPs are shown in Figure 4.9.2b and c respectively. Strong LD was detected in the following pairs (-2578, -460), (-2578, +405) and (-460, +405), with *D'* being over 0.93 for all pairs. It was noteworthy that the pair (-2578, +405) scored the highest *D'* (0.98), although these 2 SNPs were not physically next to each other. Pair-wise correlation between VEGFA-2578 and -460 was high ($r^2 = 0.85$), whereas the correlations between +405 and the 2 promoter SNPs were only moderate (r^2 å 0.40). A high *D'* and low r^2 can occur if the alleles that tend to occur on the same haplotype have very different allele frequencies. No LD was observed between +936 and any other SNPs within the upstream region of the VEGFA gene.

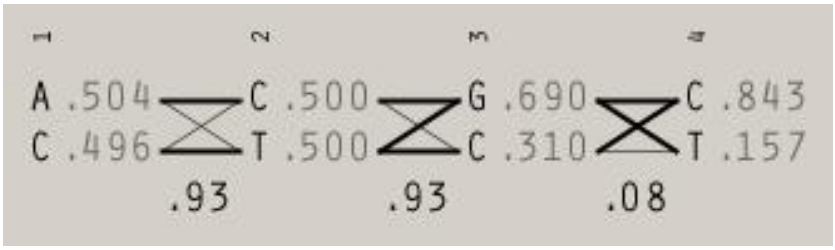
It was noted that the A (-2578), C (-460) and G (+405) alleles were strongly linked with each other, with the majority of A (-2578) and C (-460) alleles being components of the A-C-G haplotype, whereas the C (-2578) and T (-460) alleles were highly associated with each other, and together with the +405 SNP they tended to occur in the C-T-C and C-T-G haplotypes (Figure 4.9.2d). The frequencies of A-C-G, C-T-C and C-T-G haplotypes were 0.483, 0.297 and 0.182 respectively. Haplotypes across the 4 SNPs are listed in Table 4.9.2b. In the promoter region marked with -2578 and -460, the major haplotypes were A-C and C-T only.

Figure 4.9.2b-c Pair-wise LD of VEGFA SNPs measured by *D'* and r^2



Numbers in boxes represent *D'* and r^2 values (%) between paired SNPs in Figure b and c respectively.

Figure 4.9.2d Details of allelic linkage between neighbouring SNPs of VEGFA-2578, -460, +405 and +936



One, 2, 3 and 4 represent VEGFA-2578, -460, +405 and +936 respectively. Numbers next to alleles shows the frequencies of the alleles. Numbers at bottom shows the *D'* values between the neighbouring SNPs. Thick/thin lines represent the different strength of linkage, with thin lines showing the connection > 1% but Ò10%, while thick lines showing the connection > 10.0%.

Table 4.9.2b Frequency of haplotypes across the VEGFA-2578, -460, +405 and +936

Haplotype	Frequency	Haplotype	Frequency
A-C-C-C	0.0	C-C-C-C	0.009
A-C-C-T	0.0	C-C-C-T	0.0
A-C-G-C	**0.399**	C-C-G-C	0.007
A-C-G-T	**0.084**	C-C-G-T	0.001
A-T-C-C	0.0	**C-T-C-C**	**0.241**
A-T-C-T	0.003	**C-T-C-T**	**0.056**
A-T-G-C	0.017	**C-T-G-C**	**0.169**
A-T-G-T	0.001	C-T-G-T	0.013

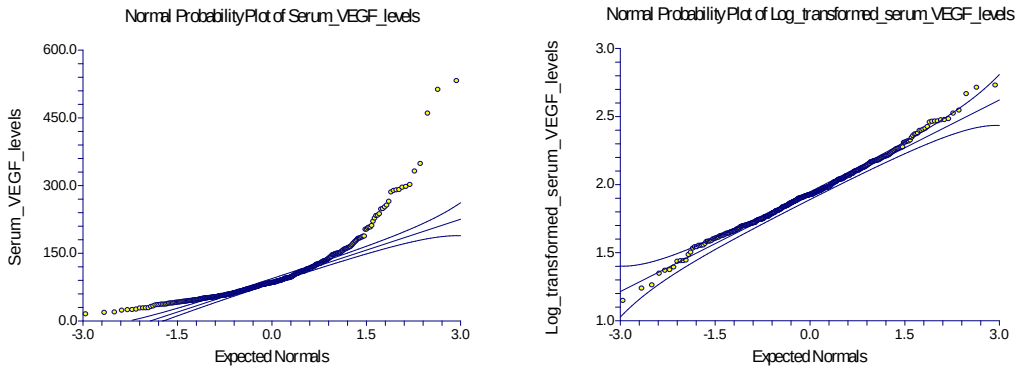
Common haplotypes are marked in black (frequency threshold = 0.05).

4.9.3 Serum level of VEGF-A

Determination and distribution

Serum VEGF-A levels were determined in 414 patients. Its distribution was rejected for normality. However, after log transformation, the values were normally distributed (Figure 4.9.3a). The median level was 82.72 (IQR 57.46 ï 119.13) pg/ml.

Figure 4.9.3a Distribution of serum VEGF-A levels before (left) and after (right) log transformation



Serum VEGF-A level, cigarette smoking and patient demographics

No difference of serum VEGF-A levels between smokers and non-smokers was seen (ever-smoker vs. non-smoker, 83.36 vs. 82.28 pg/ml, p = 0.77), although there was some insignificant effects from heavy cigarette consumption (pack-years > 30 vs. the remaining subjects, 88.51 vs. 81.70 pg/ml, p = 0.32). No significant association of age, sex, BMI or RA duration with serum VEGF-A levels was found.

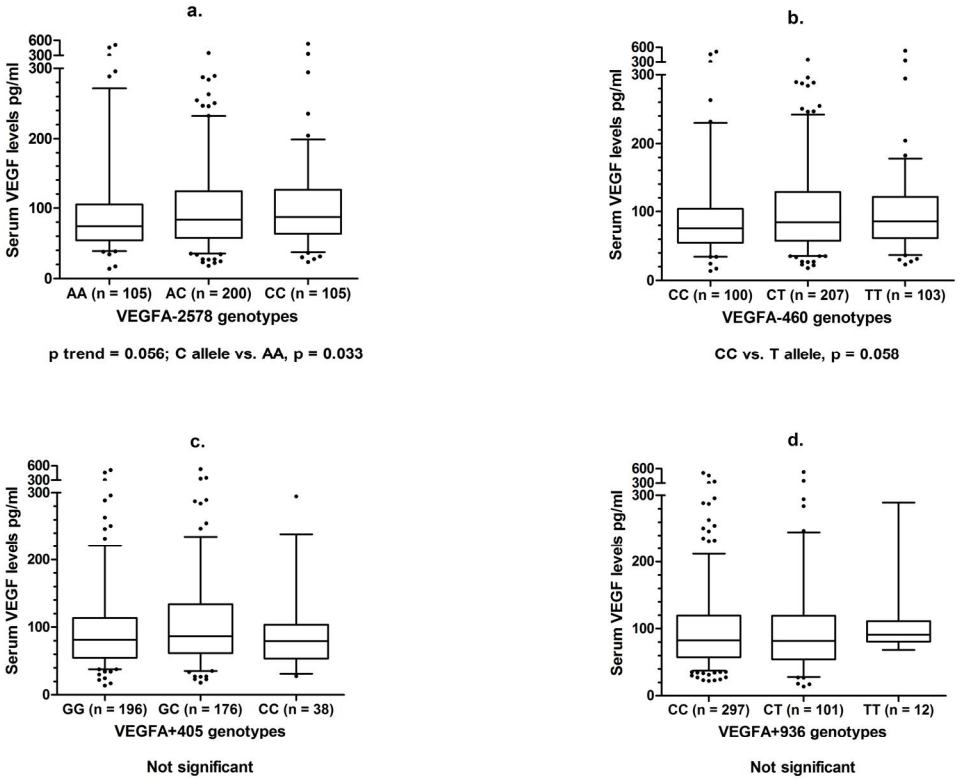
4.9.4 VEGFA polymorphism and serum VEGF-A level

Analysis in the whole population

VEGFA genotypes and serum VEGF-A data were both available in 410 patients. Serum levels of VEGF-A in each genotypic group of the studied VEGFA SNPs are shown in Figure 4.9.4a-d. Polymorphisms in the promoter region (-2578 and -460) showed a relationship with serum VEGF-A. Statistical analysis indicated that the association with -2578 was significant, whereas the one with -460 was not. Stratified by -2578 genotypes, VEGF-A levels displayed an increasing trend that was associated with the number of copies of the C allele (AA vs. AC vs. CC, 74.0 vs. 83.54 vs. 87.18 pg/ml, p trend = 0.056). Alternatively, a dominant model suggested that the levels were significantly higher in patients carrying the C allele of VEGFA-2578, compared with that in AA subjects (85.74 vs. 74.0 pg/ml, p = 0.033). This association remained significant (p = 0.045) after adjustment for age, sex and RA duration in multiple regression where the levels had been log transformed. Haplotype analysis with HAPSTAT program suggested a relationship between A-C-G (-2578/-460/+405) and decreased serum VEGF-A levels, although it was

not significant (vs. the remaining haplotypes, dominant model, p = 0.058). Status of ever-smoking, RF or anti-CCP did not differentiate the above observation.

Figure 4.9.4a-d A box-and-whisker diagram of serum VEGF-A levels stratified by VEGFA-2578, -460, +405 and +936 polymorphisms in RA



Boxplot with whiskers with 5 ï 95 percentile.

Stratification by inflammation status

At the time of recruitment, 52.1% of patients presented with increased CRP levels (Ó 10 mg/l), suggesting that obvious inflammation was present in these patients. The remaining patients showed normal CRP levels (< 10 mg/l), indicating an absence of obvious inflammation. The characteristics of all patients, and patients stratified by CRP status (< 10

mg/l vs. Ó 10 mg/l) are displayed in Table 4.9.4a. No differences of age, gender distribution, BMI, age of RA onset, RA duration and smoking status were seen between stratified groups. Not surprisingly, increased positivity for autoantibodies, higher disease activity and worse functional outcome were associated with the group with increased CRP. No difference in nodular or erosive status was found between these 2 subsets.

Table 4.9.4a Characteristics of all patients and patients stratified by CRP status

	All patients	Patients with normal CRP (< 10 mg/l), n = 195	Patients with increased CRP (Ó10 mg/l), n = 213	p[À]
Age, year	62.0 (54.0 ï 69.0)	62.0 (53.0 ï 69.0)	62.0 (55.0 ï 70.0)	NS
Male: female	133: 276	62:134	71:142	NS
BMI, kg/m^2	27.44 (24.61 ï 30.55)	27.35 (24.55 ï 30.27)	27.60 (24.66 ï 30.62)	NS
Age of onset, year	50.0 (41.0 ï 58.0)	49.0 (41.1 ï 57.8)	50.0 (41.0 ï 59.0)	NS
RA duration, year	9.0 (3.0 ï 17.0)	10.0 (3.5 ï 17.0)	9.0 (3.0 ï 18.0)	NS
RF	233/407 (57.2)	96/195 (49.2)	137/212 (64.6)	0.0017
Anti-CCP	307/408 (75.2)	137/195 (70.3)	170/213 (79.8)	0.025
Nodular disease	58/409 (14.2)	30/196 (15.3)	28/213 (13.1)	NS
Erosive status	293/401 (73.1)	142/193 (73.6)	151/208 (72.6)	NS
ESR, mm/hr	19.0 (10.0 ï 34.25)	14.0 (7.0 ï 22.0)	29.0 (14.0 ï 46.0)	< 0.0001
*DAS28	4.19 (1.39)	3.67 (1.30)	4.66 (1.30)	< 0.0001
HAQ score	1.625 (1.0 ï 2.0)	1.50 (0.75 ï 1.875)	1.625 (1.219 ï 2.156)	0.0004
Ever smoked	271/406 (66.7)	122/193 (63.2)	149/213 (70.0)	NS
Current smoker	70/406 (17.2)	28/193 (14.5)	42/213 (19.7)	NS

Values are median (IQR) or n (%) of positive case; * Values are mean (SD); NS, not significant; [À]Values without adjustment for confounding factors.

We postulated that the level of VEGF-A influenced by particular VEGFA variants may be differentially expressed relative to inflammation status. Thus, we analysed the association of VEGFA-2578 polymorphism with serum VEGF-A levels in patients with normal CRP

(< 10mg/l) and increased CRP (Ó 10 mg/l) separately (Table 4.9.4b). No difference in VEGFA-2578 distribution was seen between patients with normal and increased CRP. In patients with normal CRP, no difference in VEGF-A levels between genotypes was seen, although it appeared that patients carrying the C allele may have slightly higher VEGF-A compared to the AA genotype. In contrast, in patients with increased CRP there was significant difference in VEGF-A levels between genotypes, with CC associated with the highest levels (AA vs. AC vs. CC, p trend = 0.041; AA vs. C allele, p = 0.027).

Comparison of VEGF-A levels in the same genotypes between patients with normal CRP and increased CRP showed an interesting finding that the levels of VEGF-A were the same in patients carrying VEGFA-2578 AA and AC, whereas the levels significantly increased along with inflammation in CC patients (Table 4.9.4b). This suggested that regulation of VEGF-A expression may be related to inflammation only in patients carrying specific alleles in the VEGFA gene. Addition of other polymorphisms into the analysis provided no stronger associations.

Table 4.9.4b Relationship between VEGFA-2578 polymorphism and serum levels of VEGF-A stratified by CRP status

Patients with normal CRP, n = 195		Patients with increased CRP, n = 213	
Genotypes (n)	VEGF-A levels (IQR)	Genotypes (n)	VEGF-A levels (IQR)
-2578 AA (50)	72.98 (53.83 ï 104.12)	-2578 AA (54)	74.24 (54.69 ï 108.43)^{A}
-2578 AC (92)	83.84 (57.51 ï 111.30)	-2578 AC (108)	83.50 (57.63 ï 149.41)^{A}
-2578 CC (54)	78.76 (54.63 ï 108.31)^{ÿ}	-2578 CC (51)	94.28 (67.65 ï 130.02)^{Aÿ}

CRP status cut-point, < 10 mg/l as normal levels, Ó10 mg/l as increased levels; ^{A} AA vs. AC vs. CC, p trend = 0.041 (as additive model), AA vs. C allele, p = 0.027 (as dominant model), age, sex and RA duration adjusted; ^{ÿ} p = 0.010, age, sex and RA duration adjusted.

4.10 VEGFA POLYMORPHISM, SERUM VEGF-A LEVEL AND DISEASE ACTIVITY

4.10.1 Brief introduction

The pathology of RA is characterized by the proliferation of synovial cells and pannus formation. To cope with the increased requirement for oxygen and nutrients in the pathologic progression, angiogenesis is needed to be highly active, particularly during the early stage of the disease (Ealeolog, 2009; Koch, 1998; Yoo et al, 2008). It has been seen that the VEGF-A expression is increased in RA patients, compared with the general population. Previous studies have demonstrated that the VEGF-A level is associated with RA activity and severity (Lee et al, 2001; Ballara et al, 2001; Ozgonenel et al, 2010; Kurosaka et al, 2010). Polymorphism in the VEGFA gene has also been associated with susceptibility to RA (Han et al, 2004), but the relationship of VEGFA genetic variants with RA activity and outcome measures remains unclear. Thus, it was of great interest to investigate whether genetic variants were associated with disease activity and/or outcome in this population of RA patients.

4.10.2 Serum VEGF-A, inflammatory markers and RA clinical measures

Serum VEGF-A and ESR/CRP

In the total population, serum VEGF-A level was correlated with systemic inflammatory markers including ESR and CRP ($r = 0.12$, $p = 0.015$; $r = 0.14$, $p = 0.004$ respectively, see Figure 4.10.2a and b). The previous data suggested that VEGF-A expression may respond

to inflammation only in patients carrying VEGFA-2578 CC genotype, we therefore postulated that there would be correlations of serum VEGF-A with CRP and ESR only in patients with VEGFA-2578 CC, but not in others. The results were supportive of this idea since there was a significant correlation of serum VEGF-A level with CRP level, and a significant correlation with ESR level in VEGFA-2578 CC subjects, whereas no significant correlation was found in patients carrying -2578 AC or AA genotypes (Table 4.10.2a).

Serum VEGF-A and MMPs

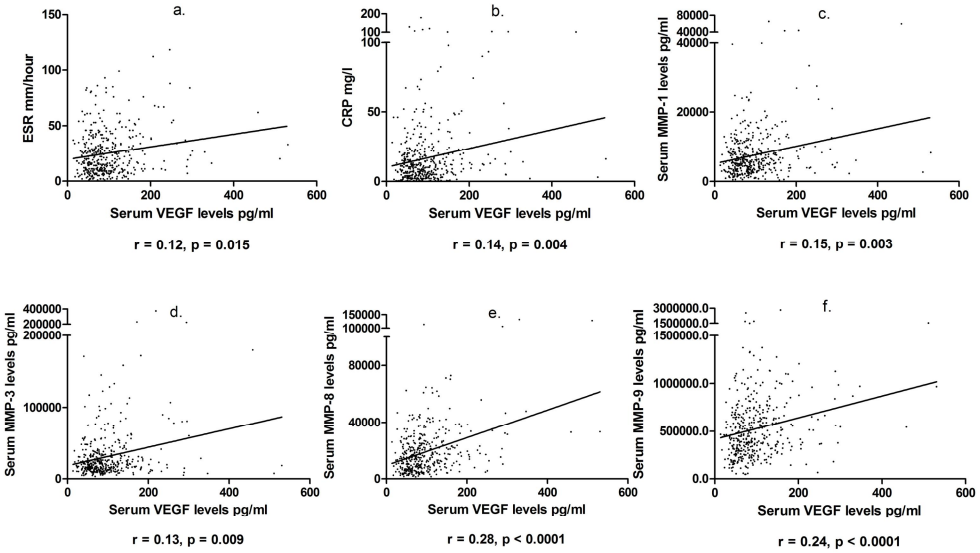
Serum VEGF-A level was also shown to be correlated with major destructive enzymes of the MMP family (serum levels) such as MMP-1, -3, -8 and -9 ($r = 0.15$, $p = 0.003$; $r = 0.13$, $p = 0.009$; $r = 0.28$, $p < 0.0001$; $r = 0.24$, $p < 0.0001$ respectively, see Figure 4.10.2c, d, e and f), but not with RF or anti-CCP, in the total population.

Table 4.10.2a Correlations between serum VEGF-A level and CRP and ESR levels stratified by VEGFA-2578 polymorphism

In patients with VEGFA-2578 AA genotype		
	CRP level	ESR level
VEGF-A level	0.081 (0.41)	0.076 (0.44)
In patients with VEGFA-2578 AC genotype		
	CRP level	ESR level
VEGF-A level	0.12 (0.11)	0.086 (0.23)
In patients with VEGFA-2578 CC genotype		
	CRP level	ESR level
VEGF-A level	0.25 (0.012)	0.20 (0.045)

Values are r (p), obtained from Spearman correlation.

Figure 4.10.2a-f Correlations of serum VEGF-A levels with ESR, CRP, and serum levels of MMP-1, -3, -8 and -9 in RA



The lines show least-squares regression line (trend line); r values are obtained from Spearman correlation.

Correlation between proteins important in angiogenesis

Apart from VEGF-A, serum levels of angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB, PECAM-1 were also measured. These proteins are believed to be important in angiogenesis. The median levels of angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB and PECAM-1 were 422.12 (IQR 259.52 ï 660.76) pg/ml, 440.31 (IQR 341.01 ï 573.57) pg/ml, 203.47 (IQR 135.84 ï 286.99) pg/ml, 1401.29 (IQR 1135.83 ï 1709.55) pg/ml, 94.53 (IQR 70.86 ï 126.19) pg/ml, 4376.81 (IQR 2497.68 ï 7167.25) pg/ml, 2551.62 (IQR 1633.42 ï 3600.49) pg/ml and 3911.85 (IQR 3235.57 ï 4674.04) pg/ml respectively. The relationships between these proteins levels are shown in Table 4.10.2b.

Data indicated that these proteins were strongly correlated with each other in patients with RA, although there were some exceptions.

Table 4.10.2b Correlations between serum levels of proteins important in angiogenesis

	Ang.-2*	Follistatin	G-CSF	HGF	IL-8	Leptin	PDGF-BB	PECAM-1
VEGF-A	0.32 (<0.0001)	0.50 (<0.0001)	0.42 (<0.0001)	0.50 (<0.0001)	0.25 (<0.0001)	0.23 (<0.0001)	0.56 (<0.0001)	0.35 (<0.0001)
Ang.-2*	-	0.34 (<0.0001)	0.28 (<0.0001)	0.35 (<0.0001)	0.34 (<0.0001)	0.23 (<0.0001)	0.19 (0.0002)	0.41 (<0.0001)
Follistatin	-	-	0.75 (<0.0001)	0.53 (<0.0001)	0.30 (<0.0001)	0.18 (0.0002)	0.39 (<0.0001)	0.47 (<0.0001)
G-CSF	-	-	-	0.64 (<0.0001)	0.46 (<0.0001)	NS	0.26 (<0.0001)	0.44 (<0.0001)
HGF	-	-	-	-	0.66 (<0.0001)	0.28 (<0.0001)	0.40 (<0.0001)	0.69 (<0.0001)
IL-8	-	-	-	-	-	0.16 (0..0012)	NS	0.65 (<0.0001)
Leptin	-	-	-	-	-	-	0.25 (<0.0001)	0.29 (<0.0001)
PDGF-BB	-	-	-	-	-	-	-	0.24 (<0.0001)

Values are r (p), obtained from Spearman correlation; * Angiopoietin-2; NS, not significant.

Serum VEGF-A and RA clinical measures

Furthermore, VEGF-A levels were found to be associated with disease activity (as measured by DAS28, $p < 0.0001$) and functional outcome (as measured by HAQ and MJS, $p = 0.001$ and 0.007 respectively), independent of age, sex and RA duration. No association with erosive or nodular disease was found.

4.10.3 VEGFA polymorphism and RA clinical measures

VEGFA polymorphism and age at onset of RA

The T allele at the VEGFA+936 polymorphism was associated with earlier age at onset of RA, compared to the CC genotype (T allele (n = 116) vs. CC (n = 294), 46.5 ± (SD 14.3) vs. 50.5 ± (SD 12.4), p = 0.011, adjusted for sex and ever-smoking). Sex and ever-smoking also had effects, with females and non-smokers associated with earlier onset (females vs. males, 48.1 ± (SD 13.1) vs. 51.7 ± (SD 12.6), p = 0.056; non-smokers vs. ever-smokers, 47.2 ± (SD 13.6) vs. 50.4 ± (SD 12.6), p = 0.077, borderline associations).

VEGFA polymorphism, smoking and RF

An interesting association between cigarette smoking and the VEGFA+405 polymorphism was found, with a trend showing that the highest proportion of smokers occurred in patients carrying the GG genotype (GG vs. GC vs. CC, 73.1 vs. 62.3 vs. 52.5, Appendix 8.6.3c, p trend = 0.003). Other studied polymorphisms were not involved. Adjustment for age, sex and RA duration made little difference to the above result (data not shown). This trend was also observed in another independent cohort of RA (data not shown). It implied that polymorphisms in the VEGFA gene might be somehow related to tobacco addiction.

Interestingly, we also found that the VEGFA+405 G allele was associated with decreased RF positive RA (G allele vs. CC, OR 0.47, 95% CI 0.23 ï 0.97, adjusted for age, sex and RA duration) and decreased RF level (G allele vs. CC, 27.0 vs. 60.5, p = 0.048, adjusted

for age, sex and RA duration). Since it was noticed that decreased RF+ status was found in a group of patients (carrying a G allele) with a high proportion of smokers, we postulated that this association may occur only in non-smoking subjects. This hypothesis was supported when patients were stratified into never and ever smokers (Table 4.10.3a). This suggested that the G allele of VEGFA+405 may have a protective role against RF production, but this function would be significantly lost in the presence of cigarette smoke. However, this result should be treated with some caution as the number with the VEGFA+405 CC genotype was relatively small. Analysis including other polymorphisms did not provide additional effects.

Table 4.10.3a Relationship between RF status and VEGFA+405 polymorphism in all patients and patients stratified by ever-smoked status

	All patients		Never-smoked		Ever-smoked	
	RF-	RF+	RF-	RF+	RF-	RF+
+405 CC	11	29 (72.5)	5	14 (73.7)	6	15 (71.4)
+405 GC	82	100 (54.7)	39	28 (41.8)	41	71 (63.4)
+405 GG	90	110 (55.0)	34	20 (37.0)	56	90 (61.6)
G allele	172	210 (55.0)	73	48 (39.7)	97	161 (62.4)
		*p = 0.042		*p = 0.006		NS

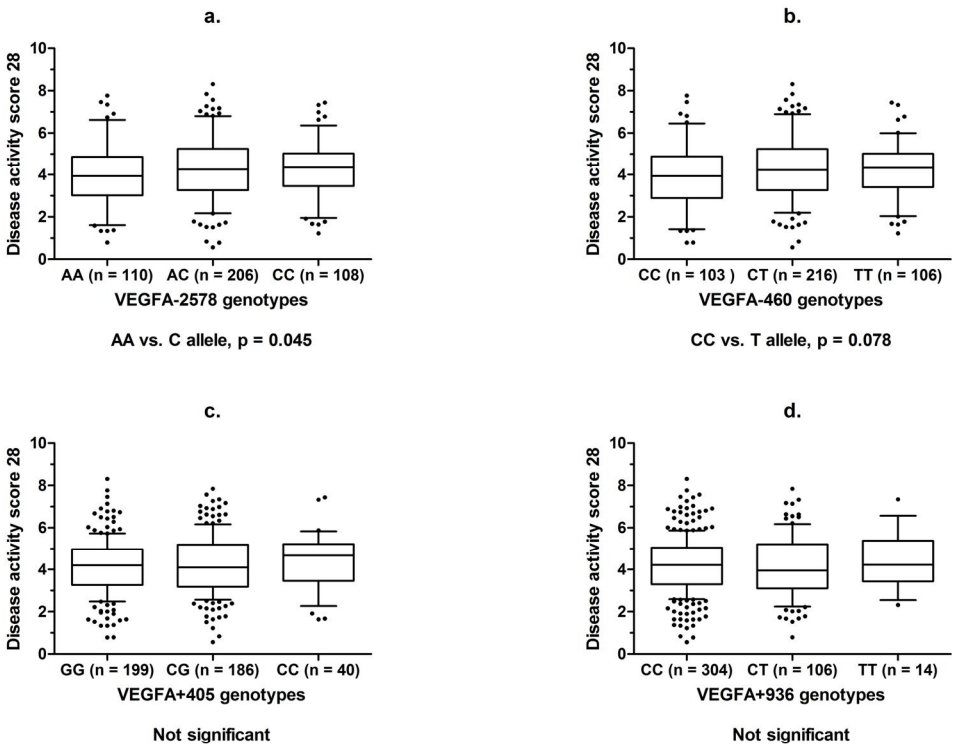
Values are n (%); * CC vs. G allele (as dominant model), adjusted for age, sex and RA duration; NS, not significant.

VEGFA polymorphism and disease activity

Figure 4.10.3a-d shows the DAS28 stratified by the genotypes of studied VEGFA polymorphisms in all patients. There were trends suggesting that patients carrying double

copies of A (-2578) and C (-460) alleles tended to have the lowest DAS28. Significance was achieved with the -2578 polymorphism (AA vs. AC vs. CC, 3.95 vs. 4.26 vs. 4.33, p = 0.045), but not with the -460 (CC vs. CT vs. TT, 3.95 vs. 4.24 vs. 4.32, p = 0.078). Haplotype analysis revealed no stronger associations.

Figure 4.10.3a-d Disease activity score (DAS28) stratified by VEGFA-2578, -460, +405 and +936 polymorphisms in RA



Boxplot with whiskers with 5 ï 95 percentile.

Using multivariate multiple regression, VEGFA-2578 AA genotype was shown to be associated with lower DAS28 after adjustment for age, sex, RA duration, and serum levels of VEGF-A, MMP-1 and -3 (Table 4.10.3b, model 1). Interestingly, this model implied that the VEGFA SNP association was independent of the VEGF-A serum level association.

Other polymorphisms did not reach significance in similar models. However, haplotype analysis with HAPSTAT program demonstrated that A-C-G (-2578/-460/+405), the most frequent haplotype (48.3%), was associated with lower DAS28 (vs. the remaining haplotypes, dominant model, p = 0.017), playing a protective role against high disease activity. To investigate whether VEGFA-2578 polymorphism influenced the disease activity over time, we analysed the relationship between VEGFA-2578 (AA vs. C allele) and the mean time-averaged DAS28 over 24 months (DAS28 at baseline, 12th month and 24th month) on 296 patients using a model similar to the above one (Table 4.10.3b, model 2). We observed a similar trend, but the p value was not significant. Serum VEGF-A level remained significant, suggesting a predictive value of this marker in terms of the disease activity over time in RA.

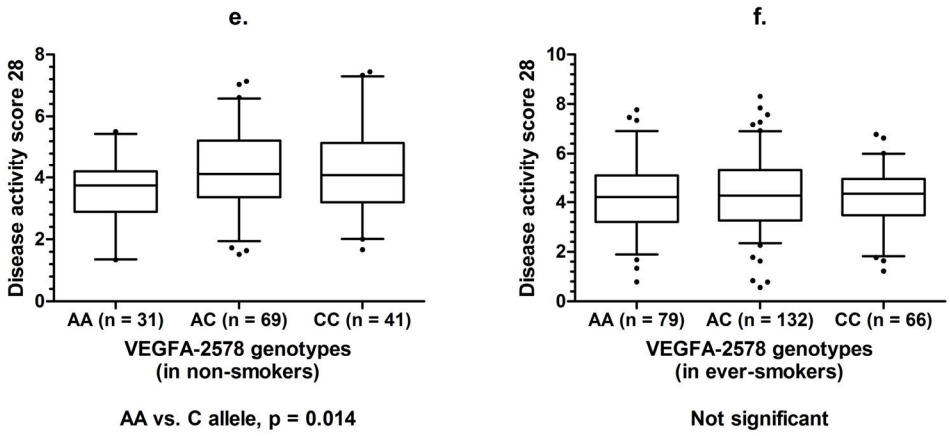
Table 4.10.3b Relationship between VEGFA-2578 and DAS28 in multivariate multiple regression

Independent variable	Regression coefficient Model 1	p	Independent variable	Regression coefficient Model 2	p
VEGFA-2578*	0.31	0.046	VEGFA-2578*	0.20	0.20
VEGF-A levels	0.0031	0.004	VEGF-A levels	0.0025	0.034
MMP-1 levels	2.1×10^{-5}	0.030	MMP-1 levels	1.5×10^{-5}	0.18
MMP-3 levels	6.4×10^{-6}	0.002	MMP-3 levels	4.5×10^{-6}	0.038
Age	0.018	0.006	Age	0.016	0.022
Female	0.37	0.012	Female	0.38	0.017
RA duration	-0.0053	0.45	RA duration	0.0031	0.67

* VEGFA-2578 AA vs. C allele (as dominant model); Model 1, baseline DAS28, all patients; Model 2, mean time-averaged DAS28 over 24 months (baseline, 12th month and 24th month) on 296 patients; Serum VEGF-A, MMP-1 and -3, baseline levels.

We further investigated whether the above relationship primarily occurred in a particular subset of patients (e.g. ever-smoked+/-, RF+/-, anti-CCP+/-). Interestingly, we observed that the association of VEGFA-2578 AA genotype with a decrease of diseae activity (DAS28) occurred only in patients who had never smoked (AA vs. C allele, 3.55 ± 1.14 vs. 4.22 ± 1.37, p = 0.014). In contrast, in those who had ever smoked, no such association existed (AA vs. C allele, 4.17 ± 1.47 vs. 4.30 ± 1.38). Figures 4.10.3 e and f show the DAS28 stratified by the genotypes at VEGFA-2578 polymorphism in patients who had never smoked and in patients who had ever smoked respectively. Taking these together, reduced disease activity only occurred among patients who had never smoked and were carrying the AA genotype at -2578 polymorphism. This suggested that the protective role of AA against high disease activity was conditional, being limited to the condition in non-smokers.

Figure 4.10.3e-f Disease activity score (DAS28) stratified by the genotypes at VEGFA-2578 in patients who had never smoked (figure e) and in patients who had ever smoked (figure f)



Boxplot with whiskers with 5 ï 95 percentile.

Using multivariate multiple regression, the association of the VEGFA-2578 AA genotype with reduced DAS28 in non-smokers was shown to be independent of age, sex, RA duration and serum levels of VEGF-A, MMP-1 and -3 (Table 4.10.3c model 1). Also, serum levels of VEGF-A, MMP-1 and -3 were significant in this model. In contrast, all of these markers were not associated with disease activity in patients who had ever smoked (Table 4.10.3c model 2). Analysis of the relationship with the mean time-averaged DAS28 over 24 months indicated similar results (Tbale 4.10.3d).

Table 4.10.3c Relationship between VEGFA-2578 and DAS28 (baseline) in multivariate multiple regression, analysed separately by ever-smoked status

Independent variable	Regression coefficient	p	Independent variable	Regression coefficient	p
Model 1 (never-smoked)			Model 2 (ever-smoked)		
VEGFA-2578*	0.73	0.0044	VEGFA-2578*	0.16	0.40
VEGF-A levels	0.0048	0.0045	VEGF-A levels	0.0023	0.090
MMP-1 levels	5.5×10^{-5}	0.0023	MMP-1 levels	1.5×10^{-5}	0.21
MMP-3 levels	8.3×10^{-6}	0.0012	MMP-3 levels	3.0×10^{-6}	0.34
Age	0.039	0.69	Age	0.022	0.0088
Female	0.22	0.44	Female	0.48	0.0070
RA duration	-0.0039	0.72	RA duration	-0.0052	0.56

* VEGFA-2578 AA vs. C allele (as dominant model); Serum VEGF-A, MMP-1 and -3, baseline levels.

Table 4.10.3d Relationship between VEGFA-2578 and DAS28 (over time) in multivariate multiple regression, analysed separately by ever-smoked status

Independent variable	Regression coefficient	p	Independent variable	Regression coefficient	p
Model 1 (never-smoked)			Model 2 (ever-smoked)		
VEGFA-2578*	0.75	0.0057	VEGFA-2578*	-0.020	0.92
VEGF-A levels	0.0038	0.053	VEGF-A levels	0.0022	0.16
MMP-1 levels	3.9×10^{-5}	0.11	MMP-1 levels	1.2×10^{-5}	0.36
MMP-3 levels	5.5×10^{-6}	0.041	MMP-3 levels	2.3×10^{-6}	0.51
Age	0.0098	0.41	Age	0.014	0.10
Female	0.47	0.14	Female	0.39	0.030
RA duration	0.0053	0.69	RA duration	0.0044	0.62

* VEGFA-2578 AA vs. C allele (as dominant model); Available samples n = 291, non-smokers n = 95, ever-smokers n = 196; DAS28 over time, mean time-averaged DAS28 over 24 months (baseline, 12th month and 24th month); Serum VEGF-A, MMP-1 and -3, baseline levels.

VEGFA polymorphism and functional outcome

It was found that heterozygous VEGFA-2578 and -460 were associated with the highest HAQ score (VEGFA-2578: AA vs. AC vs. CC, 1.50 (IQR 0.75 ï 2.0) vs. 1.63 (IQR 1.13 ï 2.13) vs. 1.50 (IQR 0.94 ï 2.0), p = 0.043; VEGFA-460: CC vs. CT vs. TT, 1.50 (IQR 0.69 ï 2.0) vs. 1.63 (IQR 1.13 ï 2.13) vs. 1.50 (IQR 0.94 ï 2.0), p = 0.019). The associations also fit to dominant models where VEGFA-2578 C allele and -460 T allele associated with higher HAQ score (C allele vs. AA, 1.63 (IQR 1.0 ï 2.13) vs. 1.50 (IQR 0.72 ï 2.0), p = 0.045; T allele vs. CC, 1.63 (IQR 1.0 ï 2.13) vs. 1.50 (IQR 0.69 ï 2.0), p = 0.02 respectively). However, these associations became insignificant after adjustment for RA duration. Haplotype analysis indicated an association between haplotype A-C-G (-2578/-

460/+405) and lower HAQ (vs. the remaining haplotypes, dominant model, p = 0.013, without adjustment for confounding factors). No association of VEGFA polymorphism with MJS, erosive status or nodular disease was seen.

4.11 VEGFA POLYMORPHISM AND CO-MORBIDITY

4.11.1 Brief introduction

RA has been strongly associated with morbidity and mortality. It is known to be associated with a reduction in life expectancy, much of which can be attributed to co-morbid conditions such as CVD. The most common cardiovascular condition, IHD, is increased in RA populations, and its clinical presentation appears to be different to that in the normal population, with RA patients more likely to experience unrecognized MI and sudden death (Maradit-Kremers et al, 2005). VEGF-A is a fundamental promoter of angiogenesis, and may also play roles in atherosclerosis. A recent study reported that the VEGFA-2578 CC genotype was associated with less atherosclerosis (Howell et al, 2005). The role of VEGFA polymorphisms in the development of CVD in RA has not been investigated previously. In order to do this, we examined the association of VEGFA SNPs (-2578/-460/+405/+936) with the presence of IHD or previous MI in our cohort patients with RA. Since smoking has a causal role in the development of atherosclerotic change in CVD, we also looked for any evidence of interaction between smoking and VEGFA. Other co-morbid conditions recorded in these patients were also investigated.

4.11.2 VEGFA polymorphism, smoking and IHD/MI

Patient characteristics stratified by IHD/MI status

VEGFA genotyping (-2578/-460/+405/+936), and the records of smoking, IHD and previous MI were complete in 418 patients. Evidence of IHD and previous MI was found in 18.7% (n = 78) and 11.0% (n = 46) of patients respectively. Of the 46 patients with previous MI, 13 had no medical history of MI and were identified on the basis of their ECG (Q-wave development in absence of any conduction defect, suggesting full thickness MI).

The patient characteristics in the total population and stratified by the presence of IHD are displayed in Table 4.11.2a. Patients with IHD were older and more likely to be male (30.1% vs. 13.1%, $p < 0.0001$). They also had a later onset of RA in this population. There was no significant difference in RA duration between patients with or without IHD. Increased frequencies of RF status and CRP abnormality (CRP Ó10 mg/l) were observed in patients with IHD, and although insignificant, ESR ($p = 0.074$) and DAS28 ($p = 0.091$) values were also elevated in these patients. Furthermore, evidence of worse functional outcome measured by HAQ score was seen. Patients with IHD were more likely to have suffered from other co-morbid conditions including diabetes type 2, asthma, CVA, hypertension and hypercholesterolemia. The use of MTX was less in patients with IHD compared to those without IHD.

To do a more detailed analysis, we compared patient characteristics within RA-IHD patients, stratified by previous MI status (Table 4.11.2b). Patients who had a previous heart

attack were more likely to be males and had longer RA duration. Interestingly, we found that MI was more likely to occur in autoantibody (RF and anti-CCP) positive RA, although for anti-CCP it was not significant ($p = 0.074$). By assessing the whole patientsô data (including Table 4.11.2a and b) we suggested that RF may be a risk factor particularly for MI, since its frequency was increased only in MI patients but not in IHD patients without MI, compared to patients without IHD. No differences of inflammation, disease activity and functional outcome measures were seen between RA-IHD patients with and without MI. However, erosive status and nodular disease were enhanced in patients with MI, although for erosive disease it was not significant ($p = 0.075$). CVA and MI highly co-existed with each other, and cases of hypertension may be increased in patients with MI (insignificant, $p = 0.081$).

Table 4.11.2a Demographic and clinical characteristics of RA patients stratified by the presence of IHD

Variable	All patients	Patients without IHD (n = 340)	Patients with IHD (n = 78)	p[ÿ]
Demographics				
Age, years	62.0 (54.0 ï 69.0)	61.0 (54.0 ï 68.0)	68.0 (58.8 ï 74.3)	0.001
Male:Female	136:282	95:245	41:37	< 0.0001
BMI, kg/m^2	27.3 (24.5 ï 30.5)	27.3 (24.4 ï 30.5)	27.3 (25.2 ï 30.6)	NS
CSUM (Ómedian)	196/400 (49.0)	160/328 (48.8)	36/72 (50.0)	NS
RA characteristics				
Age of onset	50.0 (41.0 ï 58.0)	49.0 (40.8 ï 57.0)	55.0 (45.0 ï 65.0)	0.001
Duration, years	9.0 (4.0 ï 18.0)	10.0 (3.0 ï 18.0)	8.0 (3.9 ï 19.3)	NS
RF	239/418 (57.2)	185/340 (54.4)	54/78 (69.2)	0.02
Anti-CCP	306/406 (75.4)	251/332 (75.6)	55/74 (74.3)	NS
ESR, mm/hr	19.0 (10.0 ï 35.0)	18.0 (10.0 ï 34.0)	26.0 (10.0 ï 43.0)	NS
CRP (Ó10 mg/l)	224/417 (53.7)	169/339 (49.9)	55/78 (70.5)	0.001
*DAS28	4.2 (1.4)	4.2 (1.4)	4.4 (1.4)	NS
Erosive status	303/411 (73.7)	251/334 (75.1)	52/77 (67.5)	NS
MJS	7.0 (3.0 ï 15.0)	7.5 (3.0 ï 15.0)	7.0 (3.0 ï 17.0)	NS
HAQ score	1.6 (1.0 ï 2.0)	1.6 (0.9 ï 2.0)	1.8 (1.3 ï 2.3)	0.01
Nodular disease	54/418 (12.9)	40/340 (11.8)	14/78 (18.0)	NS
Cigarette smoking				
Ever smoked	278/418 (66.5)	211/340 (62.1)	67/78 (85.9)	0.0001
Current smoker	73/418 (17.5)	60/340 (17.6)	13/78 (16.7)	NS
[A]Cigarettes per day	15 (8 - 20)	12 (8 - 20)	20 (10 - 20)	0.007
[A]Years of smoking	30.0 (17.8 ï 41.0)	27.5 (16.8 ï 40.0)	36.0 (25.0 ï 42.5)	0.011
[A]Total pack-years	18.3 (8.3 ï 33.0)	17.0 (7.2 ï 30.0)	28.0 (15.5 ï 45.5)	0.0004
Co-morbidities				
MI	46/418 (11.0)	ï	46/78 (59.0)	ï
Diabetes type 1	11/418 (2.6)	7/340 (2.1)	4/78 (5.1)	NS
Diabetes type 2	22/418 (5.3)	9/340 (2.6)	13/78 (16.7)	< 0.0001
Asthma	44/418 (10.5)	29/340 (8.5)	15/78 (19.2)	0.004

CVA	15/418 (3.6)	6/340 (1.8)	9/78 (11.5)	< 0.0001
Solid cancers	31/398 (7.8)	28/323 (8.7)	3/75 (4.0)	NS
Hypertension	163/418 (39.1)	115/340 (33.8)	48/78 (61.5)	< 0.0001
Hypercholesterolemia	70/418 (17.2)	41/340 (12.1)	29/78 (37.2)	< 0.0001
Haemanalysis				
Hemoglobin, g/dl	13.0 (12.1 ï 13.9)	13.0 (12.1 ï 13.9)	13.3 (12.1 ï 14.1)	NS
Leukocyte, × 10^9/l	7.5 (6.2 ï 9.3)	7.5 (6.1 ï 9.1)	7.6 (6.5 ï 9.5)	NS
Platelet, × 10^9/l	293 (240 - 366)	297 (241 ï 370)	285 (289 - 347)	NS
Serum VEGF-A level	82.9 (57.2 ï 119)	82.6 (57.1 - 120)	83.3 (61.9 - 114)	NS
Drug treatment				
DMARDs use	389/418 (93.1)	317/340 (93.2)	72/78 (92.3)	NS
MTX use	247/418 (59.1)	212/340 (62.3)	35/78 (44.9)	0.007
Steroid use	40/418 (9.6)	29/340 (8.5)	11/78 (14.1)	NS
Biologic agent use	60/418 (14.4)	53/340 (15.6)	7/78 (9.0)	NS

Values are n (%) or median (IQR); * Values are mean (SD); CSUM, Carstairs score upper-median; [À]Excluded the non-smoking samples; [ÿ] p values show significant differences between patients with and without IHD (un-adjusted); NS, not significant.

Table 4.11.2b Demographic and clinical characteristics of RA-IHD patients stratified by previous MI

Variable	All RA-IHD patients	Patients without MI (n = 32)	Patients with MI (n = 46)	p[ÿ]
Demographics				
Age, years	68.0 (58.8 ï 74.3)	63.0 (55.5 ï 76.3)	68.5 (60.8 ï 73.5)	NS
Male:Female	41:37	12:20	29:17	0.026
BMI, kg/m^2	27.3 (25.2 ï 30.6)	27.3 (24.3 ï 32.3)	27.4 (25.4 ï 30.1)	NS
CSUM (Ómedian)	36/72 (50.0)	11/29 (37.9)	25/43 (58.1)	NS
RA characteristics				
Age of onset	55.0 (45.0 ï 65.0)	55.8 (47.3 ï 62.8)	52.5 (42.8 ï 65.3)	NS
Duration, years	8.0 (3.9 ï 19.3)	5.5 (1.4 ï 14.0)	10.5 (5.0 ï 20.3)	0.05
RF	54/78 (69.2)	18/32 (56.3)	36/46 (78.3)	0.038
Anti-CCP	55/74 (74.3)	19/30 (63.3)	36/44 (81.8)	NS
ESR, mm/hr	26.0 (10.0 ï 43)	25.0 (10.0 ï 40.0)	26.5 (14.3 ï 43.3)	NS
CRP (Ó10 mg/l)	55/78 (70.5)	23/32 (71.9)	32/46 (69.6)	NS
*DAS28	4.4 (1.4)	4.59 (1.58)	4.33 (1.34)	NS
Erosive status	52/77 (67.5)	18/32 (56.3)	34/45 (75.6)	NS
MJS	7.0 (3.0 ï 17.0)	7.0 (2.3 ï 17.8)	6.5 (3.0 ï 13.3)	NS
HAQ score	1.8 (1.3 ï 2.3)	1.8 (1.3 ï 2.2)	1.9 (1.3 ï 2.3)	NS
Nodular disease	14/78 (18.0)	2/30 (6.7)	12/45 (26.7)	0.029
Cigarette smoking				
Ever smoked	67/78 (85.9)	25/32 (78.1)	42/46 (91.3)	NS
Current smoker	13/78 (16.7)	7/30 (23.3)	6/45 (13.3)	NS
[A]Cigarettes per day	20 (10 - 20)	14 (8 - 20)	20 (10 - 25)	NS
[A]Years of smoking	36.0 (25.0 ï 42.5)	36.5 (13.8 ï 43.5)	36.0 (25.8 ï 42.5)	NS
[A]Total pack-years	28.0 (15.5 ï 45.5)	19.0 (5.6 ï 44.5)	28.4 (15.0 ï 47.1)	NS
Co-morbidities				
Diabetes type 1	4/78 (5.1)	1/32 (3.1)	3/46 (6.5)	NS
Diabetes type 2	13/78 (16.7)	7/32 (21.9)	6/46 (13.0)	NS
Asthma	15/78 (19.2)	6/32 (18.8)	9/46 (19.6)	NS
CVA	9/78 (11.5)	1/32 (3.1)	8/46 (17.4)	0.05

Solid cancers	3/75 (4.0)	1/30 (3.3)	2/45 (4.4)	NS
Hypertension	48/78 (61.5)	16/32 (50.0)	32/46 (69.6)	NS
Hypercholesterolemia	29/78 (37.2)	10/32 (31.3)	19/46 (41.3)	NS
Haemanalysis				
Hemoglobin, g/dl	13.3 (12.1 ï 14.1)	13.4 (11.9 ï 14.4)	13.3 (12.1 ï 14.1)	NS
Leukocyte, × 10^9/l	7.6 (6.5 ï 9.5)	7.8 (6.7 ï 9.8)	7.3 (6.1 ï 9.5)	NS
Platelet, × 10^9/l	285 (289 - 347)	286 (245 - 340)	285 (199 - 360)	NS
Serum VEGF-A level	83.3 (61.9 - 114)	87.5 (65.3 - 106)	80.6 (55.5 - 119)	NS

Values are n (%) or median (IQR); * Values are mean (SD); CSUM, Carstairs score upper-median; [À]Excluded the non-smoking samples; [ÿ] p values show significant differences between patients with and without IHD (un-adjusted); NS, not significant.

VEGFA polymorphism and IHD/MI

Associations between VEGFA genotypes and the presence of IHD/MI, without adjustment for confounders, are shown in Table 4.11.2c. The associations of VEGFA-2578, -460 and +405 with IHD/MI were best explained by dominant models in which the A, C and G alleles respectively provided an increased risk, although for +405 it was not statistically significant. The VEGFA+936 polymorphism was not associated with IHD or MI.

When adjusted for age, sex and RA duration in logistic regression analyses, VEGFA-2578 A showed the strongest association with IHD (OR 2.9, 95% CI 1.4 ï 6.2) compared with VEGFA-460 C (OR 2.2, 95% CI 1.1 ï 4.5) and VEGFA+405 G (OR 2.1, 95% CI 0.7 ï 6.1). The OR for age, sex and RA duration adjusted associations of -2578 A, -460 C and +405 G with MI were 8.4 (95% CI 2.0 ï 35.8), 3.9 (95% CI 1.3 ï 11.3) and 5.0 (95% CI 0.7 ï 38.3) respectively.

Since there is strong LD between VEGFA-2578, -460 and +405, the associations involving -460 and +405 may be due to the greater association of -2578 with IHD and MI. This was supported by logistic regression analysis that included the -2578 A allele, -460 C allele and +405 G allele together as independent variables, and used forward stepwise selection to determine which factor(s) was most strongly associated with IHD and MI. The VEGFA-2578 A allele maintained significance in relation to both IHD (OR 2.96, 95% CI 1.28 ï 6.83) and MI (OR 8.39, 1.97 ï 35.77), while the other 2 SNPs lost significance in these models (adjusted for age, sex and RA duration).

Common haplotypes of VEGFA-2578, -460 and +405, and their relationship with the presence of IHD/MI are presented in Table 4.11.2d. The A-C-G haplotype displayed a significant difference in distribution between patients with and without IHD/MI, suggesting an increased risk associated with this haplotype. No significant difference in the frequency of C-T-C or C-T-G was found between patients with and without IHD/MI.

Table 4.11.2c Frequency of IHD and MI in RA patients stratified by VEGFA SNP genotypes

	IHD			MI		
	Negative	Positive (%)	Odds ratio (95% CI)	Negative	Positive (%)	Odds ratio (95% CI)
VEGFA-2578 (A/C)						
CC	98	9 (8.4)	1.0 (referent)	105	2 (1.9)	1.0 (referent)
AC	154	47 (23.4)	3.19 (1.52 ï 6.69)	167	34 (16.9)	8.69 (2.35 ï 32.10)
AA	88	22 (20.0)	2.63 (1.17 ï 5.93)	100	10 (9.1)	4.41(1.08 ï 17.99)
A allele	242	69 (22.2)	2.97 (1.45 ï 6.09)	267	44 (14.1)	7.02 (1.93 ï 25.60)
VEGFA-460 (T/C)						
TT	93	11 (10.6)	1.0 (referent)	100	4 (3.8)	1.0 (referent)
CT	165	45 (21.4)	2.23 (1.15 ï 4.48)	179	31 (14.8)	3.91 (1.42 ï 10.84)
CC	82	22 (21.2)	2.22 (1.03 ï 4.79)	93	11 (10.6)	2.75 (0.89 ï 8.47)
C allele	247	67 (21.3)	2.22 (1.14 ï 4.33)	272	42 (13.4)	3.48 (1.28 ï 9.45)
VEGFA+405 (C/G)						
CC	36	4 (10.0)	1.0 (referent)	39	1 (2.5)	1.0 (referent)
CG	146	32 (18.0)	1.80 (0.63 ï 5.14)	159	19 (10.7)	3.22 (0.59 ï 17.57)
GG	158	42 (21.0)	2.17 (0.77 ï 6.13)	174	26 (13.0)	3.99 (0.74 ï 21.47)
G allele	304	74 (19.6)	1.98 (0.72 ï 5.46)	333	45 (11.9)	3.59 (0.68 ï 18.89)
VEGFA+936 (T/C)						
TT	11	3 (21.4)	1.0 (referent)	13	1 (7.1)	1.0 (referent)
CT	89	14 (13.6)	0.53 (0.14 ï 1.99)	95	8 (7.8)	0.80 (0.13 ï 4.98)
CC	240	61 (20.3)	0.84 (0.24 ï 2.87)	264	37 (12.3)	1.28 (0.23 ï 7.13)
ï						

Values are n (%).

Table 4.11.2d Frequency of VEGFA haplotypes in RA patients with and without IHD or MI

	All patients	IHD			MI		
VEGFA haplotype (-2578/-460/+405)	EM freq.	Negative EM freq.	Positive EM freq.	OR (95% CI)	Negative EM freq.	Positive EM freq.	OR (95% CI)
A-C-G	0.483	0.469	0.544	2.37 (1.22 ï 4.62)[À]	0.476	0.541	4.10 (1.45 ï 11.59)[À]
C-T-C	0.297	0.309	0.242	0.73 (0.49 ï 1.09)[ÿ]	0.307	0.217	0.62 (0.37 ï 1.04)[ÿ]
C-T-G	0.182	0.190	0.147	0.74 (0.46 ï 1.20)[ÿ]	0.185	0.161	0.85 (0.47 ï 1.53)[ÿ]
Rare	0.038						

EM freq., frequency estimated by the expectation/maximization algorithm; Maximum EM iteration = 500, EM convergence tolerance = 0.0001; Frequency threshold = 0.05; Best odds ratio achieved in [À]dominant model, [ÿ] additive model.

Smoking and IHD/MI

Cigarette smoking is a well known risk factor for CVD in the normal population and in RA. In our RA population, an increased frequency of ever-smoking was observed in patients with IHD, compared with those without IHD (Table 4.11.2a). Both intensity and duration of smoking (measured by cigarettes per day and the number of years of smoking respectively) were associated with the incidence of IHD (Table 4.11.2a). The total amount of smoking measured by pack-years showed the strongest association (Table 4.11.2a).

After adjustment for age, sex and RA duration, it was found that RA patients who ever smoked had a 2.5-fold increased likelihood of IHD (95% CI 1.28 ï 4.95, p = 0.007) and a 4-fold increased likelihood of MI (95% CI 1.45 ï 12.37, p = 0.009), compared to RA patients who had never smoked.

VEGFA-smoking interaction and IHD/MI

We found evidence of a strong interaction between smoking and the SNPs within the VEGFA promoter with regard to both IHD and MI (Table 4.11.2e). A strongly enhanced risk was observed only in patients with both a VEGFA risk allele and a history of smoking. The AP due to interaction for the VEGFA-2578 A allele plus smoking, and the -460 C allele plus smoking were significant for both IHD and MI, although the AP for the +405 G allele plus smoking did not reach significance. Patients who had ever smoked but did not carry a VEGFA risk allele, or those who carried a risk allele but did not smoke, did not have a significantly increased risk of having IHD or MI compared to the patients who neither smoked nor carried risk alleles.

To test the effect of pack-years of smoking in VEGEA-smoking interaction relative to IHD/MI, we carried out an analysis which only recruited patients carrying VEGFA risk alleles, and in which pack-years were divided into 3 categories, 0< n Ò15, 15 < n Ò30 and n > 30, as light-, medium- and heavy-smoking respectively (Table 4.11.2f). The amount of smoking quantified in pack-years demonstrated a dose effect with regard to the occurrence of IHD and MI in the presence of VEGFA risk alleles. Among those with pack-years > 30 (heavy smokers), the presence of IHD and previous MI were found to be approximately 40% and 30% respectively, whereas in non-smokers they were only about 9% and 4% respectively.

Table 4.11.2e Association of VEGFA polymorphisms and ever having smoked with IHD and MI in RA patients

	IHD			MI		
	Negative	Positive (%)	Odds ratio (95% CI)	Negative	Positive (%)	Odds ratio (95% CI)
Smoke/VEGFA-2578 A						
-/-	38	2 (5.0)	1.0 (referent)	40	0 (0.0)	1.0 (referent)
-/+	91	9 (9.0)	1.59 (0.38 ï 6.77)	96	4 (4.0)	3.77 (0.26 ï 17.15)
+/-	60	7 (10.4)	1.91 (0.43 ï 8.45)	65	2 (3.0)	3.09 (0.14 ï 11.84)
+/+	151	60 (28.4)	6.15 (1.65 ï 22.87)	171	40 (19.0)	19.13 (1.84 ï 101.7)
			AP: 0.63 (0.27 ï 0.98)			AP: 0.76 (0.52 ï 0.99)
Smoke/VEGFA-460 C						
-/-	36	2 (5.3)	1.0 (referent)	38	0 (0.0)	1.0 (referent)
-/+	93	9 (8.8)	1.48 (0.35 ï 6.29)	98	4 (3.9)	3.52 (0.24 ï 15.96)
+/-	57	9 (13.6)	2.41 (0.56 ï 10.32)	62	4 (6.1)	5.54 (0.37 ï 25.19)
+/+	154	58 (27.4)	5.53 (1.52 ï 20.61)	174	38 (17.9)	16.99 (1.63 ï 90.12)
			AP: 0.54 (0.07 ï 1.0)			AP: 0.52 (0.13 ï 0.91)
Smoke/VEGFA+405 G						
-/-	18	1 (5.3)	1.0 (referent)	19	0 (0.0)	1.0 (referent)
-/+	111	10 (8.3)	1.16 (0.20 ï 6.89)	117	4 (3.3)	1.49 (0.07 ï 6.64)
+/-	18	3 (14.3)	2.33 (0.31 ï 17.56)	20	1 (4.8)	2.85 (0.05 ï 7.61)
+/+	193	64 (24.9)	4.11 (0.76 ï 22.23)	216	41 (16.0)	7.48 (0.69 ï 38.96)
			AP: 0.57 (-0.28 ï 1.43)			AP: 0.56 (-0.02 ï 1.14)

Values are n (%); AP, the attributable proportion due to interaction.

Table 4.11.2f A dose effect of amount of smoking quantified in pack-years in the presence of VEGFA risk alleles relative to IHD/MI in RA

IHD				MI			
VEGFA-2578 (A+)	Negative	Positive	p trend	VEGF-2578 (A+)	Negative	Positive	p trend
0	91	9 (9.0)		0	96	4 (4.0)	
0 < n Ò15	72	16 (18.2)		0 < n Ò15	78	10 (11.4)	
15 < n Ò 30	40	13 (24.5)		15 < n Ò 30	43	10 (18.9)	
n > 30	37	28 (43.1)	< 0.0001	n > 30	45	20 (30.8)	< 0.0001
VEGFA-460 (C+)				VEGF-460 (C+)			
0	94	9 (8.7)		0	99	4 (3.9)	
0 < n Ò15	73	16 (18.0)		0 < n Ò15	79	10 (11.2)	
15 < n Ò 30	41	13 (24.1)		15 < n Ò 30	44	10 (18.5)	
n > 30	39	26 (40.0)	< 0.0001	n > 30	47	18 (27.7)	< 0.0001
VEGFA+405 (G+)				VEGF+405 (G+)			
0	112	10 (8.2)		0	118	4 (3.3)	
0 < n Ò15	95	18 (15.9)		0 < n Ò15	102	11 (9.7)	
15 < n Ò 30	52	14 (21.2)		15 < n Ò 30	56	10 (15.2)	
n > 30	44	29 (39.7)	< 0.0001	n > 30	53	20 (27.4)	< 0.0001

Values are n (%); VEGFA-2578 A, -460 C and +405 G are risk alleles for IHD and MI.

Multivariate analysis: factors associated with IHD/MI

We carried out multivariate logistic regression analysis using models containing the VEGFA risk allele/smoking interaction terms as well as other potential IHD/MI associated factors such as hypertension, hypercholesterolemia, asthma, diabetes, CVA, RF, anti-CCP, ESR, CRP, DAS28, nodular disease, erosive disease, HAQ, male, age and the use of MTX. The ñbestò models obtained from forward selection (cut-point = 0.10) analyses are shown in Table 4.11.2g. For both IHD and MI, independent associations were found with the VEGFA-2578 A/smoking interaction term, hypercholesterolemia, CVA and male sex. The presence of type 2 diabetes and CRP (Ó10 mg/l), and older age were associated with IHD, while HAQ score and the presence of hypertension and asthma were associated with MI. RF positive status was also shown to be nearly associated with MI, but not with IHD. MTX use was negatively associated with MI, and showed a trend towards negative association with IHD. Variables not significant in these models were VEGFA-460 C/smoking and VEGFA+405 G/smoking interaction terms, anti-CCP status, ESR, DAS28, and nodular and erosive diseases.

Table 4.11.2g Multivariate stepwise logistic regression analysis of variables associated with IHD and MI

IHD (model 1[À])				MI (model 2[ÿ])			
Variable	Regression coefficient	OR (95% CI)	p	Variable	Regression coefficient	OR (95% CI)	p
*Smoke + VEGFA-2578 A (+/-)	1.347	3.85 (1.93 ï 7.66)	0.0001	*Smoke + VEGFA-2578 A (+/-)	2.129	8.40 (2.52 ï 27.99)	0.0005
Hypercholesterolemia (+/-)	1.222	3.39 (1.68 ï 6.85)	0.0006	Hypercholesterolemia (+/-)	0.902	2.47 (1.0 ï 6.07)	0.050
Age, per year	0.047	1.05 (1.01 ï 1.08)	0.0050	HAQ score	0.656	1.93 (1.07 ï 3.46)	0.030
CRP Ó10mg/l (+/-)	1.044	2.84 (1.48 ï 5.45)	0.0017	Hypertension (+/-)	1.043	2.84 (1.17 ï 6.86)	0.021
Diabetes type 2 (+/-)	1.947	7.01 (2.16 ï 22.74)	0.0012	Asthma (+/-)	1.106	3.02 (1.02 ï 8.95)	0.046
CVA (+/-)	1.614	5.02 (1.27 ï 19.76)	0.021	CVA (+/-)	2.50	12.14 (2.52 ï 58.56)	0.0019
Male (+/-)	0.687	1.99 (1.07 ï 3.70)	0.030	Male (+/-)	1.725	5.62 (2.35 ï 13.51)	0.0001
MTX use (+/-)	-0.575	0.56 (0.32 ï 0.99)	0.096	MTX use (+/-)	-0.764	0.47 (0.22 ï 0.97)	0.040
				RF (+/-)	0.862	2.37 (0.94 ï 5.97)	0.068

* Patients who have ever smoked and carry a VEGFA-2578 A allele, compared with all remaining patients; [À] Patients with IHD vs. without IHD; [ÿ] Patients with MI vs. all non-MI patients.

Serum VEGF-A level, IHD/MI and other co-morbidity

No association was found between serum VEGF-A level and IHD/MI status (Table 4.11.2a and b). We showed that serum VEGF-A level was not significantly influenced by smoking status. However, it was influenced by VEGFA-2578 polymorphism, with A allele associated with low levels. Analysis stratified by VEGFA-2578 A/smoking interaction term suggested that serum VEGF-A expression was not significantly affected by this interaction (interaction+ vs. the remaining, 81.5 (IQR 54.7 ï 117.1) vs. 83.7 (IQR 59.9 ï 121.8), p = 0.64).

No significant association between serum VEGF-A and other co-morbid conditions recorded in RA was found.

4.11.3 VEGFA polymorphism and hypertension

Patient characteristics stratified by hypertension status

VEGFA genotyping (-2578/-460/+405/+936) and hypertension status were completely recorded in 426 subjects. Evidence of hypertension occurred in 166 patients, which was 39% of the whole group. The patient characteristics in all patients and those stratified by the presence of hypertension are shown in Table 4.11.3a. Patients with hypertension were older and more likely to be male (49.6% vs. 33.9%, p = 0.045). Hypertension was also associated with higher BMI and lower socioeconomic status measured by Carstairs score upper-median (CSUM). There was a significant older age of onset of RA in patients with hypertension. These patients were associated with a higher disease activity and a more

severe functional outcome, measured by DAS28 and HAQ respectively. MJS was also higher in patients with hypertension, but this was not statistically significant ($p = 0.075$). No difference of RF and anti-CCP status, and erosive and nodular diseases was found between patients stratified by the presence of hypertension. Ever-smoking may be a risk factor for hypertension. However, in un-adjusted analysis it was not significant ($p = 0.098$). The presence of IHD, MI, type 1 and 2 diabetes, and hypercholesterolemia were shown to be significantly associated with the presence of hypertension. Also, it was shown that the concentration of platelets in blood was lower in patients with hypertension.

Table 4.11.3a Demographic and clinical characteristics of RA patients stratified by the presence of hypertension

Variable	All patients	Patients without hypertension (n = 260)	Patients with hypertension (n = 166)	p[ÿ]
Demographics				
Age, years	62.0 (54.5 ï 69.0)	59.0 (52.0 ï 66.0)	66.5 (59.0 ï 72.3)	< 0.0001
Male:Female	139:286	70:189	69:97	0.002
BMI, kg/m^2	27.4 (24.5 ï 30.5)	26.8 (23.6 ï 29.6)	28.4 (25.9 ï 31.2)	< 0.0001
CSUM (Ómedian)	193/396 (48.7)	106/243 (43.6)	87/153 (56.9)	0.01
RA characteristics				
Age of onset	50.0 (41.0 ï 58.0)	48.9 (39.0 ï 56.0)	51.5 (43.3 ï 63.4)	0.0003
Duration, years	9.0 (3.0 ï 18.0)	9.0 (2.6 ï 17.0)	10.0 (4.0 ï 18.0)	NS
RF	239/423 (56.5)	148/258 (57.4)	91/165 (55.2)	NS
Anti-CCP	310/413 (75.1)	190/251 (75.7)	120/162 (74.1)	NS
ESR, mm/hr	20.0 (10.0 ï 36.5)	17.0 (10.0 ï 33.0)	25.0 (14.0 ï 29.8)	0.006
CRP (Ó10 mg/l)	226/422 (53.6)	135/257 (52.5)	91/165 (55.2)	NS
*DAS28	4.2 (1.4)	4.1 (1.4)	4.4 (1.4)	0.005
Erosive status	306/417 (73.4)	191/254 (75.2)	115/163 (70.6)	NS
MJS	7.0 (3.0 ï 15.0)	6.0 (3.0 ï 14.0)	8.0 (3.0 ï 16.0)	NS
HAQ score	1.6 (1.0 ï 2.0)	1.5 (0.8 ï 1.9)	1.8 (1.3 ï 2.1)	< 0.0001
Nodular disease	54/394 (13.7)	32/239 (13.4)	22/155 (14.2)	NS
Cigarette smoking				
Ever smoked	279/419 (66.6)	162/255 (63.5)	117/164 (71.3)	NS
Current smoker	73/399 (18.3)	53/242 (21.9)	20/157 (12.7)	0.021
[A]Cigarettes per day	15 (8 - 20)	15 (8 - 20)	15 (9.5 - 20)	NS
[A]Years of smoking	30.0 (17.0 ï 41.0)	29.0 (16.8 ï 39.3)	32.0 (19.0 ï 43.0)	NS
[A]Total pack-years	18.6 (8.4 ï 33.5)	17.6 (7.2 ï 32.1)	22.0 (9.7 ï 41.5)	NS
Co-morbidities				
IHD	78/421 (18.5)	30/257 (11.7)	48/164 (29.3)	< 0.0001
MI	46/421 (10.9)	14/257 (5.4)	32/164 (19.5)	< 0.0001
Diabetes type 1	10/421 (2.4)	3/257 (1.2)	7/164 (4.3)	0.042

Diabetes type 2	22/421 (5.2)	6/257 (2.3)	16/164 (9.8)	0.0008
Asthma	42/420 (10.0)	25/257 (9.7)	17/163 (10.4)	NS
CVA	15/421 (3.6)	6/257 (2.3)	9/164 (5.5)	NS
Solid cancers	31/394 (7.9)	18/239 (7.5)	13/155 (8.4)	NS
Hypercholesterolemia	71/421 (16.9)	19/257 (7.4)	52/164 (31.7)	< 0.0001
Haemanalysis				
Hemoglobin, g/dl	13.0 (12.1 ï 13.9)	13.1 (12.2 ï 13.9)	12.9 (11.9 ï 13.9)	NS
Leukocyte, × 10^9/l	7.5 (6.2 ï 9.2)	7.3 (6.0 ï 9.1)	7.7 (6.4 ï 9.4)	NS
Platelet, × 10^9/l	293 (239 - 366)	299 (245 - 382)	286 (230 - 334)	0.012
Serum VEGF-A level	82.9 (57.3 ï 119)	83.4 (57.4 - 116)	82.2 (57.1 - 126)	NS

Values are n (%) or median (IQR); * Values are mean (SD); [Â]Excluded the non-smoking samples; [ÿ] p values show significant differences between patients with and without IHD (un-adjusted); NS, not significant.

VEGFA polymorphism and hypertension

Association between promoter polymorphisms in the VEGFA gene and the presence of hypertension, without adjustment for confounders, are displayed in Table 4.11.3b. The associations of VEGFA-2578 and -460 with hypertension were best explained by dominant models in which the A and C alleles respectively provided an increased risk, although for -460 it was not significant. The 5ô-UTR SNP (+405) and the 3ô-UTR SNP (+936) were not associated with the presence of hypertension. When adjusted for age, sex, RA duration, BMI and CSUM in logistic regression analyses, the association between VEGFA promoter SNPs (-2578 and -460) and hypertension was lost.

Although the association was not significant in the whole group of patients, we provided some evidence showing this association may be restricted to RF positive patients (Table

4.11.3c), and it was significant (only for -2578 SNP) after adjustment for age, sex, RA duration, BMI and CSUM. However, further adjustment including IHD/MI largely reduced its significance.

Table 4.11.3b Frequency of hypertension in RA patients stratified by VEGFA SNP genotypes in all patients

	Hypertension		
	Negative	Positive (%)	Odds ratio (95% CI)
VEGFA-2578 (A/C)			
CC	75	33 (30.6)	1.0 referent
AC	116	90 (43.7)	1.75 (1.07 ï 2.86)
AA	69	43 (38.4)	1.41 (0.81 ï 2.46)
A allele	185	133 (41.8)	1.62 (1.02 ï 2.58)
VEGFA-460 (T/C)			
TT	72	33 (31.4)	1.0 referent
CT	123	92 (42.8)	1.62 (0.99 ï 2.65)
CC	65	41 (38.7)	1.37 (0.78 ï 2.41)
C allele	188	133 (41.4)	1.53 (0.96 ï 2.44)
VEGFA+405 (C/G)			
CC	25	15 (37.5)	1.0 referent
CG	116	68 (37.0)	0.97 (0.48 ï 1.94)
GG	119	83 (41.0)	1.15 (0.58 ï 2.29)
ï			
VEGFA+936 (T/C)			
TT	10	4 (28.6)	1.0 referent
CT	68	37 (35.2)	1.28 (0.39 ï 4.13)
CC	182	125 (40.7)	1.60 (0.52 ï 4.95)
C allele	250	162 (39.3)	1.51 (0.49 ï 4.64)

Values are n (%).

Table 4.11.3c Association of VEGFA-2578 with the presence of hypertension (HT) restricted to RF positive patients

	RF- patients			RF+ patients		
	HT-	HT+	OR (95% CI)	HT-	HT+	OR (95% CI)
-2578 CC	30	18 (37.5)	1.0 referent	44	15 (25.4)	1.0 referent
-2578 AC	49	37 (43.0)	1.3 (0.6 ï 2.6)	66	53 (44.5)	2.3 (1.2 ï 4.6)
-2578 AA	31	19 (38.0)	1.0 (0.5 ï 2.3)	38	23 (37.7)	1.8 (0.8 ï 3.8)
A allele	80	56 (41.2)	1.2 (0.6 ï 2.3)	104	76 (42.2)	2.1 (1.1 ï 4.0)
		NS			*p = 0.047	

Values are n (%); * CC vs. A allele (as dominant model), adjusted for age, sex, RA duration, BMI and CSUM; NS, not significant.

4.12 SUMMARY OF FINDINGS FOR 4.9 – 4.11

- No difference in allelic distribution of VEGFA polymorphisms between RA patients and healthy UK Caucasians was found.

- Strong LD was detected across the region marked by VEGFA-2578 (A/C), -460 (C/T) and +405 (G/C) polymorphisms, in the promoter region and 5ô-UTR of the VEGFA gene. A-C-G, C-T-C and C-T-G were the most frequent haplotypes.

- VEGFA-2578 C allele was associated with high serum VEGF-A level, compared to AA genotype. No clear evidence showing other polymorphisms associated with levels of VEGF-A was seen.

- The above association only occurred in patients with increased CRP (Ó10mg/l), suggesting that increased VEGF-A expression may be a response to inflammation. Furthermore, this increase was only observed in patients carrying the VEGFA-2578 CC genotype, suggesting an allele-specific mechanism.

- We confirmed the previous findings that serum VEGF-A level was correlated with inflammatory markers and major destructive enzymes in the MMP family, and that it was associated with disease activity and functional outcome measured by DAS28, and HAQ and MJS respectively.

- Most serum levels of investigated angiogenic proteins were correlated with each other.

- The T allele at VEGFA+936 was associated with earlier age at onset of RA, compared to the CC genotype.

- An increased proportion of smokers occurred in individuals carrying the VEGFA+405 G allele, especially in those carrying 2 copies. Interestingly, this allele was associated with decreased production of RF in the absence of smoking, but the association was lost in smokers.

- VEGFA-2578 AA genotype was associated with low disease activity, which was independent of the VEGF-A serum level association. Haplotype A-C-G may be responsible for this association if there are multiple influential sites in the gene sequence.

- The above association only occurred in patients who had never smoked, suggesting the AA genotype at VEGFA-2578 playing a protective role only in non-smokers.

- VEGFA promoter polymorphisms (-2578 and -460) were found to be associated with the risk of IHD and MI in RA, with VEGFA-2578 SNP showing the strongest relationship. Interestingly, it was shown that these associations may be due to a novel smoking-gene interaction where a dramatic increase of IHD/MI occurred in patients who had ever smoked and carried VEGFA risk alleles, compared to other combinations.

- Within the above interaction, the amount of smoking, measured by pack-years, showed a dose effect relationship in terms of the increased incidence of IHD/MI.

- There were independent risk factors associated with IHD and/or MI in RA, including male, age, RF, increased CRP, HAQ, hypercholesterolemia, hypertension, CVA, diabetes type 2, asthma and VEGFA-2578-smoking interaction. The use of MTX was found to be a protective factor, independent of those risk factors.

- VEGFA promoter polymorphism appeared to be associated with hypertension in RF positive patients. However, this may be due to the main association of VEGFA polymorphism with IHD/MI.

- Several co-morbid conditions tended to co-exist in RA, such as MI-CVA, IHD-diabetes type 2, hypertension-diabetes type 2, and hypertension-hypercholesterolemia.

PART D: ENOS POLYMORPHISM, SEVERITY AND CO-MORBIDITY IN RA (4.13 – 4.16)

4.13 ENOS POLYMORPHISM

4.13.1 Brief introduction

Reduction in basal NO release in the circulation is known to be associated with hypertension, thrombosis, vasospasm and atherosclerosis (Quyyumi et al, 1995; Nava et al, 1995; Nava et al, 1995; Warren et al, 1994; Tikkanen et al, 1995). In contrast, markedly elevated NO levels are reported to be associated with endotoxic shock and exaggerated inflammatory reaction (Moilanen et al, 1995). We hypothesised that functionally important polymorphism in the eNOS gene could influence the enzyme expression/activity, subsequently the amount of NO generated by endothelial cells and eventually the disease severity and/or co-morbid condition in RA.

4.13.2 ENOS polymorphism

Studied SNP

Polymorphism rs1799983 (G/T) is a missense variant in exon 7 of the gene which encodes a Glu Ÿ Asp amino acid substitution at residue 298 of eNOS (+894 according to the translational start site of the gene).

Allelic and genotypic distribution

Genotypes were determined in 427 patients. The allelic frequencies were 63.35% (n = 541) and 36.65% (n = 313) for G and T allele respectively. The genotypic distributions were 40.75% (n = 174), 45.20% (n = 116) and 14.05% (n = 60) for GG, GT and TT respectively. Genotype distribution was distributed in accordance with a close fit to HWE.

4.14 ENOS POLYMORPHISM AND DISEASE SEVERITY

4.14.1 Brief introduction

Polymorphism in the eNOS gene has been associated with RA susceptibility (Melcher et al, 2007; Vazgiourakis et al, 2007). However, there are few publications concerning the relationship with disease activity/severity. In the present study, we looked at whether the missense SNP eNOS+894 was associated with disease outcome in our patients with RA.

4.14.2 ENOS polymorphism and RA clinical measures

No association of the eNOS+894 polymorphism with any disease clinical measures was observed.

4.15 ENOS POLYMORPHISM AND CO-MORBIDITY

4.15.1 Brief introduction

The eNOS+894 polymorphism has been associated with hypertension (Rossi et al, 2003), impaired coronary collateral development (Gulec et al, 2008), heart failure (Velloso et al, 2010), coronary artery disease (Cam et al, 2005; Casas et al, 2004; Aroon et al, 1998) and the incidence of MI (Hibi et al, 1998; Shimasaki et al, 1998) in the general population. It was particular interesting to see whether this polymorphism was associated with hypertension, IHD and/or MI in our patients of RA. The relationships with other co-morbid conditions were also investigated.

4.15.2 ENOS polymorphism, smoking and IHD/MI

ENOS polymorphism and IHD/MI

The patient characteristics stratified by IHD/MI status were described before (Section 4.11.2). The relationship between eNOS+894 genotypes and the presence of IHD/MI, without adjustment for confounders, are shown in Table 4.15.2a. Patients carrying the TT genotype were associated with the highest occurrence of IHD and MI, whereas carriage of the G allele showed a reduced risk, although for MI it was only a borderline association.

When adjusted for age and sex in logistic regression analyses, the significance of the association between eNOS+894 SNP and IHD/MI was lost (TT vs. G allele, $p = 0.12$ and 0.24 for IHD and MI respectively; TT vs. GT vs. GG, $p = 0.16$ and 0.20 for IHD and MI

respectively). Further adjustment for other independent risk factors for IHD and/or MI revealed similar results.

ENOS-smoking combination and IHD/MI

Although the association between eNOS polymorphism and IHD/MI was not significant after adjustment, we looked at whether there was excess effect due to interaction with smoking (Table 4.15.2b). Results showed some evidence of an interaction based on AP. However, the 95% CI for IHD was not significant, whereas 95% CI for MI was not available (due to no MI case in the smoke-/TT+ combination).

Table 4.15.2a Frequency of IHD and MI in RA patients stratified by eNOS+894 genotypes

	IHD			MI		
	Negative	Positive (%)	Odds ratio (95% CI)	Negative	Positive (%)	Odds ratio (95% CI)
eNOS+894 (G/T)						
TT	43	16 (27.1)	1.0 (referent)	49	10 (16.9)	1.0 (referent)
GT	157	34 (17.8)	0.58 (0.29 ï 1.14)	170	21 (11.0)	0.59 (0.27 ï 1.33)
GG	145	26 (15.2)	0.48 (0.24 ï 0.97)	157	14 (8.2)	0.43 (0.18 ï 1.02)
G allele	302	60 (16.6)	0.53 (0.28 ï 0.99)	327	35 (9.7)	0.51 (0.24 ï 1.08)

Values are n (%).

Table 4.15.2b Association of eNOS+894 polymorphism and ever having smoked with IHD and MI in RA patients

	IHD			MI		
	Negative	Positive (%)	Odds ratio (95% CI)	Negative	Positive (%)	Odds ratio (95% CI)
Smoke/TT genotype						
-/-	115	9 (7.3)	1.0 (referent)	120	4 (3.2)	1.0 (referent)
-/+	15	2 (11.8)	1.96 (0.44 ï 8.71)	17	0 (0.0)*	0.77 (0.04 ï 14.83)
+/-	184	51 (21.7)	3.39 (1.64 ï 7.04)	204	31 (13.2)	4.12 (1.50 ï 11.36)
+/+	28	14 (33.3)	6.19 (2.48 ï 15.45)	32	10 (23.8)	8.65 (2.68 ï 27.88)
			AP: 0.30 (-0.25 ï 0.96)			AP: 0.55 (95% CI: NA)

Values are n (%); AP, the attributable proportion due to interaction; NA, not available; * Replacement of 0 with 1 reveals an insignificant AP with 95% CI (0.44 (-0.17 ï 1.05)).

4.16 SUMMARY OF FINDINGS FOR 4.13 – 4.15

- Patients carrying the TT genotype of eNOS+894 were associated with higher occurrence of IHD and MI, compared to those carrying a G allele. However, the significance was reduced (became insignificant) after adjustment for confounding factors including sex and age.

- With regard to the above association, our initial data suggested that there may be an interaction of eNOS+894 SNP with smoking, although it was not significant.

PART E: MMP POLYMORPHISM, SERUM MMP LEVEL, SEVERITY AND CO-MORBIDITY IN RA (4.17 – 4.21)

4.17 MMP POLYMORPHISM AND SERUM MMP LEVEL

4.17.1 Brief introduction

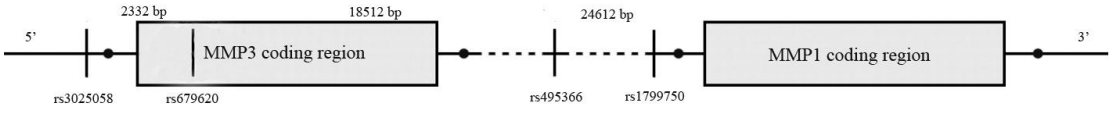
MMP-1 (collagenase-1) and MMP-3 (stromelysin-1) are the most widely studied MMPs in RA. A relationship between the MMP3 5A/6A (rs3025058) polymorphism and circulating levels of MMP-3 in RA has been found in 2 studies (Mattey et al, 2004; Tsukahara et al, 2008) although another study found no relationship (Dörr et al, 2004). The latter study also found no relationship between the MMP1 1G/2G (rs1799750) polymorphism and circulating MMP-1 levels, but no other studies have investigated this. We believed it would be of interest to further evaluate the relationship between polymorphisms in the MMP1 and 3 region and the circulating levels of MMP-1 and -3. In addition to MMP1 1G/2G and MMP3 5A/6A polymorphisms we selected 2 variants (rs495366 and rs679620) on the basis of recent studies (Cheng et al, 2009; Beyzade et al, 2003) showing the likely importance of these polymorphisms in relation to MMP-1 levels (Cheng et al, 2009) and MMP-3 function (Beyzade et al, 2003). Other MMPs such as -2 (gelatinase-1), -8 (neutrophil collagenase) and -9 (gelatinase-2) may be also important in RA. The relationship between polymorphisms in MMP2 gene and the circulating MMP-2 levels have not been investigated yet. Thus, it was also interesting to see whether MMP2 promoter SNP (rs243865) influences the circulating levels of MMP-2.

4.17.2 MMP polymorphism

Studied SNP

MMP1 and 3 are neighboring genes on the long arm of chromosome 11 (11q22.3). Rs3025058 (MMP3 5A/6A polymorphism) is a deletion/insertion polymorphism which occurs in the MMP3 promoter and at -1612 relative to the transcriptional start site. Polymorphism rs67620 A/G, located in exon 2 of the MMP3 gene, results in an amino acid change (Glu Ÿ Lys) at residue 45. Rs495366 G/A is located in the intergenic region of the MMP1 and 3 genes. Rs1799750 (MMP1 1G/2G polymorphism) is also a deletion/insertion polymorphism at -1607 relative to the transcriptional start site, in the promoter region of the MMP1 gene. Figure 4.17.2a shows the locations of MMP1 and 3 polymorphisms, and their physical distance between each other. MMP2 gene is located on the long arm of chromosome 16 (16q12.2). Polymorphism rs243865 is normally known as MMP2-1306 C/T, named after its position relative to the transcription start site.

Figure 4.17.2a Diagram of genetic region across human MMP1 and 3 genes highlighting the locations of the polymorphisms investigated



The positions of rs3025058, rs679620, rs495366 and rs1799750 at chromosome 11 are 102715952, 102713620, 102695108 and 102670496 respectively. Physical distances in bp between neighboring polymorphisms are shown. Dots placed before and after the MMP3 and MMP1 coding regions demonstrate the transcription start and end sites.

Allelic and genotypic distribution

Genotypes were determined on 430 subjects for rs3025058 (5A/6A), on 423 subjects for rs679620 (A/G), on 419 subjects for rs495366 (G/A), on 429 subjects for rs1799750 (1G/2G), and on 417 subjects for rs243865 (C/T).The allelic and genotypic frequencies of these polymorphisms in RA patients are shown in Table 4.17.2a. The Chi-Square goodness-of-fit test showed that the genotypic distribution of the 5 polymorphisms were all in HWE.

Table 4.17.2a Distribution of allelic and genotypic frequencies of rs3025058 (5A/6A), rs679620 (A/G), rs495366 (G/A), rs1799750 (1G/2G) and rs243865 (C/T) polymorphisms in RA patients

Polymorphism	Genotypic distribution	Allelic distribution
Rs3025058 (5A/6A)	5A5A: 112 (26.1)	5A: 454 (52.8)
(n = 430)	5A6A: 230 (53.5)	6A: 406 (47.2)
	6A6A: 88 (20.5)	
Rs679620 (A/G)	AA: 110 (26.0)	A: 446 (53.3)
(n = 423)	AG: 226 (53.4)	G: 390 (46.7)
	GG: 82 (20.6)	
Rs495366 (G/A)	GG: 215 (51.3)	G: 609 (72.7)
(n = 419)	GA: 179 (42.7)	A: 229 (27.3)
	AA: 25 (6.0)	
Rs1799750 (1G/2G)	1G1G: 129 (30.1)	1G: 457 (53.3)
(n = 429)	1G2G: 199 (46.4)	2G: 401 (46.7)
	2G2G: 101 (23.5)	
Rs243865 (C/T)	CC: 227 (54.4)	C: 632 (75.8)
(n = 417)	CT: 178 (42.7)	T: 202 (24.2)
	TT: 12 (2.9)	

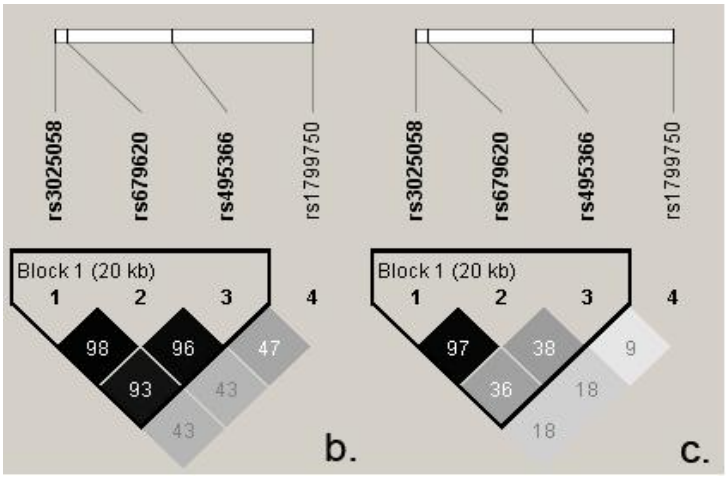
Values are n (%).

Linkage disequilibrium and haplotype

LD and haplotype were analysed among polymorphisms in the MMP1-3 genetic region. Analysis with the Haploview program demonstrated that the MMP3 SNPs rs3025058 and rs679620, and the intergenic SNP rs495366 were in the same LD block with *D'* being over 0.93 for all pairs (Figure 4.17.2b). Pair-wise correlation (r^2) between rs3025058 and rs679620 was very high (0.97, Figure 4.17.2c), whereas the correlation between rs495366 and the other 2 SNPs was only moderate (r^2 å 0.40, Figure 4.17.2c). A high *D'* and low r^2 can occur if the alleles that tend to occur on the same haplotype have very different allele frequencies. MMP1 SNP rs1799750 was not included in this LD block. However, there was still a moderate LD between rs1799750 and the LD block (*D'* = 0.44, Figure 4.17.2d), although the pair-wise correlations between rs1799750 and the other SNPs were low (r^2 < 0.2, Figure 4.17.2c). According to the location of the SNPs, this newly identified LD block included at least part of the MMP3 promoter, the whole MMP3 gene and part of the intergenic region of the MMP1 and 3 genes.

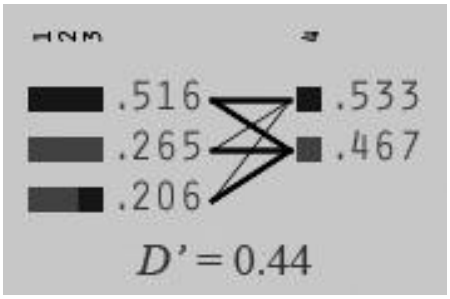
It was noted that the most frequent haplotype within the LD block (5A-A-G) was linked with the majority of 1G alleles of rs1799750 (73%), thereby forming the 5A-A-G-1G haplotype, with a frequency of 38.3% (Figure 4.17.2d). Frequencies of other haplotypes across the 4 polymorphisms are also listed (Table 4.17.2b).

Figure 4.17.2b-c Pair-wise LD of MMP1 and 3 polymorphisms measured by *D'* and r^2



Numbers in boxes represent *D'* and r^2 values (%) between paired polymorphisms in Figure b and c respectively. Polymorphisms within black border are in the same LD block.

Figure 4.17.2d Details of allelic linkage between common haplotypes within the LD block and rs1799750



One, 2, 3 and 4 represent rs3025058, rs679620, rs495366 and rs1799750 respectively. Numbers next to alleles/haplotypes show the frequencies. The value 0.44 shows the *D'* between the LD block and rs1799750. Thick/thin lines represent the different strength of linkage, with thin lines showing the connection > 1% but Ò10%, while thick lines showing the connection > 10%. Black colour represents 5A, A, G and 1G alleles for rs3025058 (as 1), rs679620 (as 2), rs495355 (as 3) and rs1799750 (as 4) respectively, whereas grey colour represents 6A, G, A and 2G alleles for rs3025058 (as 1), rs679620 (as 2), rs495355 (as 3) and rs1799750 (as 4) respectively.

Table 4.17.2b Frequency of haplotypes across rs3025058, rs679620, rs495366 and rs1799750

Haplotype	Frequency	Haplotype	Frequency
5A-A-G-1G	**0.383**	6A-A-G-1G	0.001
5A-A-G-2G	**0.133**	6A-A-G-2G	0.002
5A-A-A-1G	0.003	6A-A-A-1G	0.0
5A-A-A-2G	0.003	6A-A-A-2G	0.0
5A-G-G-1G	0.001	**6A-G-G-1G**	**0.071**
5A-G-G-2G	0.0	**6A-G-G-2G**	**0.135**
5A-G-A-1G	0.0	**6A-G-A-1G**	**0.073**
5A-G-A-2G	0.002	**6A-G-A-2G**	**0.192**

Common haplotypes are marked in black (frequency threshold = 0.05).

4.17.3 Serum level of MMP

Determination and distribution

Serum levels of MMP-1, -2, -3 as well as -8 and -9 were determined on 413, 415, 411, 412 and 414 patients respectively. MMP-2 levels were distributed close to normality (Figure 4.17.3b), while others were clearly not normally distributed. For the latter MMPs, their values were normally distributed (MMP-1 and -8) or close to a normal distribution (MMP-3 and -9) after a log transformation (Figure 4.17.3a, c-e). The median levels for MMP-1, -2, -3, -8 and -9 were 5587.8 (IQR 3383.6 ï 9405.3) pg/ml, 175344.8 (IQR 150811.3 ï 202218.1) pg/ml, 20487.2 (IQR 13426.1 ï 33915.2) pg/ml, 15061.3 (IQR 8014.4 ï 24849.7) pg/ml and 462987.0 (IQR 302560.4 ï 668509.9) pg/ml respectively.

Figure 4.17.3a Distribution of serum MMP-1 levels before (left) and after (right) log transformation in RA patients

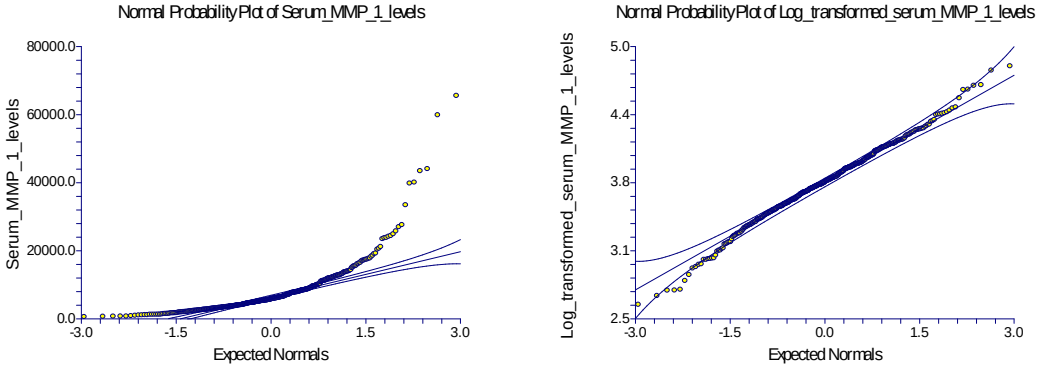


Figure 4.17.3b Distribution of serum MMP-2 levels in RA patients

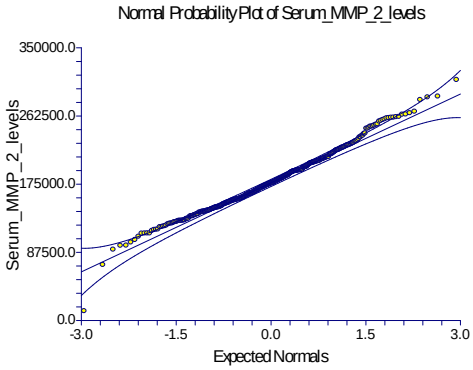


Figure 4.17.3c Distribution of serum MMP-3 levels before (left) and after (right) log transformation in RA patients

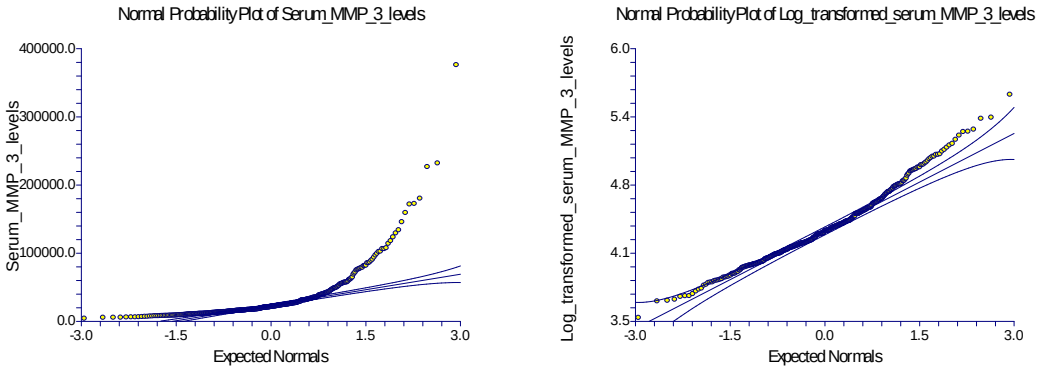


Figure 4.17.3d Distribution of serum MMP-8 levels before (left) and after (right) log transformation in RA patients

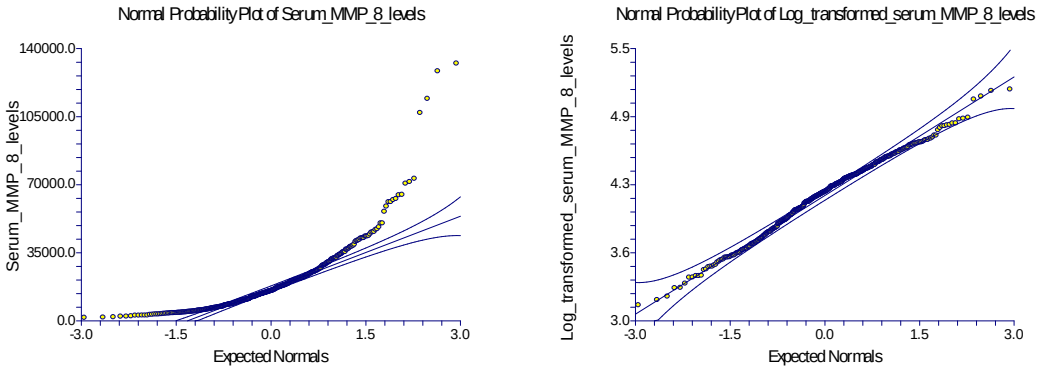
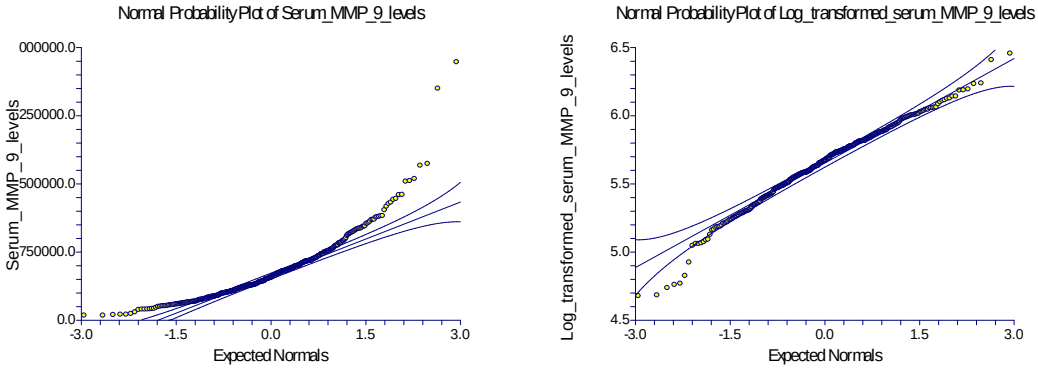


Figure 4.17.3e Distribution of serum MMP-9 levels before (left) and after (right) log transformation in RA patients



Correlation between MMPs

Serum MMP-1 was correlated with serum MMP-2 and -3, while it had weak or no relationship with serum MMP-8 and -9. Serum MMP-2 and -3 were correlated with each other, and their relationship with serum MMP-8 and -9 was much weaker. It was interesting to see that serum levels of MMP-8 and -9 were highly correlated, with r value reached 0.70.

Table 4.17.3a Correlations between serum levels of MMP-1, -2, -3, -8 and -9 in studied RA population

	Serum MMP-1	Serum MMP-2	Serum MMP-3	Serum MMP-8
Serum MMP-2	0.25 (< 0.0001)	ï	ï	ï
Serum MMP-3	0.20 (< 0.0001)	0.18 (0.0002)	ï	ï
Serum MMP-8	0.10 (0.047)	NS	0.10 (0.038)	ï
Serum MMP-9	NS	-0.10 (0.042)	NS	0.70 (< 0.0001)

Values are r (p), obtained from Spearman correlation; NS, not significant.

Serum MMP level, cigarette smoking and patient demographics

The relationship between smoking (including status and quantitative measures) and serum levels of MMP-1, -2, -3, -8 and -9 was investigated. Stratification by ever-smoked status (never vs. ever smoking) revealed no significant relationships. However, interestingly, we found that some MMP levels were particularly associated with current smoking, while there was not much difference between never and past smokers. For MMP-2 and -3, decreased levels were observed in current smokers (current smokers vs. the remaining patients, MMP-2: 156595.8 (IQR 140945.4 ï 185712.5) vs. 176701.7 (IQR 153259.6 ï 202846.1) pg/ml, p = 0.0016; MMP-3: 16380.1 (IQR 12192.7 ï 30266.8) vs. 21184.2 (IQR 13697.8 ï 34343.7) pg/ml, p = 0.080, borderline association). For MMP-9, an increased level was found in those patients (current smokers vs. the remaining patients, 564277.3 (IQR 412057.8 ï 799009.9) vs. 424269.1 (IQR 283565.9 ï 643137.1) pg/ml, p = 0.0004).

The relationships between MMP levels and parameters such as age, sex, BMI and RA duration were also investigated. Interestingly, we found that there were a positive correlation of MMP-2 with age (r = 0.31, p < 0.0001) and a negative correlation of MMP-2

with RA duration (r = -0.13, p = 0.0076). Analysis by multivariate multiple regression indicated that both age and RA duration were independently associated with MMP-2 level (adjusted for sex, $p < 0.0001$ and $p = 0.023$ respectively). MMP-3 level in male patients was higher than that in female patients (25265.8 vs. 17037.0 pg/ml, p = 0.050, adjusted for age and RA duration).

4.17.4 MMP1, 2, 3 polymorphism and serum MMP level

Polymorphism in MMP1-3 loci and serum level of MMP-1 and -3

The serum levels of MMP-1 were not only associated with the MMP1 promoter polymorphism rs1799750 but also with the markers of the LD block, including rs495366, rs679620 and rs3025058 (Figure 4.17.4a-d). In order to test whether the latter associations were independent from the former association, multivariate multiple regression analyses were carried out (adjusted for age, sex and RA duration) with rs1799750 and each of the other 3 SNPs as independent variables, and MMP-1 level (log transformed) as the dependent variable. In each model, both the association with rs1799750 and the LD block polymorphism remained significant, indicating the independence of these associations (Table 4.17.4a, models 1, 2 and 3). There was also an association of the intergenic SNP (rs495366) independent of the MMP3 SNPs (Table 4.17.4a, models 4 and 5).

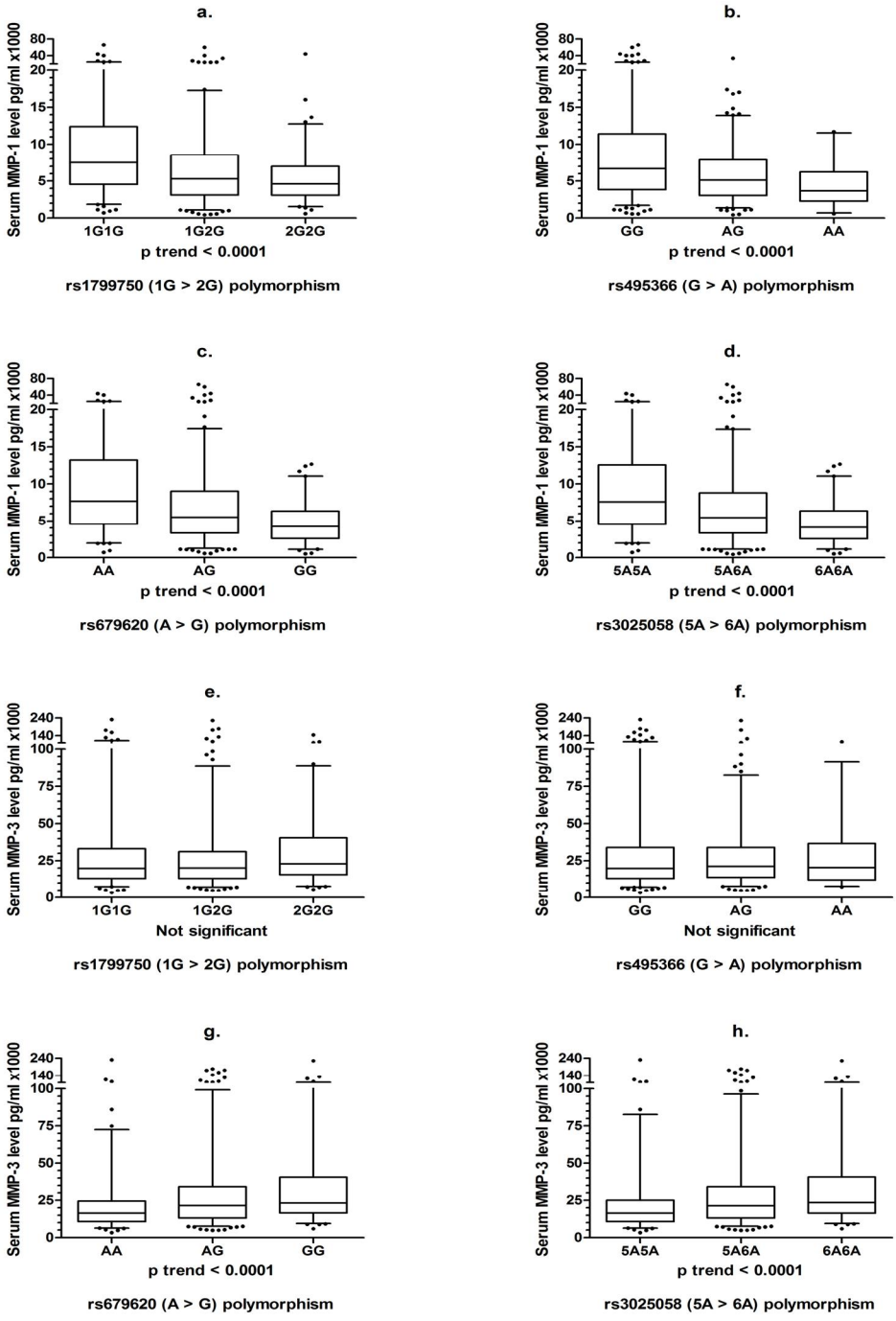
Analysis of triple combinations of SNPs revealed a model in which rs1799750, rs495366 and rs3025058 were each independently associated with MMP-1 levels (Table 4.17.4a, model 6). In model 6, we show the rs3025058 SNP as being independent of rs1799750 and rs495366. However, replacement of rs3025058 with rs679620 in this model gives

almost identical results. Analyses were also carried out which investigated possible interactions between SNPs by including a SNP x SNP interaction term, as well as the main effects, in each analysis. None of the interaction terms were found to be significant.

Our data suggest that there is at least another functional site if not multiple sites, located probably within or close-by the LD block, which has functional effects on MMP-1 expression, apart from the rs1799750 polymorphism. The independent association of rs1799750 and rs3025058 with MMP-1 levels was confirmed in a second cohort of patients (n = 291) with established RA (data not shown).

In un-adjusted analyses of single SNPs, the circulating levels of MMP-3 were associated with the MMP3 SNPs rs3025058 and rs679620, but not with the intergenic SNP rs495366 or the MMP1 SNP rs1799750 (Figure 4.17.4e-h). The 6A allele of rs3025058 and the G allele of rs679620 were associated with the highest levels of circulating MMP-3. Interestingly, in joint analysis of 2 SNP combinations (adjusted for age, sex and RA duration) we found that the MMP1 SNP rs1799750 was associated with MMP-3 levels after adjustment for the intergenic SNP (Table 4.17.4a, model 1). However, the MMP1 SNP was no longer associated when analysed jointly with either of the MMP3 SNPs (Table 4.17.4a, models 2 and 3). In contrast, the intergenic SNP demonstrated an association with MMP-3 levels (Table 4.17.4a, models 4 and 5) when adjusted for either MMP3 SNP, each of which remained highly significant ($p < 0.0001$). The association of rs3025058 with MMP-3 levels was confirmed in a second cohort of patients (n = 291, data not shown).

Figure 4.17.4a-h Relationship between genotypes of rs1799750, rs495366, rs679620 and rs3025058 and serum levels of MMP-1 and -3



Boxplot with whiskers with 5 ï 95 percentile.

Table 4.17.4a Test of independence of MMP SNP associations with MMP serum levels in multiple regression analyses

Independent variable	Response variable MMP-1 pg/ml (log transformed) Regression coefficient (SE)	p	Response variable MMP-3 pg/ml (log transformed) Regression coefficient (SE)	p
Model 1				
rs1799750 (1G1G > 1G2G > 2G2G)	0.077 (0.024)	0.0017	- 0.047 (0.018)	0.009
rs495366 (GG > AG > AA)	0.110 (0.029)	0.0003	- 0.012 (0.021)	0.57
Model 2				
rs1799750 (1G1G > 1G2G > 2G2G)	0.066 (0.025)	0.0096	- 0.012 (0.018)	0.50
rs679620 (AA > AG > GG)	0.110 (0.027)	< 0.0001	- 0.090 (0.019)	< 0.0001
Model 3				
rs1799750 (1G1G > 1G2G > 2G2G)	0.066 (0.026)	0.0110	- 0.011 (0.018)	0.53
rs3025058 (5A5A > 5A6A > 6A6A)	0.110 (0.027)	< 0.0001	- 0.091 (0.019)	< 0.0001
Model 4				
rs495366 (GG > AG > AA)	0.067 (0.034)	0.047	0.068 (0.025)	0.007
rs679620 (AA > AG > GG)	0.090 (0.030)	0.0029	- 0.132 (0.022)	< 0.0001
Model 5				
rs495366 (GG > AG > AA)	0.067 (0.029)	0.02	0.065 (0.025)	0.01
rs3025058 (5A5A > 5A6A > 6A6A)	0.085 (0.025)	0.0009	- 0.131 (0.022)	< 0.0001
Model 6*				
rs1799750 (1G1G > 1G2G > 2G2G)	0.064 (0.021)	0.002	- 0.012 (0.019)	0.57
rs495366 (GG > AG > AA)	0.060 (0.028)	0.034	0.063 (0.025)	0.01
rs3025058 (5A5A > 5A6A > 6A6A)	0.058 (0.026)	0.028	- 0.125 (0.023)	< 0.0001

Multiple regression analyses adjusted for age, sex and RA duration; SE, standard error; * Replacement of rs3025058 with rs679620 in this model gives almost identical results.

We next tested the haplotypic associations with MMP-1 and MMP-3 levels (Table 4.17.4b). Analysis with the HAPSTAT program indicated that the 5A-A-G-1G (rs3025058, rs679620, rs495366, rs1799750) was associated with the highest levels of MMP-1 ($p < 0.0001$, in both additive and dominant models, vs. the remaining haplotypes). This association could be explained mainly by the 2 SNP 5A-1G haplotype since over 98% of patients with this haplotype carried 5A-A-G-1G. The 6A-G-A-2G haplotype was correlated with the lowest levels of MMP-1 ($p < 0.0001$, in both additive and dominant models, vs. the remaining haplotypes). Furthermore, the 6A-G-A-1G haplotype was also associated with low MMP-1 levels compared to the other haplotypes, excluding 6A-G-A-2G (p Ò0.02 in both additive and dominant models). This supports the idea that the polymorphisms within the LD block region have a strong effect on the regulation of MMP1 expression, independent of the rs1799750 polymorphism.

The 4 SNP haplotype associated with the highest MMP-3 levels was 6A-G-G-2G (p Ò 0.004 in additive and dominant models, vs. the remaining haplotypes), although the association was stronger after exclusion of the rs1799750 SNP (6A-G-G, $p < 0.0001$). Due to the high LD and correlation between rs3025058 and rs679620, the association could be explained mainly by the 2 SNP (rs3025058-rs495366) 6A-G haplotype or the (rs679620-rs495366) G-G haplotype, both of which gave highly significant associations ($p < 0.0001$). This association was lost if the rs495366 G allele was replaced by the A allele to give 6A-A or G-A haplotypes ($p > 0.5$). The lowest MMP-3 levels were associated with the 5A-A-G-1G haplotype, although this could be explained mainly by the 2 SNP haplotype 5A-G (rs3025058-rs495366).

Table 4.17.4b Association of haplotypes in MMP1-3 loci with serum MMP-1 and -3 levels

		Response variable MMP-1 pg/ml (log transformed)		Response variable MMP-3 pg/ml (log transformed)	
Haplotype	Frequency	Regression coefficient (SE)	p	Regression coefficient (SE)	p
4 SNP					
6A-G-A-1G	0.073	-0.127 (0.053)	0.015	-0.087 (0.049)	0.077
6A-G-G-1G	0.071	-0.011 (0.058)	0.85	0.190 (0.049)	< 0.0001
5A-A-G-1G	0.383	0.159 (0.026)	< 0.0001	-0.067 (0.024)	0.005
6A-G-A-2G	0.192	-0.147 (0.034)	< 0.0001	0.042 (0.032)	0.18
6A-G-G-2G	0.133	-0.048 (0.041)	0.24	0.100 (0.035)	0.004
5A-A-G-2G	0.135	-0.031 (0.039)	0.43	-0.059 (0.036)	0.10
3 SNP*					
6A-A-1G	0.073	-0.130 (0.052)	0.013	-0.085 (0.050)	0.085
5A-G-1G	0.383	0.152 (0.025)	< 0.0001	-0.058 (0.024)	0.015
6A-G-1G	0.074	-0.004 (0.057)	0.95	0.190 (0.049)	0.0001
5A-A-2G	0.005	0.036 (0.153)	0.82	-0.242 (0.139)	0.082
6A-A-2G	0.193	-0.149 (0.033)	< 0.0001	0.043 (0.032)	0.18
5A-G-2G	0.133	-0.035 (0.040)	0.38	-0.063 (0.036)	0.082
6A-G-2G	0.135	-0.051 (0.040)	0.20	0.100 (0.035)	0.004
2 SNP[À]					
5A-1G	0.385	0.150 (0.025)	< 0.0001	-0.058 (0.024)	0.014
6A-1G	0.147	-0.068 (0.038)	0.077	0.050 (0.035)	0.15
5A-2G	0.140	0.029 (0.038)	0.44	-0.073 (0.035)	0.035
6A-2G	0.328	-0.123 (0.027)	< 0.0001	0.082 (0.026)	0.001
2 SNP[ÿ]					
5A-A	0.008	0.009 (0.131)	0.94	-0.181 (0.120)	0.13
6A-A	0.270	-0.145 (0.028)	< 0.0001	0.007 (0.027)	0.79
5A-G	0.516	0.130 (0.025)	< 0.0001	0.083 (0.023)	0.003
6A-G	0.206	-0.031 (0.031)	0.32	0.124 (0.028)	< 0.0001

* 3 SNP haplotype rs3025058-rs495366-rs1799750; [À]2 SNP haplotype rs3025058-rs1799750; [ÿ] 2 SNP haplotype rs3025058-rs495366; SE, standard error. The results are based on additive models of inheritance (dominant models demonstrated less significant results, and therefore are not shown).

Previous studies had shown significant correlations of MMP-1, and particularly MMP-3 serum levels with levels of inflammation in RA (Taylor et al, 1994; Keyszer et al, 1999; Cheung et al, 2000; Cunnane et al, 2001; Green et al, 2003). We were therefore interested to see whether the relationship between variations across the MMP1-3 loci and the levels of MMP-1 and -3 was independent of the level of systemic inflammation. In multiple regression models we included CRP as an independent variable together with each SNP or combination of SNPs. We found that CRP was strongly associated with MMP-1 levels, but each SNP was also independently associated. By analysis of 2 and 3 SNPs jointly, together with CRP levels, the best overall model associated with MMP-1 levels was found to consist of rs3025058, rs1799750 and CRP (Table 4.17.4c). This was confirmed in the second cohort of patients with established disease (n = 291, data not shown).

MMP-3 was also strongly associated with CRP, but single marker analyses of the MMP3 SNPs, rs3025058 and rs679620, also showed associations with MMP-3 independent of CRP. In 2 SNP analyses we found that the intergenic SNP rs495366 was associated (p Ò 0.01) with MMP-3 levels after adjustment for rs3025058 or rs679620, and independently of CRP levels (Table 4.17.4c). Replication of the independent association of rs3025058 and CRP with MMP-3 levels was confirmed in the second cohort of patients (n = 291, data not shown).

Table 4.17.4c Polymorphisms in MMP1-3 loci are associated with MMP-1 and MMP-3 serum levels independently of CRP levels

	Response variable MMP-1 pg/ml (log transformed)		Response variable MMP-3 p/ml (log transformed)	
Independent variable	Regression coefficient (SE)	p	Regression coefficient (SE)	p
rs1799750 (1G1G > 1G2G > 2G2G)	0.060 (0.020)	0.003		
rs3025058 (5A5A > 5A6A > 6A6A)	0.096 (0.022)	< 0.0001	-0.136 (0.022)	< 0.0001
rs495366 (GG > AG > AA)			0.066 (0.025)	0.01
CRP (mg/l)	0.0028 (0.0006)	< 0.0001	0.0043 (0.0006)	< 0.0001

Multiple regression analyses adjusted for age, sex and RA duration; SE, standard error.

MMP2 polymorphism and serum level of MMP-2

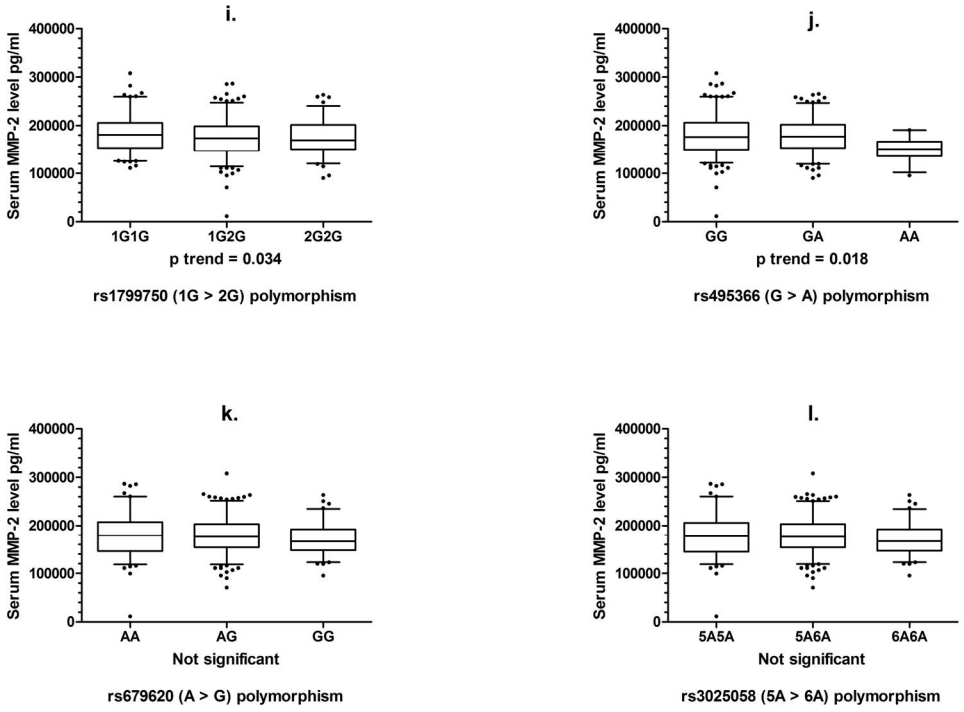
No association was found between MMP2 promoter SNP rs243865 (C/T) and serum levels of MMP-2 in this RA population (CC vs. T allele, 175109.8 vs. 186198.4 pg/ml, p = 0.23).

Polymorphisms in MMP1-3 loci and serum levels of MMP-2

In un-adjusted analysis, 1G allele of rs1799750 (MMP1 SNP) and G allele of rs495366 (intergenic SNP) were found to be associated with increased levels of MMP-2 (Figure 4.17.4i and j). Although it was not significant, MMP3 polymorphisms including rs679620 and rs3025058 also showed a trend in which high MMP-2 levels were related to A and 5A alleles respectively (Figure 4.17.4k and l). Haplotype analysis with HAPSTAT suggested that 5A-A-G-1G (rs3025058-rs679620-rs495366-rs1799750) was associated with

increased MMP-2 (p = 0.028, additive model, vs. the remaining haplotypes), whereas 6A-G-A-2G was associated with decreased MMP-2 (p = 0.025, additive model, vs. the remaining haplotypes). Exclusion of MMP3 polymorphisms gave more significant associations (G-1G associated with high MMP-2, p = 0.0066, additive model; A-2G associated with low MMP-2, p = 0.020, additive model). Further analysis in multivariate regression revealed that SNP rs495366 appeared to be associated with MMP-2 levels independently of current smoking, and serum MMP-1 and -3 levels (Table 4.17.4d).

Figure 4.17.4i-l Relationship between genotypes of rs1799750, rs495366, rs679620 and rs3025058 and serum levels of MMP-2



Boxplot with whiskers with 5 ï 95 percentile.

Table 4.17.4d Relationship between rs495366 polymorphism and serum MMP-2 levels in multivariate multiple regression

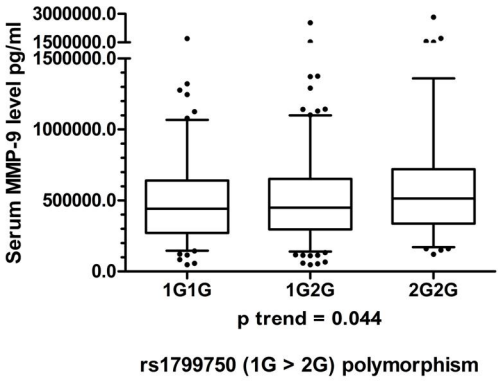
	Serum MMP-2	
Independent variable	Regression coefficient	p
Rs495366*	17137.32	0.026
Serum MMP-1 levels[A]	22211.19	< 0.0001
Serum MMP-3 levels[A]	14119.08	0.017
Current smokers[¶]	-9618.09	0.044
Age	1066.98	< 0.0001
Female	3706.74	0.36
RA duration	-399.75	0.040

* Rs495366 AA vs. G allele (as dominant model); [A]log transformed; [¶] vs. the remaining subjects.

MMP1 SNP rs1799750 and serum level of MMP-9

MMP1 polymorphism rs1788750 was associated with serum MMP-9 levels, with 1G allele associated with low levels (1G1G vs. 1G2G vs. 2G2G, p trend = 0.044, Figure 4.17.4n). Other polymorphisms in the MMP1-3 gene loci did not seem to be involved. Using multivariate multiple regression, we found that this association (p = 0.022) was independent from CRP, MMP-1, age, sex and RA duration.

Figure 4.17.4n Relationship between MMP1 SNP rs1799750 and serum MMP-9 levels



Boxplot with whiskers with 5 ï 95 percentile.

4.18 MMP POLYMORPHISM, SERUM MMP AND DISEASE SEVERITY

4.18.1 Brief introduction

Much of the joint damage in RA is believed to be mediated by abnormal release of MMPs in rheumatoid synovium stimulated by persistent inflammation (MacNaul et al, 1990; Konttinen et al, 1991; Gravallese et al, 1991; Konttinen et al, 1999; Cunnane et al, 2001). We have found a relationship between polymorphisms in the MMP1-3 genetic loci and the serum levels of MMP-1 and -3, and have further indicated that the associations are independent of inflammatory status. Other previous studies have examined the relationship between MMP polymorphisms (including SNPs in MMP1, 3, 7, 9, 12 and13 genes) and the development and/or severity of RA (Constantin et al, 2002(1); Constantin et al, 2002(2); Lee et al, 2003; Mattey et al, 2004; Dörr et al, 2004; Nemec et al, 2007; Ye et al, 2007; Tsukahara et al, 2008; Scherer et al, 2010). No association has been found between these

polymorphisms and susceptibility to RA, apart from one study which showed a lower frequency of the MMP3 rs3025058 5A allele in RA patients compared with controls (Scherer et al, 2010). With regard to disease severity evidence of an association has been found, but results between studies are conflicting. On the base of our observation, it was of interest to further evaluate the association of variants of MMP1 and 3 and serum levels of MMP-1 and -3 with disease activity/outcome measures in this group of RA patients.

4.18.2 Serum MMP and RA clinical measures

No difference in MMP levels was found between patients stratified by RF or anti-CCP status. The correlations between serum levels of MMP-1, -2, -3, -8 and -9, and inflammatory markers including ESR and CRP and disease measures including DAS28, MJS and HAQ are shown in Table 4.18.2a. Serum MMP-1 was shown to be correlated with both inflammatory markers and clinical measures, although the correlations with ESR and MJS were not statistically significant ($r = 0.08$, $p = 0.11$; $r = 0.10$, $p = 0.059$ respectively). However, in further analyses adjusted for sex, age and RA duration, all these relationships reached significance and provided much stronger signals (data not shown). MMP-3 level was primarily correlated with inflammatory markers and with disease activity, but no significant correlations were achieved with functional outcome measures. No correlations were found between MMP-2, -8 and -9 and these listed disease measures. We also tested their relationships with nodular and erosive diseases. In the whole population no association was found with nodular disease, although decreased MMP-1 was associated with nodular disease particularly in female patients (4460.2 (IQR 3097.1 ï 7256.8) vs. 5842.5 (IQR 3359.9 ï 9905.0), $p = 0.034$). Interestingly, we found that serum MMP-2 was decreased in patients with erosive disease, compared to those without joint

erosions (172220.3 (IQR 149212.9 ï 196414.8) vs. 182885.6 (IQR 156451.3 ï 211862.7), p = 0.021). Further analysis suggested that the association only occurred in autoantibody positive patients (RF+: p = 0.008; Anti-CCP+: p = 0.004) or patients who had ever smoked (p = 0.010) (statistics remained significant after adjusting for sex, age and RA duration, data not shown).

Table 4.18.2a Correlations between serum MMP levels and inflammatory and disease measures in RA

	ESR	CRP	DAS28	MJS	HAQ
MMP-1	NS	0.16 (0.0009)	0.10 (0.048)	NS	0.10 (0.038)
MMP-2	NS	NS	NS	NS	NS
MMP-3	0.14 (0.0045)	0.28 (< 0.0001)	0.12 (0.013)	NS	NS
MMP-8	NS	NS	NS	NS	NS
MMP-9	NS	NS	NS	NS	NS

Values are r (p), obtained from Spearman correlation; NS, not significant.

4.18.3 MMP polymorphism and RA clinical measures

Polymorphism rs495366 and age at onset of RA

The AA genotype at the intergenic SNP rs495366 was associated with earlier age at onset of RA, compared to the G allele (AA (n = 24) vs. G allele (n = 376), 41.7 ± (SD 12.9) vs. 50.1 (SD 13.0), p = 0.0048). In a multivariate multiple regression model that contained age, ever-smoking, VEGFA+936 SNP (T allele vs. CC) and rs495366 (AA vs. G allele) together as independent variables, it was shown that the T allele at VEGFA+936, the AA

genotype at rs495366 and females were independently associated with earlier age at onset of RA (Table 4.18.3a).

Table 4.18.3a Association of the AA genotype at rs495366 with earlier age at onset of RA in a multivariate multiple regression model, independent of age, sex and VEGFA+936 SNP

	Response variable: age at onset of RA	
Independent variable	Regression coefficient (SE)	p
Rs495366*	-8.26 (2.69)	0.0023
VEGFA+936[A]	-3.77 (1.41)	0.0082
Female	-3.08 (1.40)	0.028
Ever-smoking	2.36 (1.39)	0.091

SE, standard error; * AA vs. G allele (as dominant model); [À]T allele vs. CC (as dominant model).

Polymorphism in MMP1-3 loci and disease activity

We investigated whether there was a relationship between variations across the MMP1-MMP3 loci and the level of disease activity (measured by DAS28). Analysis of each of the MMP1 and MMP3 SNPs and haplotypes (adjusted for age, sex and RA duration) did not find any significant associations although there was a trend of increasing DAS28 with 5A allele number for the MMP3 rs3025058 SNP (p trend = 0.070) and with A allele number for the MMP3 rs679620 SNP (p trend = 0.10). When these SNPs were analysed singly in separate multiple regression models adjusted for levels of MMP-1 and MMP-3 (log transformed) as independent variables we found that the DAS28 was associated with each MMP3 SNP (rs3025058, 5A5A vs. 5A6A vs. 6A6A, p = 0.035; rs679620, AA vs. AG vs. GG, p = 0.038) as well as independently with the levels of both MMP-1 and MMP-3 (Table

4.18.3b, model 1 and 2 respectively). The association of each SNP with the DAS28 remained when CRP was also included in these models as an independent variable, although the association of MMP-1 with disease activity was lost. Table 4.18.3b shows the model containing rs3025058 SNP, MMP-3 and CRP levels (model 3). A very similar model was seen with rs679620 polymorphism (data not shown). Haplotype analysis did not reveal any additional associations.

Further evidence of an association between MMP3 polymorphism and DAS28 was provided by analysis of the mean time-averaged DAS28 measurements on 301 patients followed up for 24 months (DAS28 at baseline, 12^{th} month and 24^{th} month). Once again, both MMP3 SNPs were associated with the mean time-averaged DAS28 (p Ò 0.015), independently of baseline MMP-3 levels (log transformed) (p Ò 0.001, Table 4.18.3c). As at baseline, there was an increasing time-averaged DAS28 with increasing rs3025058 5A allele dose or rs679620 A allele dose. Baseline MMP-1 levels were not significantly associated in these models.

Table 4.18.3b Association of MMP3 polymorphism with DAS28 in multivariate multiple regression models, independent from MMP-1, MMP-3 and CRP levels

	Response variable: DAS28	
Independent variable	Regression coefficient (SE)	p
Model 1		
rs679620 (AA > AG > GG)	0.212 (0.102)	0.038
Serum MMP-1	0.021 (0.01)	0.038
Serum MMP-3	0.009 (0.002)	< 0.0001
Model 2		
rs3025058 (5A5A > 5A6A > 6A6A)	0.215 (0.102)	0.035
Serum MMP-1	0.021 (0.01)	0.035
Serum MMP-3	0.008 (0.002)	< 0.0001
Model 3*		
rs3025058 (5A5A > 5A6A > 6A6A)	0.216 (0.092)	0.025
Serum MMP-3	0.004 (0.002)	0.028
CRP	0.024 (0.003)	< 0.0001

Multiple regression analyses adjusted for age, sex and RA duration; SE, standard error; * Replacement of rs3025058 with rs679620 gives similar results.

Table 4.18.3c Association of MMP3 polymorphism with mean time-averaged DAS28 over 2 years (on 301 patients) in multivariate multiple regression models, independent from baseline MMP-3

	Response variable: Mean time-averaged DAS28 (over 2 years)	
Independent variable	Regression coefficient (SE)	p
Model 1		
rs679620 (AA > AG > GG)	0.228 (0.103)	0.028
Model 2		
rs3025058 (5A5A > 5A6A > 6A6A)	0.213 (0.102)	0.037
Model 3		
rs679620 (AA > AG > GG)	0.266 (0.102)	0.010
Baseline serum MMP-3	0.008 (0.002)	0.0007
Model 4		
rs3025058 (5A5A > 5A6A > 6A6A)	0.249 (0.101)	0.015
Baseline serum MMP-3	0.007 (0.002)	0.001

Multiple regression analyses adjusted for age, sex and RA duration at baseline; SE, standard error.

Polymorphism in MMP1-3 loci and erosive disease

We analysed the relationship between variations across the MMP1 and 3 genes and the development of erosive disease. The presence of at least one erosion was found in 312/423 (73.8%) of patients for whom radiographs were available. In the whole group of patients no significant association of erosive status with any genotypes or haplotypes were found. However, we observed a general trend where patients carrying genetic markers associated with high levels of MMP-1 were more likely to have a higher frequency of erosive disease

(Table 4.18.3d). It has been suggested that when erosiveness is used as an outcome measure, such analyses should be restricted to patients early in the disease since it suffers from a ceiling effect i.e. the majority of patients will have developed erosions in more advanced disease (van der Helm-van Mil 2010). Since this was a cross-sectional study it was not possible to follow the development of erosions prospectively in individual patients. We therefore re-analysed the data by only including patients with RA durations up to particular time points (2 years, 4 years, 6 years and > 6 years). We found that analysis of the data in patients with up to 6 years RA duration (n = 167) revealed significant associations of erosive status with both MMP1 and MMP3 genotypes (Table 4.18.3d). We found no significant associations in patients with RA duration greater than 6 years.

Significant trends were found with rs1799750 (1G1G vs. 1G2G vs. 2G2G) and rs3025058 (5A5A vs. 5A6A vs. 6A6A) genotypes in patients with RA duration Ò 6 years. To test whether these 2 polymorphisms were associated with erosive status independently, a multivariable logistic regression model containing both rs1799750 and rs3025058 as independent variables and erosive status as response variable was carried out. The model was adjusted for sex, age and RA duration. The results showed that the association of rs1799750 with erosive status remained significant (p = 0.032), whilst the association with rs3025058 was no longer significant. The association involving rs3025058 may thus be explained by its linkage with the rs1799750 polymorphism.

Table 4.18.3d Relationship between polymorphism in MMP1-3 loci and erosive development

	All patients		Group 1: patients with disease duration Ò6 yrs		Group 2: patients with disease duration > 6 yrs	
Genotype	Erosions		Erosions		Erosions	
rs1799750						
1G1G	96/123 (78.0)		35/47 (74.5)		61/76 (80.3)	
1G2G	145/198 (73.2)		50/80 (62.4)		95/118 (80.5)	
2G2G	68/98 (69.4)		17/39 (43.6)		51/59 (86.4)	
		NS	p trend = 0.004			NS
rs495366						
GG	149/207 (72.0)		53/84 (63.1)		96/123 (78.0)	
GA	132/177 (74.6)		46/77 (59.7)		86/100 (86.0)	
AA	20/25 (80.0)		2/5 (40.0)		18/20 (90.0)	
		NS		NS		NS
rs679620						
AA	81/105 (77.1)		28/40 (71.8)		53/66 (80.3)	
AG	160/222 (72.1)		58/96 (60.4)		102/126 (81.0)	
GG	62/86 (72.1)		15/30 (50.0)		47/56 (83.9)	
		NS		NS		NS
rs3025058						
5A5A	84/107 (78.5)		29/39 (74.4)		55/68 (80.9)	
5A6A	163/226 (72.1)		59/98 (60.2)		104/128 (81.3)	
6A6A	62/87 (71.3)		14/30 (46. 7)		48/57 (84.2)	
		NS	p trend = 0.019			NS

Values are n of positive case (%); NS, not significant.

Analysis of haplotypes indicated that carriage of the 5A-A-G-1G (rs3025058-rs679620-rs495366-rs1799750) haplotype provided an increased risk for the development of erosive disease during the early years of disease. In contrast, 6A-G-G-2G may be a protective

haplotype (Table 4.18.3e). Haplotypes which carried the 1G allele but not 5A, or the 5A allele but not 1G, were not associated with an increased risk of erosive disease in early years of disease.

The DAS28 was not significantly associated with erosive disease, although in patients with RA duration Ò6 years it was higher in subjects with erosions (4.28 vs. 4.07). Similarly, the circulating levels of MMP-1 and -3 were not associated with erosive status (as mentioned before), although in patients with RA duration Ò6 years a higher levels of MMP-1 was observed in those with erosions, compared to those without (6523.25 vs. 5324.7 pg/ml). When the DAS, MMP-1 or -3 levels were included as independent variables along with the 5A-A-G-1G haplotype in logistic regression models, only the haplotype was found to be significantly associated (p Ò0.004) with erosive disease in patients with RA duration Ò6 years.

Table 4.18.3e Relationship between haplotypes across rs3025058, rs679620, rs495366 and rs1799750, and the development of erosive disease

All patients				
Haplotype	Case, control frequencies	Chi square	Odds ratio (95% CI)	p value
5A-A-G-1G	0.395, 0.331	2.863	1.33 (0.96 ï 1.83)	0.091
6A-G-A-2G	0.197, 0.185	0.165	1.08 (0.73 ï 1.60)	0.684
6A-G-G-2G	0.123, 0.170	3.105	0.68 (0.44 ï 1.03)	0.078
5A-A-G-2G	0.129, 0.145	0.358	0.88 (0.56 ï 1.36)	0.550
6A-G-G-1G	0.080, 0.062	0.690	1.24 (0.68 ï 2.29)	0.406
6A-G-A-1G	0.065, 0.088	1.270	0.69 (0.40 ï 1.21)	0.260
Group 1: patients with RA duration Ò6 yrs (n = 167)				
Haplotype	Case, control frequencies	Chi square	Odds ratio (95% CI)	p value
5A-A-G-1G	0.456, 0.303	6.771	1.83 (1.15 ï 2.91)	0.004
6A-G-A-2G	0.183, 0.216	0.624	0.80 (0.46 ï 1.38)	0.330
6A-G-G-2G	0.120, 0.202	3.651	0.56 (0.31 ï 0.95)	0.020
5A-A-G-2G	0.108, 0.123	0.437	0.75 (0.38 ï 1.48)	0.493
6A-G-G-1G	0.057, 0.071	0.093	0.83 (0.34 ï 1.98)	0.770
6A-G-A-1G	0.066, 0.041	0.412	1.24 (0.47 ï 3.30)	0.337
Group 2: patients with RA duration > 6 yrs (n = 256)				
Haplotype	Case, control frequencies	Chi square	Odds ratio (95% CI)	p value
5A-A-G-1G	0.362, 0.380	0.095	0.94 (0.59 ï 1.52)	0.758
6A-G-A-2G	0.201, 0.138	1.822	1.55 (0.81 ï 2.95)	0.177
6A-G-G-2G	0.144, 0.153	0.051	0.89 (0.47 ï 1.66)	0.821
5A-A-G-2G	0.124, 0.117	0.026	1.05 (0.52 ï 2.14)	0.872
6A-G-G-1G	0.089, 0.091	0.003	0.93 (0.42 ï 2.03)	0.955
6A-G-A-1G	0.070, 0.119	2.457	0.53 (0.26 ï 1.09)	0.117

Possible MMP1-3-loci-smoking interaction and erosive disease

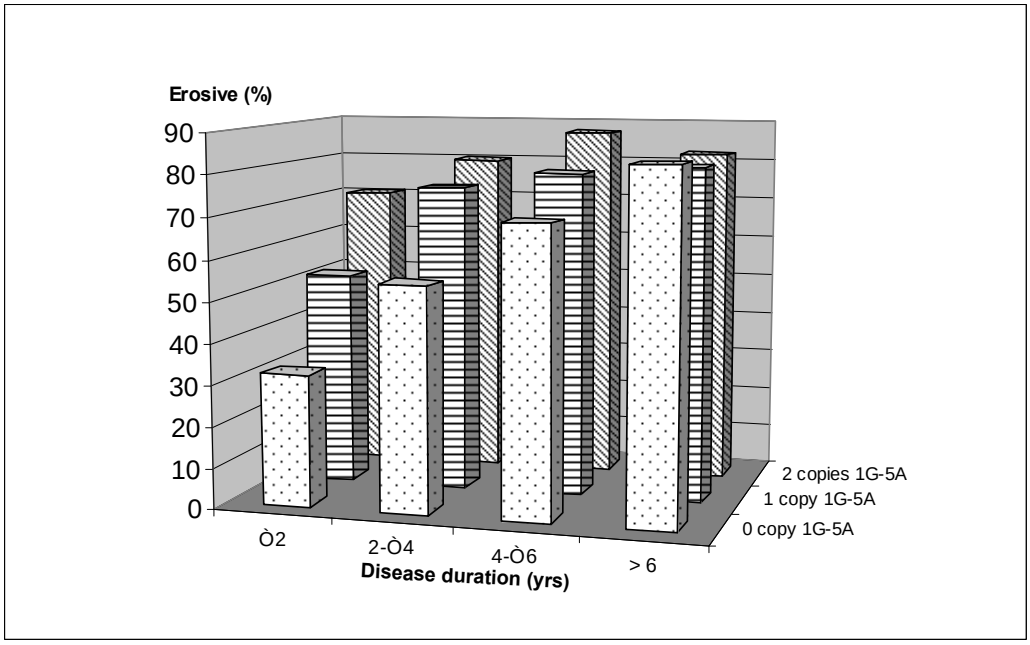
As described above, analysis of individual polymorphisms suggested that the 1G allele at rs1799750 and the 5A allele at rs3025058 were associated with higher rate of erosive

damage in patients with earlier disease (RA duration Ò6 yrs), while other polymorphisms were not significantly involved. Patients carrying 2 copies of the risk alleles were associated with the highest rate, which was best explained by an additive model of genetic heritability. Meanwhile, haplotype analysis in these patients indicated the 5A-A-G-1G as the risk haplotype. This could be explained by the 2 SNP 5A-1G haplotype since over 98% of patients with this haplotype carried 5A-A-G-1G. To reduce complexity we used the 2 SNP haplotype to investigate the possible interaction between smoking and sequence variation within the MMP1-3 loci relative to erosive course in RA. The number of copies of 5A-1G was determined on each patient based on the genotype information (for analyses using additive model). It should be noted that all of the patients carrying 5A6A at rs3025058 and 1G2G at rs1799750 were categorized into those with 1 copy of 5A-1G, since the majority (84%) of these subjects was estimated to be carrying a copy of 5A-1G due to the LD between rs3025058 and rs1799750 (calculation method shown in Appendix 8.5).

In the above analysis, individual polymorphism was not significantly associated with erosive course in the whole population (Table 4.18.3d). Based on an additive model we tested whether the number of copies of 5A-1G (2 copies vs. 1 copy vs. 0 copy) would increase the significance of association. A significant p value (0.047) was achieved after adjustment for sex, age, RA duration and smoking (ever-smoked) in a multivariant regression model. This model also suggested that smoking showed an independent effect (p = 0.054). Thus, it was interesting to see the combination effect of 5A-1G (copy numbers, as additive model) with smoking in relation to the occurrence of erosive disease in all patients (Table 4.18.3f). Since we found that the association mostly occurred during the earlier disease (RA duration Ò6 years), we also particularly looked at the 5A-1G effect in

those patients (Table 4.18.3f). Figure 4.18.3a shows the RA-duration-restricted relationship between the number of copies of 5A-1G haplotype and the occurrence of erosive damage.

Figure 4.18.3a Relationship between the number of copies of 5A-1G haplotype and the occurrence of erosive disease, stratified by RA duration (n Ò2, 2 < n Ò4, 4 < n Ò6, n > 6, years)



In the total group the results suggested that patients with the combination of smoking with 2 copies of 5A-1G were associated with a significantly increased likelihood of erosive disease compared to non-smokers with 0 copies of 5A-1G. However, the interaction effect was not significant (see AP (95% CI) value, Table 4.18.3f). In patients with RA duration Ò 6 years, a greater proportion of risk due to the risk combination was detected, and analysis provided a significant 95% CI for AP. These data suggest a possible interaction between smoking and 2 copies of 5A-1G haplotype in relation to erosive disease, especially in patients with early disease.

Table 4.18.3f Occurrence of erosive disease stratified by the copy number of 5A-1G haplotype and ever-smoking in RA

All patients					
	In never-smokers			In ever-smokers	
5A-1G copy	Erosions	OR (95% CI)	5A-1G Copy	Erosions	OR (95% CI)
0	34/55 (61.8)	1.0 referent	0	66/88 (75.0)	1.84 (0.90 ï 3.78)
1	50/66 (75.8)	1.91 (0.88 ï 4.14)	1	107/143 (74.8)	1.84 (0.95 ï 3.54)
2	13/19 (68.4)	1.29 (0.44 ï 3.81)	2	37/43 (86.0)	3.60 (1.33 ï 9.69)
Additive interaction				AP (95% CI)	
1 copy of 5A-1G with smoking				ND	
2 copies of 5A-1G with smoking				0.41 (-0.25 ï 1.08)	
Patients with RA duration Ò6 years (n = 162)					
	In never-smokers			In ever-smokers	
5A-1G copy	Erosions	OR (95% CI)	5A-1G Copy	Erosions	OR (95% CI)
0	9/21 (42.9)	1.0 referent	0	17/33 (51.5)	1.40 (.047 ï 4.10)
1	15/24 (62.5)	2.15 (0.67 ï 6.90)	1	38/56 (67.9)	2.74 (1.0 ï 7.51)
2	7/11 (63.6)	2.19 (0.52 ï 9.27)	2	15/17 (88.2)	8.16 (1.68 ï 39.6)
Additive interaction				AP (95% CI)	
1 copy of 5A-1G with smoking				ND	
2 copies of 5A-1G with smoking				0.57 (0.22 ï 1.24)	

Values are number of positive case (%); AP, the attributable proportion due to interaction; ND, not detected.

4.19 MMP POLYMORPHISM, SERUM MMP AND CO-MORBIDITY

4.19.1 Brief introduction

Polymorphisms in the MMP genes (including MMP1, 2, 3, 7, 9, 12 and 13) have been associated with CVD in the general population (Ye, 2006). Both MMP1 and 3 SNPs were found to be associated with the occurrence of coronary artery disease and particularly MI (Zhou et al, 2004; Wu et al, 2009; Román-García et al, 2009; Pearce et al, 2005; Horne et

al, 2007; Kaplan et al, 2008), with some reports suggesting an interaction with smoking (Liu et al, 2003; Liu et al, 2006). Recently, genetic variants in the MMP2 gene were associated with systolic heart failure prognosis in a Chinese report (Hua et al, 2009). In addition, research also has observed that MMP3 polymorphisms are associated with blood pressure and CVA (Taylor et al, 2008; Kaplan et al, 2008), and that MMP1 polymorphisms are associated with several cancer types such as lung cancer, and head and neck cancer (Zhou et al, 2001; O-charoenrat et al, 2006). These associations may be due to the differential levels of MMP influenced by genetic polymorphisms. In the present study, we tested whether there were associations of MMP polymorphisms and serum MMP levels with the occurrence of co-morbid conditions in patients with RA, with particular interests in the relationship with IHD, MI and hypertension.

4.19.2 MMP polymorphism, serum MMP and IHD/MI

MMP polymorphism and IHD/MI

The patient characteristics stratified by the presence of IHD and MI were described previously (Section 4.11.2). No association of MMP polymorphisms with IHD/MI in the whole population was found. However, further analysis provided some tentative evidence that polymorphism in the MMP1-3 loci may have a relationship with the occurrence of IHD and MI in patients who were RF positive or in patients who had ever smoked. MMP1 polymorphism (rs1799750) seemed not to be involved, while the other 3 studied SNPs (rs3025058, rs679620 and rs495366) showed a trend where the 6A6A genotype of rs3025058, the GG genotype of rs679620 and the AA genotype of rs495366 were related with the highest occurrence of IHD and MI (additive models). Only the associations of

rs495366 with IHD reached significance. Table 4.19.2a and b show the frequency of IHD and MI stratified by MMP1-3 loci polymorphisms in patients who were RF positive and in patients who had ever smoked respectively. Haplotype analysis did not provide significant association (data not shown). No association of MMP2 SNP rs243865 with IHD/MI in any subset of RA patients was found.

Since an increased occurrence of IHD was found to be associated with the AA genotype at rs495366 in patients who had ever smoked, we carried out an analysis to investigate whether there was excess risk to due this combination. According to AP (0.67), a possible interaction between smoking (ever-smoked status) and the AA genotype at rs495366 was seen (Table 4.19.2c). However, the 95% CI for AP was not available as there was no IHD case in the smoke-/AA genotype+ combination.

Table 4.19.2a Frequency of IHD and MI stratified by MMP1-3 loci polymorphisms in RF+ patients (n = 242)

	IHD (n = 54)		MI (n = 36)	
	Negative	Positive (%)	Negative	Positive (%)
Rs3025058 (5A/6A)				
5A5A	52	10 (16.1)	55	7 (11.3)
5A6A	97	30 (23.6)	108	19 (15.0)
6A6A	37	14 (27.5)	41	10 (19.6)
		NS		NS
Rs679620 (A/G)				
AA	50	10 (16.7)	53	7 (11.7)
AG	96	29 (23.2)	106	19 (15.2)
GG	36	14 (28.0)	40	10 (20.0)
		NS		NS
Rs495366 (G/A)				
GG	96	21 (17.9)	102	15 (12.8)
GA	75	23 (23.5)	83	15 (15.3)
AA	10	7 (41.2)	14	3 (17.6)
		p trend = 0.044*		NS
Rs1799750 (1G/2G)				
1G1G	58	17 (22.7)	63	12 (16.0)
1G2G	79	24 (23.3)	87	16 (15.5)
2G2G	49	13 (21.0)	54	8 (12.9)
		NS		NS

Values are n (%); * p for trend, based on additive model; based on dominant model (G allele vs. AA), p = 0.047; NS, not significant.

Table 4.19.2b Frequency of IHD and MI stratified by MMP1-3 loci polymorphisms in patients who had ever smoked (n = 282)

	IHD (n = 67)		MI (n = 42)	
	Negative	Positive (%)	Negative	Positive (%)
Rs3025058 (5A/6A)				
5A5A	60	15 (20.0)	67	8 (10.7)
5A6A	113	36 (24.2)	126	23 (15.4)
6A6A	39	16 (29.1)	44	11 (20.0)
		NS		NS
Rs679620 (A/G)				
AA	59	14 (19.2)	65	8 (11.0)
AG	109	36 (24.8)	122	23 (15.9)
GG	38	16 (29.6)	43	11 (20.4)
		NS		NS
Rs495366 (G/A)				
GG	114	30 (20.8)	127	17 (11.8)
GA	86	27 (23.9)	94	19 (16.8)
AA	8	7 (46.7)	12	3 (20.0)
		NS*		NS
Rs1799750 (1G/2G)				
1G1G	67	22 (24.7)	74	15 (16.9)
1G2G	99	32 (24.4)	112	19 (14.5)
2G2G	46	13 (22.0)	51	8 (13.6)
		NS		NS

Values are n (%); * p = 0.030 if based on dominant model (G allele vs. AA); NS, not significant.

Table 4.19.2c Association of MMP1-3 intergenic SNP rs495366 and ever having smoked with IHD in RA patients

	IHD		
Smoke/AA genotype	Negative	Positive (%)	Odds ratio (95% CI)
-/-	117	12 (9.3)	1.0 referent
-/+	10	0 (0.0)*	0.45 (0.09 ï 5.88)
+/-	201	56 (21.8)	2.64 (1.37 ï 5.07)
+/+	8	7 (46.7)	8.29 (2.56 ï 25.99)
			AP: 0.67 (95% CI: NA)

Values are n (%); AP, the attributable proportion due to interaction; NA, not available; * Replacement of 0 with 1 reveals a significant AP with 95% CI (0.67 (0.23 ï 1.11)).

Serum MMP and IHD/MI

No serum MMP levels were different between IHD and non-IHD patient. MMP-3 increased particularly in patients with MI (MI- vs. MI+, p = 0.020, Table 4.19.2d). Interestingly, serum MMP-3 in IHD+/MI- patients showed the lowest level, although it was not significantly lower compared to that in IHD- patients (IHD+/MI- vs. MI+, p = 0.021; IHD+/MI- vs. IHD-, p = 0.26, Table 4.19.2d). Adjustment for age, sex and RA duration made little different (data not shown).

Table 4.19.2d Levels of serum MMP-3 stratified by IHD/MI status in RA

Patient group	Serum MMP-3 level, pg/ml
IHD- (n = 354)	20323.8 (13336.6 ï 34371.0)
IHD+/MI- (n = 22)	16818.3 (12337.1 ï 31304.3)
MI+ (n = 32)	24676.0 (19317.3 ï 32192.9)

Values are median (IQR).

MMP polymorphism, serum MMP and other co-morbidity

MMP polymorphisms were not associated with other recorded co-morbidities. We observed some associations of serum MMP levels with certain co-morbid conditions. Elevated MMP-9 was associated with hypercholesteraemia (539075 (IQR 344750 - 792333) vs. 448568 (IQR 295588 - 648629) pg/ml, p = 0.0051, adjusted for age, sex and RA duration), and elevated MMP-8 was associated with CVA (26440.6 (IQR 15291.8 ï 39784.7) vs. 14882.3 (IQR 7912.2 ï 24404.8) pg/ml, p = 0.036, adjusted for age, sex and RA duration).

4.20 MMP POLYMORPHISM, SERUM MMP AND SERUM TIMP

4.20.1 Brief introduction

The action of MMPs is controlled (or limited) by their natural inhibitors, known as TIMPs, which neutralize the activity of the MMPs under normal conditions (Firestein, 1997; Matrisian, 1990). Thus, MMPs and TIMPs and especially the imbalance between these enzymes and inhibitors are thought to play an important role in the destruction and remodelling of articular tissues in patients with RA. Some MMPs and TIMPs interact with each other and form MMP-TIMP complexes. These complexes are thought to have functional roles, although the detail of function is largely unknown (Brew et al, 2000). The up-regulation of MMPs in RA is well documented. However, little is known about their tissue inhibitors and the MMP/TIMP ratios in patients with RA. In the present study, we observed that genetic polymorphisms within the MMP1-3 loci were associated with serum

levels of MMP-1, -2, -3 and -9. Furthermore, these variants were also associated with the development of erosive disease in patients with earlier RA (RA duration Ò6 years). Since TIMPs act closely with MMPs and have a highly specific inhibition role, it was interesting to see whether the genetic polymorphisms were also associated with serum levels of TIMPs and/or the ratios of MMP/TIMP. Furthermore, the associations of serum levels of TIMPs and the ratios of MMP/TIMP with disease measurements and co-morbid conditions were also investigated.

4.20.2 Serum level of TIMP

Determination and distribution

Serum levels of TIMP-1, -2, -3 and -4 (all the members in the TIMP family) were determined on 160 patients with earlier disease (RA duration Ò 6 years), since the association of polymorphisms in the MMP1-3 loci with erosive disease occurred primarily in those patients. TIMP-2 levels were normally distributed (Figure 4.20.2b), while the others were not (Figure 4.20.2a, c and d). For TIMP-1 and -4, their values were close to a normal distribution after a log transformation (Figure 4.20.2a and d). Transformations (e.g. log, square root, etc) could not improve the distribution of TIMP-3 (data not shown). The median levels for TIMP-1, -2, -3 and -4 were 168322.3 (IQR 150147.3 ï 194878.9) pg/ml, 125302.0 (IQR 117803.2 ï 138980.1) pg/ml, 12241.5 (IQR 1000.0 ï 24895.7) pg/ml, and 2111.1 (IQR 1750.8 ï 2834.5) pg/ml respectively.

Figure 4.20.2a Distribution of serum TIMP-1 levels before (left) and after (right) log transformation in RA (RA duration Ò6 years)

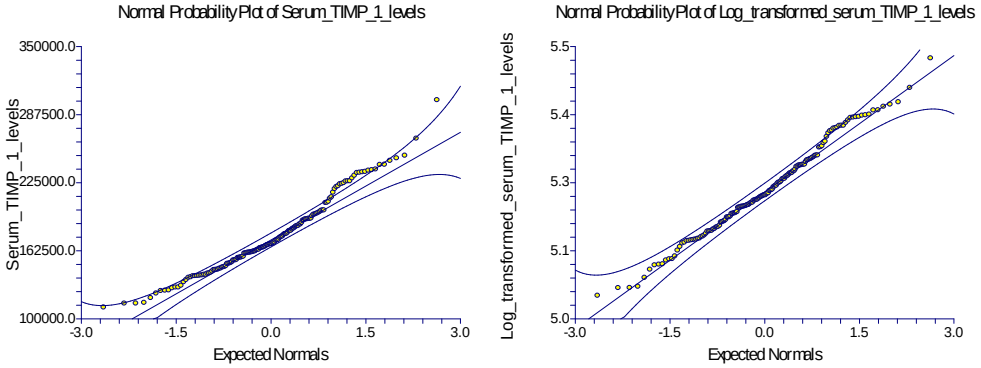


Figure 4.20.2b Distribution of serum TIMP-2 levels in RA (RA duration Ò6 years)

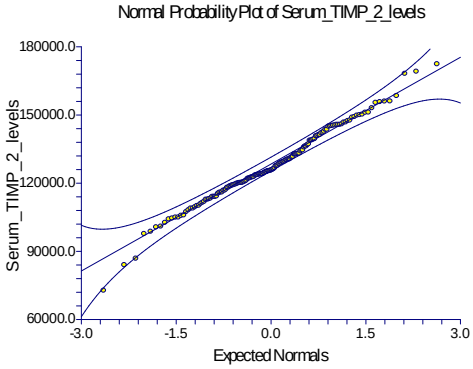


Figure 4.20.2c Distribution of serum TIMP-3 levels in RA (RA duration Ò6 years)

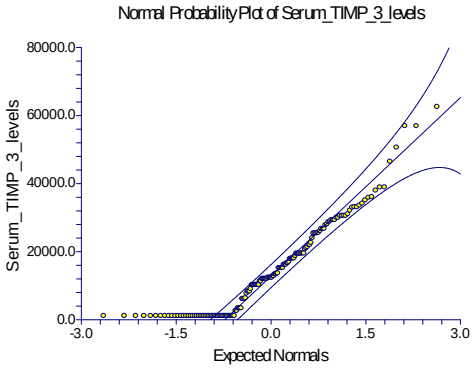
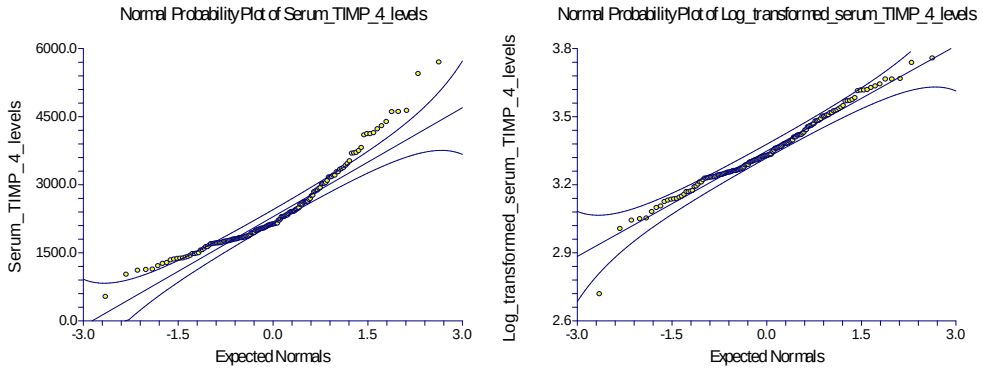


Figure 4.20.2d Distribution of serum TIMP-4 levels before (left) and after (right) log transformation in RA (RA duration Ò6 years)



Correlation between MMPs and TIMPs

The correlations between MMPs in patients with RA duration Ò 6 years (Table 4.20.2a) were similar to those in the whole population (as described in Section 4.17.3).

For TIMPs, most members were significantly associated with each other, with moderate correlations ranging from 0.20 to 0.40, except for one pair (TIMP-3, TIMP-4) which did not reach significance (Table 4.20.2a).

MMP-1 was significantly correlated with TIMP-1, -2 and -3, but not with TIMP-4 (Table 4.20.2a). MMP-2 was found to be particularly associated with TIMP-2, with a significant correlation which reached 0.40 (Table 4.20.2a). MMP-3 was correlated with TIMP-1 only, in a relatively weak relationship (Table 4.20.2a). MMP-8 and -9 were very strongly correlated with each other (r = 0.75). However, they showed slightly different relationships with TIMPs, with MMP-8 correlated only with TIMP-2 while MMP-9 correlated with both TIMP-1 and -2 (Table 4.20.2a).

Table 4.20.2a Correlations between serum levels of MMP-1, -2, -3, -8 and -9, and TIMP-1, -2, -3 and -4 in RA patients with RA duration Ò6 years

	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	TIMP-1	TIMP-2	TIMP-3
MMP-2	0.25 (0.0014)	ï	ï	ï	ï	ï	ï	ï
MMP-3	0.33 (< 0.0001)	0.16 (0.040)	ï	ï	ï	ï	ï	ï
MMP-8	0.18 (0.023)	NS	NS	ï	ï	ï	ï	ï
MMP-9	NS	NS	NS	0.75 (< 0.0001)	ï	ï	ï	ï
TIMP-1	0.23 (0.0037)	NS	0.20 (0.011)	NS	0.18 (0.026)	ï	ï	ï
TIMP-2	0.25 (0.0018)	0.40 (< 0.0001)	NS	0.25 (0.0013)	0.25 (0.0016)	0.40 (< 0.0001)	ï	ï
TIMP-3	0.30 (0.0001)	NS	NS	NS	NS	0.31 (< 0.0001)	0.20 (0.012)	ï
TIMP-4	NS	NS	NS	NS	NS	0.24 (0.0024)	0.25 (0.0016)	NS

Values are r (p), obtained from Spearman correlation; NS, not significant.

Serum TIMP level, cigarette smoking and patient demographics

No significant associations between serum levels of TIMPs (including TIMP-1, -2, -3, and 4) and smoking characteristics (including ever-smoked status, current-smoking status, cigarettes per day, years smoked and pack-years) were found in these patients with RA duration Ò6 years.

The relationships between TIMP levels and parameters such as age, sex, BMI and RA duration were also investigated. No association of TIMPs with RA duration or BMI was observed. Interestingly, serum levels of TIMP-1, -2 and -4 were positively correlated with

age, with r (p) values = 0.19 (0.015), 0.40 (< 0.0001) and 0.24 (0.0022) respectively, whereas TIMP-3 was not correlated. These relationships remained significant after adjustment for sex and RA duration (data not shown). Furthermore, we also found a relationship with sex. Serum TIMP-4 was shown to be higher in female patients (n = 109), compared to that in male subjects (n = 49) (2277.5 (IQR 1799.0 ï 3009.6) vs. 1917.1 (IQR 1616.4 ï 2389.1) pg/ml, p = 0.013, adjusted for age and RA duration).

MMP/TIMP ratio, cigarette smoking and patient demographics

Each MMP/TIMP ratio (e.g. MMP-1/TIMP-2, MMP-3/TIMP-1, etc) was obtained from each patient with RA duration Ò6 years (where both MMP and TIMP were available). In total, there were 20 combinations (5 MMPs × 4 TIMPs). Although each TIMP may have specific activity on particular MMP(s), such detail is not clear. Thus, we used all the possible 20 combinations to investigate the relationships.

No significant difference in MMP/TIMP ratios was observed between patients who had never smoked (n = 51) and who had ever smoked (n = 105), although for MMP-9/TIMP-1 and MMP-9/TIMP-4, patients who had ever smoked had higher ratios (MMP-9/TIMP-1: 2.91 (IQR 1.92 ï 4.01) vs. 2.34 (IQR 1.61 ï 2.34), p = 0.087; MMP-9/TIMP-4: 246.20 (IQR 125.68 ï 357.21) vs. 195.65 (124.86 ï 251.60), p = 0.059). Current smoking (n = 35) was associated with lower ratios of MMP-2/TIMP-1, -2, -3 and -4, compared with never or past smoking (Table 4.20.2b). No other significant association according to current-smoking stratification was found. In the present study, we also observed correlations between pack-years (excluded non-smoking samples) and ratios of MMP-9/TIMP-2 (r =

0.17, p = 0.045) and MMP-9/TIMP-4 (r = 0.19, p = 0.023). No correlations with other quantitative measures (cigarettes per day, years smoked) were found.

The relationships between ratios of MMP/TIMP and parameters including age, sex, BMI and RA duration were evaluated. No associations with age and BMI were seen. We found that several TIMP4-related ratios (including MMP-3/TIMP-4, MMP-8/TIMP-4 and MMP-9/TIMP-4) were associated with sex, with decreased values observed in females (data not shown). This could be explained by the higher TIMP-4 levels observed in these female patients. Negative correlations between RA duration, and MMP-2/TIMP-1 (r = -0.19, p = 0.023), MMP-2/TIMP-2 (r = -0.31, p = 0.0001), MMP-3/TIMP-1 (r = -0.16, p = 0.046) and MMP-3/TIMP-2 (r = -0.17, p = 0.044) were seen. These remained significant after further adjustment for age and sex (data not shown).

Table 4.20.2b Ratios of MMP-2/TIMP-1, -2, -3 and -4 stratified by never-, past- and current-smoking in RA (RA duration Ò6 years)

	Never-smoking (n = 51)	Past-smoking (n = 63)	Current-smoking (n = 35)	p*
MMP-2/TIMP-1	1.08 (0.86 ï 1.28)	1.11 (0.98 ï 1.24)	0.96 (0.79 ï 1.12)	0.0071
MMP-2/TIMP-2	1.43 (1.22 ï 1.61)	1.47 (1.32 ï 1.66)	1.34 (1.07 ï 1.58)	0.062
MMP-2/TIMP-3	16.1 (8.8 ï 119.5)	16.6 (8.6 ï 153.2)	11.7 (6.5 ï 111.3)	0.093
MMP-2/TIMP-4	80.7 (61.6 ï 109.1)	97.4 (74.9 ï 108.2)	78.8 (54.8 ï 88.4)	0.0074

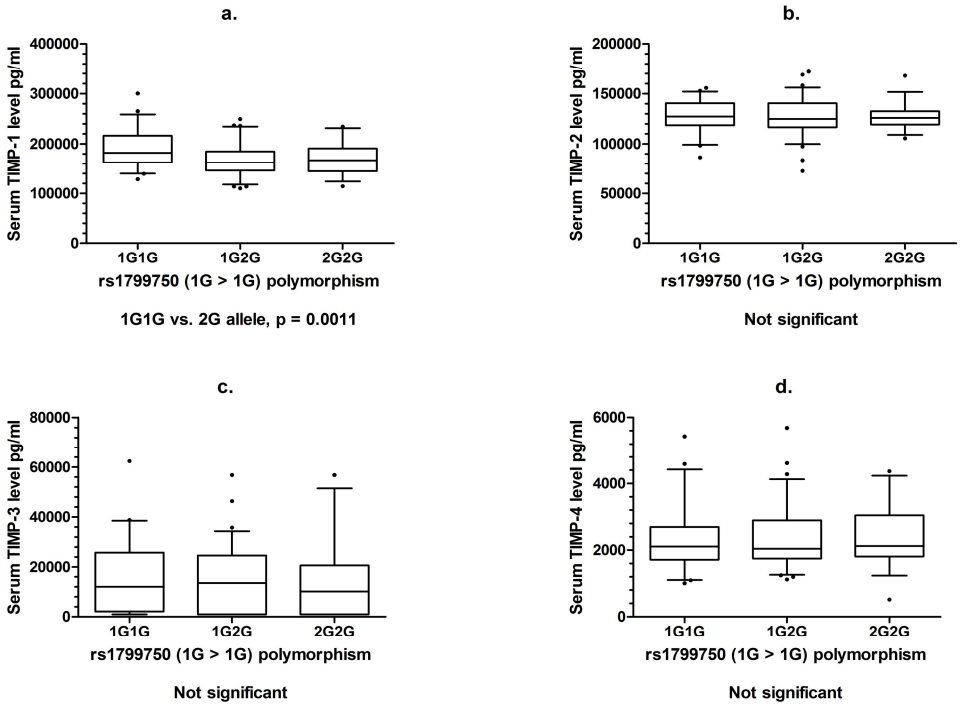
Values are median (IQR); * Current-smoking vs. never- + past-smoking (no significant difference between never and past smoking). Values adjusted for age, sex and RA duration. Some associations were borderline associations.

4.20.3 MMP1, 2, 3 polymorphism, serum TIMP level and MMP/TIMP ratio

MMP1, 2, 3 polymorphism and serum TIMP level

MMP3 SNPs rs3025058 and rs679620, MMP1-3 intergenic SNP rs495366, and MMP2 SNP rs243865 were not associated with any serum TIMP level (data not shown). MMP1 SNP rs1799750 was found to be significantly associated with serum TIMP-1 (Figure 4.20.3a), while its relationship with TIMP-2, -3 or -4 was not significant (Figure 4.20.3b, c and d). In addition, we also tested the relationship between copy numbers of 5A-1G haplotype and TIMP levels. No association was found (data not shown).

Figure 4.20.3a-d Relationship between genotypes of rs1799750 and serum levels of TIMP-1, -2, -3 and -4 in RA (RA duration Ò6 years)



Boxplot with whiskers with 5 ï 95 percentile.

MMP1, 2, 3 polymorphism and MMP/TIMP ratio

MMP1 SNP rs1799750 was found to be associated with both serum MMP-1 and TIMP-1 (as shown). Hence, we investigated whether genotypes in this polymorphism differentiate the MMP-1/TIMP-1 ratio. We found that it was not only associated with MMP-1/TIMP-1 but also with MMP-1/TIMP-2 and -4 ratios, with 1G1G associated with higher ratios compared to the 2G allele (Table 4.20.3a). However, this polymorphism was not associated with MMP-1/TIMP-3 ratio, and other MMP/TIMP ratios (Table 4.20.3a).

MMP3 SNP rs3025058 was associated with serum MMP-3, and serum MMP-1 independently of rs1799750 (as shown). Similar to rs1799750, we found that this polymorphism was associated with MMP-1/TIMP-1, -2 and -4 ratios with 5A5A associated with higher ratios compared to the 6A allele, but was not associated with MMP-1/TIMP-3 ratio (Table 4.20.3b). We also found that this polymorphism was not associated with MMP-3-related ratios, and other MMP/TIMP ratios (Table 4.20.3b). Analysis with MMP3 rs6799750 gave almost identical results compared to the rs3025058 data (data not shown).

These relationships were also investigated using the MMP1-3 intergenic polymorphism rs495366. Although it was not significant, the GG genotype was associated with the highest ratio of MMP-1/TIMP-1, -2, -4 (Table 4.20.3c). Furthermore, it was also seen that the G allele was associated with higher ratios of MMP-3/TIMP-1, -2, -3 and -4 compared to AA genotype, although for MMP-3/TIMP-3 and -4 it was not significant (Table 4.20.3c). However, these results should be treated with some caution since the number of AA genotype at rs495366 was very low (n = 4). Analysis with MMP2 SNP rs243865 did not show any association or trend (data not shown).

Since we found that 5A at rs3025058, A at rs679620 (data not shown), G at rs495366 and 1G at rs1799750 were associated with certain MMP/TIMP ratios, it was interesting to see the effects of the 5A-A-G-1G haplotype. To reduce the complexity, we used the 2 SNP 5A-1G haplotype as over 98% of patients with this haplotype carried 5A-A-G-1G (as mentioned). Patients carrying 2 copies of 5A-1G were associated with the highest ratios of MMP-1/TIMP-1, -2, -3 and -4 (all the MMP-1-related ratios), while patients without any copy of 5A-1G were related to low ratios (mostly the lowest ratios) (Table 4.20.3d). The significance levels obtained here seemed to be stronger compared to those achieved from individual SNPs, suggesting this haplotype as the best predictor. However, analysis using this haplotype did not show the association with ratios of MMP-3/TIMP.

Table 4.20.3a Relationship between genotypes of rs1799750 and ratios of MMP/TIMP in RA (RA duration Ò6 years)

MMP/TIMP ratio	Rs1799750			p
	1G1G (n = 46)	1G2G (n = 76)	2G2G (n = 36)	
MMP-1/TIMP-1	0.054	0.033	0.038	0.017*
MMP-1/TIMP-2	0.074	0.044	0.045	0.0007*
MMP-1/TIMP-3	0.77	0.56	0.95	NS
MMP-1/TIMP-4	4.38	2.69	2.15	0.0018*/0.0029[A]
MMP-2/TIMP-1	1.04	1.10	1.07	NS
MMP-2/TIMP-2	1.52	1.42	1.44	NS
MMP-2/TIMP-3	15.10	13.45	16.80	NS
MMP-2/TIMP-4	82.37	83.41	85.60	NS
MMP-3/TIMP-1	0.11	0.14	0.12	NS
MMP-3/TIMP-2	0.17	0.18	0.14	NS
MMP-3/TIMP-3	2.19	2.11	2.63	NS
MMP-3/TIMP-4	10.63	9.96	7.61	NS
MMP-8/TIMP-1	0.093	0.087	0.084	NS
MMP-8/TIMP-2	0.14	0.12	0.12	NS
MMP-8/TIMP-3	1.80	1.27	2.10	NS
MMP-8/TIMP-4	8.09	7.34	6.37	NS
MMP-9/TIMP-1	2.58	2.72	2.78	NS
MMP-9/TIMP-2	3.86	3.78	3.50	NS
MMP-9/TIMP-3	39.83	46.85	50.95	NS
MMP-9/TIMP-4	215.38	231.37	184.16	NS

Values are median; * 1G1G vs. 2G allele (as dominant model); [A]p for trend (as additive model); NS, not significant.

Table 4.20.3b Relationship between genotypes of rs3025058 and ratios of MMP/TIMP in RA (RA duration Ò6 years)

MMP/TIMP ratio	Rs3025058			p
	5A5A (n = 41)	5A6A (n = 91)	6A6A (n = 27)	
MMP-1/TIMP-1	0.054	0.037	0.025	0.0013*/0.0002[À]
MMP-1/TIMP-2	0.072	0.047	0.037	0.0014*/0.0005[A]
MMP-1/TIMP-3	0.92	0.53	0.98	NS
MMP-1/TIMP-4	4.39	2.62	2.41	0.0007*/0.0057[A]
MMP-2/TIMP-1	1.08	1.09	1.01	NS
MMP-2/TIMP-2	1.52	1.43	1.37	NS
MMP-2/TIMP-3	16.87	11.75	22.61	NS
MMP-2/TIMP-4	90.32	80.38	87.72	NS
MMP-3/TIMP-1	0.10	0.13	0.12	NS
MMP-3/TIMP-2	0.14	0.18	0.17	NS
MMP-3/TIMP-3	1.79	2.06	4.89	NS
MMP-3/TIMP-4	8.76	9.96	10.23	NS
MMP-8/TIMP-1	0.080	0.087	0.087	NS
MMP-8/TIMP-2	0.13	0.13	0.12	NS
MMP-8/TIMP-3	1.98	1.17	3.64	NS
MMP-8/TIMP-4	7.86	7.19	7.49	NS
MMP-9/TIMP-1	2.67	2.59	2.93	NS
MMP-9/TIMP-2	3.49	3.51	4.51	NS
MMP-9/TIMP-3	39.96	34.96	48.96	NS
MMP-9/TIMP-4	215.69	194.69	264.33	NS

Values are median; * 5A5A vs. 6A allele (as dominant model); [À]p for trend (as additive model); NS, not significant.

Table 4.20.3c Relationship between genotypes of rs495366 and ratios of MMP/TIMP in RA (RA duration Ò6 years)

MMP/TIMP ratio	Rs495366			p
	GG (n = 82)	GA (n = 71)	AA (n = 4)	
MMP-1/TIMP-1	0.042	0.036	0.019	NS
MMP-1/TIMP-2	0.058	0.044	0.027	NS
MMP-1/TIMP-3	0.62	0.63	0.75	NS
MMP-1/TIMP-4	3.02	2.82	1.88	NS
MMP-2/TIMP-1	1.09	1.07	0.93	NS
MMP-2/TIMP-2	1.43	1.47	1.13	NS
MMP-2/TIMP-3	14.21	16.34	15.19	NS
MMP-2/TIMP-4	83.13	85.60	67.88	NS
MMP-3/TIMP-1	0.12	0.13	0.059	0.046*
MMP-3/TIMP-2	0.17	0.18	0.085	0.049*
MMP-3/TIMP-3	2.14	2.75	1.46	NS
MMP-3/TIMP-4	9.50	10.23	5.29	NS
MMP-8/TIMP-1	0.087	0.082	0.12	NS
MMP-8/TIMP-2	0.13	0.12	0.15	NS
MMP-8/TIMP-3	1.93	1.65	3.15	NS
MMP-8/TIMP-4	7.67	6.46	9.73	NS
MMP-9/TIMP-1	2.79	2.54	2.27	NS
MMP-9/TIMP-2	3.78	3.75	3.46	NS
MMP-9/TIMP-3	46.12	41.16	66.81	NS
MMP-9/TIMP-4	207.82	205.25	213.75	NS

Values are median; * AA vs. G allele (as dominant model); NS, not significant.

Table 4.20.3d Relationship between number of copies of 5A-1G haplotype and ratios of MMP/TIMP in RA (RA duration Ò6 years)

MMP/TIMP ratio	Number of 5A-1G copies			p
	2 copies (n = 30)	1 copy (n = 76)	0 copy (n = 52)	
MMP-1/TIMP-1	0.058	0.038	0.031	0.0011*/< 0.0001[À]
MMP-1/TIMP-2	0.075	0.052	0.038	0.0004*/< 0.0001[A]
MMP-1/TIMP-3	1.34	0.53	0.83	0.023*
MMP-1/TIMP-4	4.45	2.94	2.15	0.001*/< 0.0001[A]
MMP-2/TIMP-1	1.08	1.08	1.07	NS
MMP-2/TIMP-2	1.53	1.43	1.40	NS
MMP-2/TIMP-3	18.85	11.95	16.80	NS
MMP-2/TIMP-4	88.50	82.18	84.02	NS
MMP-3/TIMP-1	0.11	0.14	0.12	NS
MMP-3/TIMP-2	0.15	0.18	0.14	NS
MMP-3/TIMP-3	3.37	1.99	3.11	NS
MMP-3/TIMP-4	9.19	10.50	8.31	NS
MMP-8/TIMP-1	0.11	0.085	0.084	NS
MMP-8/TIMP-2	0.14	0.12	0.12	NS
MMP-8/TIMP-3	2.21	1.07	2.43	NS
MMP-8/TIMP-4	8.72	7.34	6.59	NS
MMP-9/TIMP-1	2.88	2.59	2.78	NS
MMP-9/TIMP-2	3.99	3.51	3.69	NS
MMP-9/TIMP-3	58.32	39.18	58.18	NS
MMP-9/TIMP-4	227.24	208.40	201.33	NS

Values are median; * 2 copies vs. 1 copy + 0 copy (as dominant model); [A]p for trend (as additive model); NS, not significant.

4.20.4 Serum TIMP level, MMP/TIMP ratio and RA clinical measures

Serum TIMP and RA clinical measures

Serum TIMP-3 was found to be increased in RF+ patients, compared to that in RF- patients (13245.4 (IQR 3494.8 ï 25338.3) vs. 11189.4 (IQR 1000.0 ï 23854.1), p = 0.049, adjusted for age, sex and RA duration). Other TIMPs were not associated. No association between serum TIMPs and anti-CCP was found. The correlations between serum levels of TIMP-1, -2, -3 and -4, and inflammatory markers including ESR and CRP and disease measures including DAS28, MJS and HAQ are shown in Table 4.20.4a. Serum TIMP-1 was shown to be correlated with inflammatory markers, disease activity, and the functional severity measured by HAQ score. TIMP-4 was correlated with disease activity and with MJS. These correlations remained significant after adjustment for sex, age and RA duration (data not shown). No correlations were found between TIMP-2 and -3, and the listed disease measures. We also tested their relationships with erosive and nodular diseases. No significant difference was seen either stratified by erosive or nodular status.

Table 4.20.4a Correlations between serum TIMP levels and inflammatory and disease measures in RA (RA duration Ò6 years)

	ESR	CRP	DAS28	MJS	HAQ
TIMP-1	0.16 (0.048)	0.21 (0.0068)	0.18 (0.024)	NS	0.19 (0.015)
TIMP-2	NS	NS	NS	NS	NS
TIMP-3	NS	NS	NS	NS	NS
TIMP-4	NS	NS	0.17 (0.034)	0.27 (0.0005)	NS

Values are r (p), obtained from Spearman correlation; NS, not significant.

MMP/TIMP ratio and RA clinical measures

The MMP/TIMP ratio stratified by RF status was investigated. Six MMP/TIMP ratios were significantly lower in RF+ patients (n = 85) compared to those in RF- patients (n = 73). They were MMP-1/TIMP-3 (0.53 vs. 0.89, p = 0.0084), MMP-3/TIMP-1 (0.11 vs. 0.15, p = 0.0074), MMP-3/TIMP-2 (0.16 vs. 0.18, p = 0.0018), MMP-3/TIMP-3 (1.65 vs. 4.84, p = 0.020), MMP-3/TIMP-4 (9.20 vs. 11.54, p = 0.027) and MMP-8/TIMP-3 (1.34 vs. 2.41, p = 0.022). Furthermore, MMP-2/TIMP-1 (1.07 vs. 1.12, p = 0.071), MMP-2/TIMP-3 (12.7 vs. 18.4, p = 0.092) and MMP-9/TIMP-3 (35.0 vs. 62.8, p = 0.057) were also lower in RF+ patients, although they were not significant. When stratified by anti-CCP status (anti-CCP-, n = 55; anti-CCP+, n = 105), no difference was seen except for MMP-8/TIMP-3 ratio which was lower in anti-CCP+ patients (1.68 vs. 2.22, p = 0.010). All these results were adjusted for age, sex and RA duration.

The relationships between MMP/TIMP ratio, and ESR, CRP, DAS28, MJS and HAQ were investigated. MMP-2/TIMP-1 ratio was negatively correlated with inflammatory markers ESR (r = -0.14, p = 0.075 insignificant) and CRP (r = -0.17, p = 0.032), and disease activity score (DAS28, r = -0.19, p = 0.016). Also, it had a borderline correlation (negative) with HAQ (r = -0.15, p = 0.059). MMP-2/TIMP-4 was found to be negatively correlated with MJS (r = -0.19, p = 0.017) and HAQ (r = -0.24, p = 0.0023). MMP-3-related ratios were positively correlated with disease measures, with MMP-3/TIMP-1 correlated with MJS (r = 0.19, p = 0.016), MMP-3/TIMP-2 correlated with CRP (r = 0.19, p = 0.016), DAS28 (r = 0.17, p =0.038) and MJS (r = 0.20, p = 0.012), MMP-3/TIMP-3 correlated with MJS (r = 0.19, p = 0.015), and MMP-3/TIMP-4 correlated with CRP (r = 0.22, p =

0.0066). Adjustment for age, sex and RA duration made little difference in terms of p values (data not shown).

4.20.5 Serum TIMP level, MMP/TIMP ratio and co-morbidity

Serum TIMP and co-morbidity

Initial data suggested that serum levels of TIMP-1 and -2 were increased in patients with IHD (n = 31) compared to those levels in patients without IHD (n = 127), although the p values were not significant after adjustment for age, sex and RA duration (TIMP-1: 178959.9 (IQR 162785.5 ï 223176.2) vs. 166577.6 (IQR 145901.9 ï 191216.2) pg/ml, p = 0.092; MMP-2: 132640.8 (IQR 119972.4 ï 145734.1) vs. 124765.6 (IQR 116962.3 ï 136934.7) pg/ml, p = 0.16). No other trend was found.

MMP/TIMP ratio and co-morbidity

MMP-8/TIMP-3 ratio was found to be increased in patients with hypertension (n = 60) compared with that in patients without hypertension (n = 95) (2.34 (IQR 0.80 ï 12.62) vs. 1.39 (IQR 0.59 ï 4.53), p = 0.0040 adjusted for age, sex and RA duration). Similarly, elevated MMP-9/TIMP-3 ratio was found in hypertensive patients (45.72 (IQR 20.85 ï 401.27) vs. 41.22 (IQR 17.36 ï 149.96), p = 0.021 adjusted for age, sex and RA duration). Since there was strong correlation between MMP-8 and -9, we used multivariate logistic regression which contained both MMP/TIMP-3 ratios as independent variables to test the independence of these associations. Both associations lost significance in the regression model (data not shown), implying that these 2 associations were not independent of each

other. We also observed a reduction of MMP-3/TIMP-4 ratio in patients with hypercholesteraemia (n = 29), compared to the ratio in patients without hypercholesteraemia (n = 127) (7.38 (IQR 5.55 ï 10.35) vs. 10.63 (IQR 6.78 ï 17.17), p = 0.027 adjusted for age, sex and RA duration).

4.21 SUMMARY OF FINDINGS FOR 4.17 – 4.20

- MMP3 SNPs rs3025058 and rs679620, and the intergenic SNP rs495366 were within the same LD block. The 2 MMP3 SNPs were almost identical in terms of allelic/genotypic distribution. MMP1 SNP rs1799750 linked moderately with the above LD block.

- All the 4 polymorphisms in the MMP1-3 loci were found to be associated with serum levels of MMP-1. The associations of non-MMP1 polymorphisms (rs3025058, rs679620 and rs495366) with serum MMP-1 were independent of the association with rs1799750.

- 5A-A-G-1G, the most common haplotype across the 4 polymorphisms (38.3%), was associated with the highest levels of serum MMP-1, whereas 6A-G-A-2G (19.2%) was associated with the lowest levels.

- Polymorphism within the MMP3 gene (rs3025058 and rs679620) was associated with serum levels of MMP-3. The other 2 polymorphisms (rs495366 and rs1799750) may also have some effect, as indicated by multivariate analyses.

- 6A-G-G-2G (13.5%) was associated with the highest levels of MMP-3 in haplotypic analysis. The association was stronger after exclusion of the MMP1 rs1799750 SNP. 5A-A-G-1G (38.3%) was associated with the lowest MMP-3 levels, although this could be explained mainly by the 2 SNP haplotype 5A-G (rs3025058-rs495366).

- Polymorphism in the MMP1-3 loci was associated with serum MMP-2 levels, with the strongest association occurring at rs495366. The association was found to be independent of the associations of MMP-1 and -3 levels with MMP-2 levels.

- MMP1 SNP rs1799750 was found to be associated with serum levels of MMP-9.

- Both serum levels of MMP-1 and -3 were associated with inflammation and thus disease activity. MMP-1 may be more related to disease functional outcome measured by HAQ.

- Decreased serum MMP-2 was found in patients with erosive disease, restricted to those with autoantibody (RF or anti-CCP) or those who had ever smoked.

- The AA genotype at the intergenic polymorphism rs495366 was associated with earlier age at onset of RA, compared to the G allele.

- MMP3 polymorphisms (rs3025058 and rs679620) were associated with disease activity over a 2-year period. The associations were independent of inflammation (marked by CRP), and serum MMP-1 and -3.

- MMP1 rs1799750 1G and MMP3 rs3025058 5A alleles were found to be associated with an increased risk of erosive disease in patients with short RA duration (Ò6 years). However, these genetic variants did not significantly differentiate between erosive and non-erosive disease in patients with longer RA duration.

- A possible interaction between smoking (ever-smoked) and the MMP1-3 genetic variants (marked by 5A-1G haplotype) was found in terms of the development of erosive disease in the early years of RA (Ò6 years).

- There was a trend showing that genetic polymorphism in the MMP1-3 loci may be associated with the occurrence of IHD/MI in patients who were RF positive or in patients had ever smoked, with 6A (at rs3025058), G (at rs679620) and A (at rs495366) alleles related to an increased risk.

- Elevated serum MMP-3 was found in patients with previous MI particularly, but not in IHD patients without MI.

- There were correlations between MMPs and TIMPs, with MMP-1 correlated with TIMP-1, -2 and -3, MMP-2 correlated with TIMP-2, MMP-3 correlated with TIMP-1, MMP-8 correlated with TIMP-2, and MMP-9 correlated with TIMP-1 and -2.

- Current smokers were found to be associated with lower ratios of MMP-2/TIMP-1, -2, -3 and -4, compared to the remaining subjects.

- MMP1 SNP rs1799750 was associated with serum level of TIMP-1, with 1G1G associated with an increased level.

- Polymorphisms within the MMP1-3 loci were particularly associated with MMP-1-related MMP/TIMP ratios. Haplotype analysis indicated that patients carrying double copies of 5A-1G were associated with the highest ratios of MMP-1/TIMP-1, -2, -3 and -4.

- Interestingly, many MMP/TIMP ratios were lower in RF+ patients, compared to those in RF- patients.

- MMP-8/TIMP-3 and MMP-9/TIMP-3 ratios were increased in patients with hypertension, while MMP-3/TIMP-4 was reduced in patients with hypercholesteraemia.

PART F: TGFB1 POLYMORPHISM, SERUM LEVEL OF TGF-β1, SEVERITY AND CO-MORBIDITY IN RA (4.22 – 4.25)

4.22 TGFB1 POLYMORPHISM AND SERUM TGF-β1 LEVEL

4.22.1 Brief introduction

TGF-ϐ1 is a multifunctional cytokine which regulates the proliferation and differentiation of a wide variety of cell types *in vitro.* Misregulation of this cytokine has been implicated in the development of several major diseases, including cancer, atherosclerosis, fibrotic disease and autoimmune disease. Polymorphisms (including rs1800469, rs1800470 and rs1800471) in the TGFB1 gene have been associated with the expression of TGF-ϐ1 (Grainger et al, 1999; Awad et al, 1998; Yamada et al, 1998; Yokota et al, 2000; Suthanthiran et al, 2000). However, this relationship has not yet been investigated in RA individuals. Since TGF-ϐ1 is increased in RA and is considered to be an anti-inflammatory regulator in RA, it was interesting to investigate the relationship between functional SNPs and the circulating levels of TGF-ϐ1 in this group of RA patients.

4.22.2 TGFB1 polymorphism

Studied SNP

Polymorphism rs1800469 is normally known as TGFB1-509 (C/T), named after its position relative to the transcription start site. It is a common SNP in the promoter region

of the gene. SNPs rs1800470 and rs1800471 are known as +868 (C/T) and +913 (C/G) respectively according to the positions relative to the transcription start site. They are located at positions 29 and 74 of the translated sequence (exon 1) of TGFB1 and give rise to amino acid substitutions at positions 10 (Pro Ÿ Leu) and 25 (Pro Ÿ Arg) in the signal peptide of TGF-ß1 respectively. Figure 4.22.2a shows the location of each SNP and their physical distance between each other.

Figure 4.22.2a Diagram of human TGFB1 gene highlighting the location of studied polymorphisms

The positions of rs1800469, rs1800470 and rs1800471 at chromosome 19 are 41860296, 41858921 and 41858876 respectively. Physical distances in bp between SNPs are shown. Left and right dots indicate the transcription start and end site respectively.

Allelic and genotypic distribution

Genotypes were determined in 429, in 427 and in 427 patients for TGFB1-509 (C/T), +868 (C/T) and +913 (C/G) respectively. The allelic and genotypic frequencies of the 3 TGFB1 SNPs are listed in Table 4.22.2a. Genotypes of these polymorphisms were all distributed in accordance with a close fit to HWE. The genotypic/allelic distribution of TGFB1+868 in our samples was comparable with the genotyping in another RA population from a nearby region (Dudley) (TT: 161 (40.8), TC: 176 (44.6), CC: 58 (14.7), p = 0.88 vs. the frequencies in the present study). In that study, the authors suggested that there was no

difference in distribution of +868 polymorphism between RA and healthy subjects (distribution in healthy subjects, TT: 161 (40.1), TC: 186 (46.4), CC: 54 (13.5)) (Panoulas et al, 2009).

Table 4.22.2a Distribution of allelic and genotypic frequencies of TGFB1-509 (C/T), +868 (C/T) and +913 (C/G) polymorphisms in subjects with RA

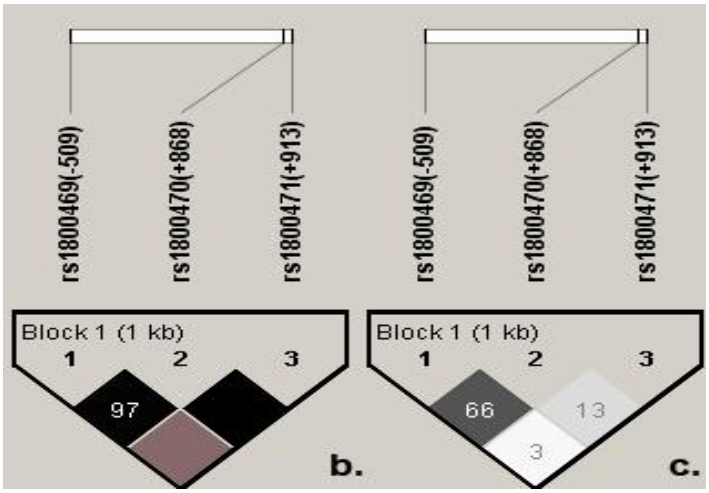
Polymorphism	Genotypic distribution	Allelic distribution
TGFB1-509 (C/T)	CC: 213 (49.7)	C: 614 (71.6)
(n = 429)	CT: 188 (43.8)	T: 244 (28.4)
	TT: 28 (6.5)	
TGFB1+868 (T/C)	TT: 174 (40.8)	T: 543 (63.6)
(n = 427)	TC: 195 (45.7)	C: 311 (36.4)
	CC: 58 (13.6)	
TGFB1+913 (G/C)	GG: 368 (86.2)	G: 793 (92.9)
(n = 427)	GC: 57 (13.4)	C: 61 (7.1)
	CC: 2 (0.5)	

Values are n (%).

Linkage disequilibrium and haplotype

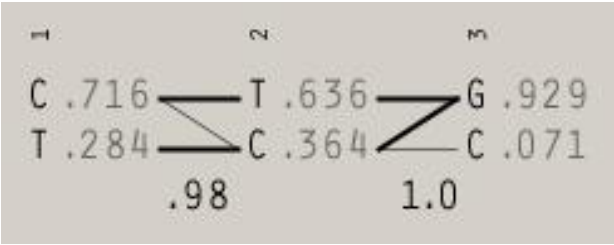
Pair-wise LD coefficients (*D'*) and correlation (r^2) between TGFB1-509, +868 and +913 are shown in Figure 4.22.2b and c respectively. They were strongly linked with each other, and formed a LD block according to the four gamete rule. C-T-G and T-C-G were the only major haplotypes across these polymorphisms (Figure 4.21.2d). Their frequencies were 0.634 and 0.281 respectively (Table 4.22.2b).

Figure 4.22.2b-c Pair-wise LD of TGFB1 SNPs measured by *D'* and r^2



Numbers in boxes represent *D'* and r^2 values (%) between paired SNPs in Figure b and c respectively. The definite *D'* values for pairs (-509, +913) and (+868, +913) are not shown, due to the very low frequencies of C allele and CC genotype in +913 polymorphism. Polymorphisms within black border are in the same LD block.

Figure 4.22.2d Details of allelic linkage between neighbouring SNPs of TGFB1-509, +868 and +913



One, 2 and 3 represent TGFB1-509, +868 and +913 respectively. Numbers next to alleles shows the frequencies of the alleles. Numbers at bottom shows the *D'* values between the neighbouring SNPs. Thick/thin lines represent the different strength of linkage, with thin lines showing the connection > 1% but Ò10%, while thick lines showing the connection > 10%.

Table 4.22.2b Frequency of haplotypes across the TGFB1-509, +868 and +913

Haplotype	Frequency	Haplotype	Frequency
C-C-C	**0.072**	T-C-C	0.0
C-C-G	0.010	T-C-G	**0.281**
C-T-C	0.0	T-T-C	0.0
C-T-G	**0.634**	T-T-G	0.004

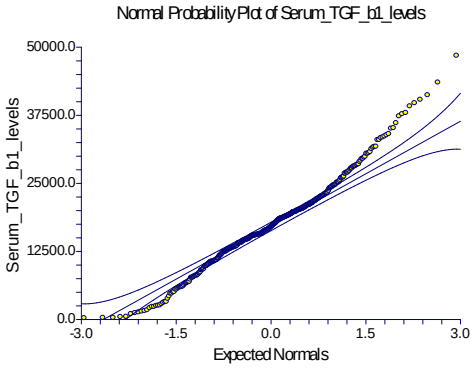
Common haplotypes are marked in black (frequency threshold = 0.05).

4.22.3 Serum level of TGF-β1

Determination and distribution

Serum TGF-ƃ1 level was determined in 415 patients. Its distribution was rejected for normality (Figure 4.22.3a). Transformations (e.g. log, square root, etc) could not improve the distribution of normality (data not shown). The median level was 16764 (IQR 12720 - 21428) pg/ml.

Figure 4.22.3a Distribution of serum TGF-ƃ1 levels in RA



Correlation between TGF-β1 and other investigated proteins

The relationships between serum TGF-ϐ1 and serum MMP-1, -2, -3, -8 and -9 were tested. TGF-ϐ1 was positively correlated with MMP-1 ($r = 0.18$, $p = 0.0002$), MMP-8 ($r = 0.17$, $p = 0.0004$) and MMP-9 ($r = 0.18$, $p = 0.0003$). Adjustment for age, sex and RA duration did not alter the significance of these correlations (data not shown).

In patients whose levels of TIMPs were measured, the relationships between serum TGF-ϐ1 and TIMP-1, -2, -3 and -4 were investigated. We found that TGF- ϐ1 was correlated with TIMP-1 ($r = 0.25$, $p = 0.0012$) and TIMP-2 ($r = 0.15$, $p = 0.061$ ($p = 0.0041$ after adjustment for age, sex and RA duration)), but not with TIMP-3 and -4.

The relationships between serum levels of TGF-ϐ1 and serum levels of angiogenic proteins (including VEGF-A, angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB and PECAM-1) were assessed. Serum TGF-ϐ1 was correlated with serum levels of VEGF-A ($r = 0.19$, $p < 0.0001$), HGF ($r = 0.13$, $p = 0.010$) and PDGF-BB ($r = 0.30$, $p < 0.0001$). Adjustment for age, sex and RA duration made little difference to the results (data not shown).

Serum TGF-β1 level, cigarette smoking and patient demographics

No difference was seen when TGF-ϐ1 was stratified by ever-smoked status. When stratified by never/past/current status, interestingly we observed that the lowest level occurred in past smokers (never vs. past vs. current, 17660 vs. 15672 vs. 18854 pg/ml), although there was no significant difference between these 3 groups after adjustment for

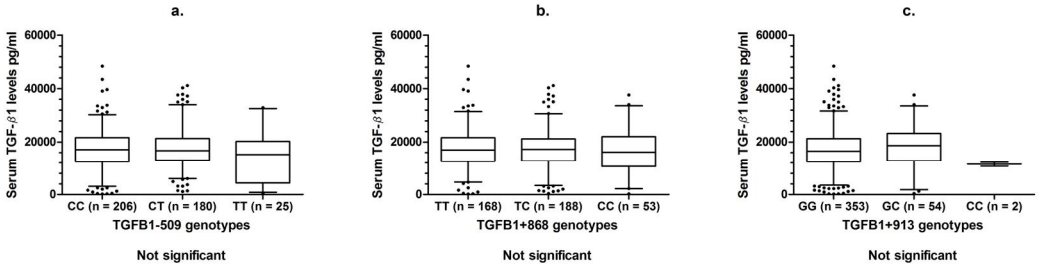
age, sex and RA duration. No correlation of TGF-ϐ1 with cigarettes per day, years smoked or pack-years was found.

No significant association of serum TGF-ϐ1 with sex or BMI was found. Interestingly, we found a negative correlation with age (r = -0.13, p = 0.0092), and a positive correlation with RA duration (r = 0.13, p = 0.0087). These 2 correlations were independent of each other in multiple regression analysis.

4.22.4 TGFB1 polymorphism and serum TGF-β1 level

Serum levels of TGF-ϐ1 in each genotypic group of the studied TGFB1 polymorphisms are shown in Figure 4.22.4a-c. These polymorphisms were not associated with serum TGF-ϐ1. Haplotype analysis with HAPSTAT program indicated that there was no haplotype across these SNPs associated with the serum level (data not shown).

Figure 4.22.4a-c A box-and-whisker diagram of serum TGF-ϐ1 levels stratified by TGFB1-509, +868 and +913 polymorphisms in RA



Boxplot with whisker with 5 ï 95 percentile.

4.23 TGFB1 POLYMORPHISM, SERUM TGF-β1 AND DISEASE SEVERITY

4.23.1 Brief introduction

TGF-ƃ1 has regulatory effects on lymphocytes, dendritic cells, macrophages, chondrocytes and osteoblasts, and therefore is considered as an important modulator in RA pathogenesis. The severity and long term outcome of RA have been related to this cytokine. A recent study showed that soluble TGF-ƃ1 serum levels were associated with the functional class evaluated in patients with RA (Muñoz-Valle et al, 2010). Other studies provided evidence based on genetic polymorphisms. Mattey et al suggested that the T allele of +868 SNP was associated with more severe disease measured by Larsen score and HAQ (Mattey et al, 2005). Kim et al found that the progression of radiographic severity, which was defined by a modified Sharp score plotted against disease duration, was significantly faster in carriers of the T allele at the -509 SNP (Kim et al, 2004). Muñoz-Valle et al observed that patients carrying the GG genotype of +913 SNP presented the highest Spanish HAQ-DI scores. However, no other reports have been published. In the present study, we included these 3 SNPs simultaneously to further evaluate their relationship with disease severity and to consider the influence of haplotypes across these SNPs.

4.23.2 Serum TGF-β1 and RA clinical measures

Serum TGF-ƃ1 was not associated with RF or anti-CCP. It was not significantly associated with inflammatory markers including ESR and CRP, disease activity measured by DAS28, or functional outcome measured by MJS and HAQ. Further analysis within specific subsets (e.g. RF+ patients, current smoker patients, etc) also revealed no significant association.

The relationships between TGF-ß1, and nodular and erosive diseases were also investigated. A decreased serum TGF-ß1 was observed in patients with nodular disease (n = 52), compared to the levels in patients without nodular disease (n = 330) (15310 (IQR 10152 - 19873) vs. 17348 (IQR 13194 - 21918) pg/ml, p = 0.041 adjusted for age, sex and RA duration). In contrast, in the whole population no association was found between TGF-ß1 and erosive disease. However, we saw a significant association restricted to RF+ patients, with increased TGF-ß1 associated with erosive development (erosion (n = 179) vs. non-erosion (n = 54), 18508 (IQR 13756 - 22264) vs. 14650 (IQR 11374 - 19671) pg/ml, p = 0.045 adjusted for age, sex and RA duration). In a forward stepwise multivariate logistic regression model which included levels of TGF-ß1, angiogenic proteins and MMPs, it was found that TGF-ß1 level was the only one positively associated with erosive disease (p = 0.028) in RF+ patients. MMP-2 level was negatively associated (p = 0.0048) in the same model.

4.23.3 TGFB1 polymorphism and RA clinical measures

No major association of TGFB1 SNPs or haplotypes with RA clinical measures was observed in this group of patients. However, patients carrying the GC/CC genotype at +913 may be associated with increased inflammation, since both ESR and CRP were higher in these subjects (ESR: GC + CC vs. GG, 25.0 (IQR 14.0 ï 43.0) vs. 19.0 (IQR 10.0 ï 34.0), p = 0.12; CRP: GC + CC vs. GG, 12.0 (IQR 7.0 ï 24.0) vs. 10.1 (IQR 4.0 ï 19.2), p = 0.050), although for ESR it was not significant.

We observed an interesting association between TGFB1+913 polymorphism and serum levels of MMP-1, with GG genotype (GG vs. C allele) associated with lower levels (GG

vs. C allele, 5309.9 (IQR 3359.9 ï 8783.8) vs. 7659.5 (IQR 4006.2 ï 12308.3) pg/ml, p = 0.0092 adjusted for age, sex, RA duration, and MMP1 rs1799750 and MMP3 rs3025058 genotypes). A similar trend was also found in relation to serum levels of MMP-2, although it was not significant (GG vs. C allele, 173665.1 (IQR 149276.1 ï 201548.9) vs. 186846 (IQR 164086.5 ï 209254.8) pg/ml, p = 0.098 adjusted for age, sex, RA duration and MMP1-3 intergenic rs495366 genotype). These results implied that the TGFB1 gene may have a regulatory effect on MMP-1 and -2 levels.

4.24 TGFB1 POLYMORPHISM, SERUM TGF-β1 AND CO-MORBIDITY

4.24.1 Brief introduction

Polymorphisms in the TGFB1 gene have been associated with cardiac events, especially MI in case-control studies (Cambien et al, 1996; Crobu et al, 2008; Koch et al 2006). However, studies with no association have also been reported (Sie et al, 2006). Investigation of the relationship between TGFB1 SNPs and CVD in an RA population has not been reported yet. It was thus of interest to determine whether TGFB1 polymorphisms were associated with the occurrence of IHD and previous MI in our cohort of RA. Since TGFB1 polymorphisms may be associated with hypertension in RA (Panoulas et al, 2009), we also looked for any association with hypertension. Other co-morbid conditions recorded in patients were also investigated.

4.24.2 Serum TGF-β1 and co-morbidity

Initial analysis suggested that there was a relationship between hypertension status and serum TGF-ϐ1, with the condition associated with lower levels (15912 (IQR 12372 - 26404) vs. 17550 (IQR 13155 - 22431) pg/ml, p = 0.050). However, significance was lost after further adjustment for age, sex and RA duration (p = 0.25).

We analysed the relationship between serum TGF-ϐ1 and the status of diabetes mellitus (including type 1 and 2). We observed a significant relationship between TGF-ϐ1 and diabetes type 2, with the disease (n = 21) associated with a reduction of serum TGF-ϐ1 (13008 (IQR 10474 - 17450) vs. 17252 (IQR 12920 - 21764) pg/ml, p = 0.031 adjusted for age, sex and RA duration). Again, we found a trend that a decreased level of TGF-ϐ1 was associated with diabetes type 1, although it was not significant (15324 (IQR 7984 - 20152) vs. 16924 (IQR 12794 - 21540), p = 0.25 adjusted for age, sex and RA duration). This may be due to the low case number of this condition (n = 11). Combination of type 1 and 2 diabetes indicated that patients with diabetes were associated with lower serum levels of TGF-ϐ1 compared to those without diabetes in RA (13398 (IQR 10453 - 17969) vs. 17348 (IQR 13037 - 21770), p = 0.013 adjusted for age, sex and RA duration).

Serum TGF-ϐ1 was not associated with the status of IHD/MI. However, there was evidence in these RA patients that this protein may be associated with overall heart diseases. We found that a reduced TGF-ϐ1 level was associated with current use of medications for heart-related diseases (based on interviews and on patientsô medical records). In patients who were taking heart medication at recruitment (n = 143) the median of serum TGF-ϐ1 was 15460 (IQR 11436 - 19752) pg/ml, while in the remaining patients

(n = 264) the median was 18500 (IQR 13853 - 22485) pg/ml (p = 0.026 adjusted for age, sex and RA duration). There was no difference in TGF-ß1 levels between patients on statins and on non-statin drugs (15456 (IQR 11490 - 20518) vs. 15484 (IQR 9780 - 19060) pg/ml).

4.24.3 TGFB1 polymorphism, smoking and IHD/MI

TGFB1 polymorphism and IHD/MI

The patient characteristics stratified by IHD/MI status were described before (Section 4.11.2). The relationship between TGFB1 genotypes and the presence of IHD/MI, without adjustment for confounders, are shown in Table 4.24.2a. Results suggested that heterozygosity of TGFB1-509 (CT genotype) and +868 (TC genotype) showed a relationship with increased occurrence of IHD and MI, although for TGFB1-509 no significant association with IHD was achieved. There was no relationship between TGFB1+913 and the presence of IHD/MI.

Since there is strong LD between TGFB1-509 and +868, the weaker association involving -509 may be due to the greater association of +868 with IHD and MI. This was supported by logistic regression analysis which contained both -509 (CT vs. CC + TT) and +868 (TC vs. TT + CC) together as independent variables and used forward stepwise selection to test for primary risk factor if factors were not independent of each other. The associations involving TGFB1+868 maintained significance in relation to both IHD (OR 1.98, 95% CI 1.17 ï 3.34) and MI (OR 3.23, 95% CI 1.63 ï 6.41), whereas the others involving -509 lost significance in these models (adjusted for age, sex and RA duration).

Although the TGFB1+868 SNP showed the strongest association, we carried out haplotype analysis to test whether there was any particular haplotype involved. Common haplotypes across TGFB1-509, +868 and +913, and their relationship with the presence of IHD/MI are presented in Table 4.24.2b. The T-C-G haplotype displayed a difference in distribution between patients with (31.5%) and without (27.8%) MI (Table 4.24.2b), although it was not significant ($p = 0.087$). The frequency of C-T-G and C-C-C was generally equal between patients with and without IHD/MI (Table 4.24.2b). Comparison between T-C-G and C-T-G suggested that the differential risk was related to the genetic region flanked by TGFB1-509 and +868 and is not associated with TGFB1+913. Exclusion of +913 SNP in the analysis made little difference (data not shown).

No significant association of TGFB1 polymorphisms with other co-morbidities including hypertension and diabetes mellitus was found.

Table 4.24.2a Frequency of IHD and MI in RA patients stratified by TGFB1 SNP genotypes

	IHD			MI		
	Negative	Positive (%)	Odds ratio (95% CI)	Negative	Positive (%)	Odds ratio (95% CI)
TGFB1-509 (C/T)						
CT	146	40 (21.5)	1.0 (referent)	159	27 (14.5)	1.0 (referent)
CC	175	35 (16.7)	0.73 (0.44 ï 1.21)	192	18 (8.6)	0.56 (0.30 ï 1.04)
TT	24	3 (11.1)	0.52 (0.16 ï 1.67)	26	1 (3.7)	0.33 (0.06 ï 1.79)
CC + TT	199	38 (16.0)	0.70 (0.43 ï 1.14)	218	19 (8.0)	0.52 (0.28 ï 0.96)
TGFB1+868 (T/C)						
TC	138	45 (23.3)	1.0 (referent)	161	32 (16.6)	1.0 (referent)
TT	146	25 (14.6)	0.53 (0.31 ï 0.91)	158	13 (7.6)	0.42 (0.22 ï 0.83)
CC	50	7 (12.3)	0.45 (0.20 ï 1.04)	56	1 (1.8)	0.13 (0.02 ï 0.70)
TT + CC	196	32 (14.0)	0.50 (0.31 ï 0.83)	214	14 (6.1)	0.34 (0.18 ï 0.64)
TGFB1+913 (G/C)						
GC	44	13 (22.8)	1.0 (referent)	52	5 (8.8)	1.0 (referent)
GG	297	65 (18.0)	0.73 (0.37 ï 1.41)	321	14 (11.3)	0.43 (0.15 ï 1.20)
CC ï	2	0 (0.0)	0.66 (0.03 ï 14.59)	2	0 (0.0)	1.91 (0.08 ï 45.04)

Values are n (%).

Table 4.24.2b Frequency of TGFB1 haplotypes in RA patients with and without IHD or MI

	All patients	IHD			MI		
TGFB1 haplotype (-509/+868/+913)	EM freq.	Negative EM freq.	Positive EM freq.	OR (95% CI)	Negative EM freq.	Positive EM freq.	OR (95% CI)
C-T-G	0.634	0.637	0.617	0.68 (0.41 ï 1.13) ÿ	0.634	0.630	0.98 (0.63 ï 1.54) ÿ
T-C-G	0.281	0.280	0.292	1.32 (0.81 ï 2.16) À	0.278	0.315	1.71 (0.94 ï 3.13) À
C-C-C	0.072	0.069	0.084	1.34 (0.68 ï 2.61) À	0.074	0.054	0.73 (0.28 ï 1.95) À
Rare	0.013						

EM freq., frequency estimated by the expectation/maximization algorithm; Maximum EM iteration = 500, EM convergence tolerance = 0.0001; Frequency threshold = 0.05; Best odds ratio achieved in Àdominant model, ÿadditive model.

TGFB1-smoking interaction and IHD/MI

Analysis was also carried out to investigate if there was potential interaction of TGFB1 ploymorphism with smoking relative to the occurrence of IHD/MI. Table 4.24.2c shows the occurrence of IHD and MI stratified by the combination of TGFB1 polymorphism (-509 and +868) with ever-smoked status in RA. Evidence of interaction was tested based on AP with 95% CI. For TGFB1-509, the values of AP (95% CI) only demonstrated weak and insignificant results. In contrast, for +868, AP showed a large proportion of effect due to interaction (in relation to IHD: 0.54; MI: 0.39), and 95% CI indicated the significance of these results. Interestingly, it was seen that the genotypes (heterozygosity) of the TGFB1 gene itsef did not have any effect on the risk for IHD (see the smoke-/genotype+ combination in Table 4.24.2c). However, patients carrying the heterozygote combination with smoking were at the highest risk for IHD. The risk was significantly higher compared to that among patients who had ever smoked but did not carry these genotypes (according to TGFB1+868: OR 2.25 (1.28 ï 3.96), p = 0.0042). A similar pattern was also seen in relation to MI, but the TGFB1 polymorphisms also showed some independent effect.

Table 4.24.2c Association of TGFB1 polymorphisms and ever having smoked with IHD and MI in RA patients

	IHD			MI		
	Negative	Positive (%)	Odds ratio (95% CI)	Negative	Positive (%)	Odds ratio (95% CI)
Smoke/-509 CT						
-/-	70	5 (6.7)	1.0 (referent)	75	0 (0.0)	1.0 (referent)
-/+	60	6 (9.1)	1.38 (0.42 ï 4.51)	62	4 (6.1)	10.87 (0.76 ï 49.87)
+/-	127	33 (20.6)	3.37 (1.31 ï 8.69)	141	19 (11.9)	20.81 (1.95 ï 109.38)
+/+	85	34 (28.6)	5.17 (1.99 ï 13.42)	96	23 (19.3)	36.77 (3.49 ï 194.74)
			AP: 0.26 (-0.20 ï 0.73)			AP: 0.17 (-0.37 ï 0.71)
Smoke/+868 TC						
-/-	71	7 (9.0)	1.0 (referent)	78	0 (0.0)	1.0 (referent)
-/+	58	5 (7.9)	0.90 (0.28 ï 2.84)	59	4 (6.3)	11.87 (0.83 ï 54.49)
+/-	125	25 (16.7)	1.94 (0.82 ï 4.60)	136	14 (9.3)	16.68 (1.52 ï 86.54)
+/+	88	40 (31.3)	4.36 (1.89 ï 10.10)	100	28 (21.9)	44.52 (4.28 ï 237.16)
			AP: 0.54 (0.20 ï 0.89)			AP: 0.39 (0 ï 0.80)

Values are n (%); AP, the attributable proportion due to interaction.

Multivariate analysis: factors associated with IHD/MI

In the present study, we have shown that VEGFA-2578-smoking interaction, older age, male, CRP Ó 10 mg/l, hypercholesterolemia, diabetes type 2, CVA and MTX use are independently associated with IHD (model 1, Table 4.11.2g), and that VEGFA-2578-smoking interaction, male, RF, HAQ score, hypercholesterolemia, hypertension, asthma, CVA and MTX use are independently associated with MI (model 2, Table 4.11.2g). Here, we further included TGFB1+868-smoking interaction into those models and demonstrated that the associations of TGFB1+868-smoking interaction with IHD/MI were independent of other factors including the VEGFA-2578-smoking interaction (Table 2.24.2d).

Table 4.24.2d Multivariate logistic regression analysis of variables associated with IHD and MI

Variable	IHD (model 1[À]) Regression coefficient	OR (95% CI)	p	Variable	MI (model 2[ÿ]) Regression coefficient	OR (95% CI)	p
*Smoke + TGFB1+868 CT (+/-)	0.716	2.05 (1.11 ï 3.74)	0.020	Smoke + TGFB1+868 CT (+/-)	0.896	2.45 (1.15 ï 5.21)	0.020
¶Smoke + VEGFA-2578 A (+/-)	1.198	3.31 (1.68 ï 6.51)	0.0005	Smoke + VEGFA-2578 A (+/-)	1.683	5.38 (1.93 ï 15.0)	0.0013
Hypercholesterolemia (+/-)	1.279	3.59 (1.84 ï 7.02)	0.0002	Hypercholesterolemia (+/-)	1.0	2.74 (1.18 ï 6.34)	0.019
Age, per year	0.040	1.04 (1.01 ï 1.07)	0.011	HAQ score	0.481	1.62 (0.93 ï 2.80)	0.081
CRP Ó10mg/l (+/-)	0.872	2.39 (1.29 ï 4.44)	0.0058	Hypertension (+/-)	0.904	2.47 (1.09 ï 5.58)	0.030
Diabetes type 2 (+/-)	1.686	5.40 (1.77 ï 16.44)	0.0030	Asthma (+/-)	0.778	2.18 (0.80 ï 5.94)	0.13
CVA (+/-)	1.80	6.05 (1.78 ï 20.59)	0.0040	CVA (+/-)	2.331	10.29 (2.60 ï 40.73)	0.0009
Male (+/-)	0.619	1.86 (1.02 ï 3.37)	0.042	Male (+/-)	1.373	3.95 (1.79 ï 8.70)	0.0007
MTX use (+/-)	-0.575	0.56 (0.32 ï 0.99)	0.096	MTX use (+/-)	-0.764	0.47 (0.22 ï 0.97)	0.040
				RF (+/-)	0.611	1.84 (0.79 ï 4.29)	0.16

* Patients who have ever smoked and carry the TGFB1+868 CT genotype, compared with all remaining patients; ¶ Patients who have ever smoked and carry a VEGFA-2578 A allele, compared with all remaining patients; [À] Patients with IHD vs. without IHD; [ÿ] Patients with MI vs. all non-MI patients.

Total risk due to VEGFA-2578, TGFB1+868 and smoking

In the present study, we have found 2 smoking-gene interactions (VEGFA-2578 A-smoking and TGFB1+868 CT-smoking) relative to an increased risk of IHD and MI in patients with RA. They seemed to be independent of each other, although smoking played significant roles in both interactions. We here investigated the total risk for IHD and MI when a RA patient had ever smoked and carried both risk variants at VEGFA-2578 (the A allele) and TGFB1+868 (the CT genotype). Table 4.24.2e and f show the occurrence of IHD and MI respectively, stratified by VEGFA-2578 (A allele- vs. A allele+), TGFB1+868 (CT- vs. CT+) and smoking (non-smoking vs. ever-smoking). Data indicated an extremely high rate of IHD (36.1%, OR = 8.14 compared to those without these risk factors) and MI (27.8%, OR = 17.55 compared to those without these risk factors) amongst individuals carrying the 3 risk factors.

Table 4.24.2e Occurrence of IHD stratified by VEGFA-2578 (A allele- vs. A allele+) and TGFB1+868 (CT- vs. CT+) in RA patients who had never smoked and in those who had ever smoked

	Non-smokers			Ever-smokers		
A/CT*	Non-IHD	IHD	OR (95% CI)	Non-IHD	IHD	OR (95% CI)
-/-	21	1 (4.5)	1.0 referent	35	1 (2.8)	0.61 (0.06 ï 6.20)
-/+	17	1 (5.6)	1.23 (0.12 ï 12.88)	25	5 (16.7)	3.09 (0.46 ï 20.55)
+/-	50	5 (9.1)	1.56 (0.24 ï 10.18)	89	25 (21.9)	4.08 (0.74 ï 22.64)
+/+	40	4 (9.1)	1.59 (0.23 ï 10.87)	62	35 (36.1)	8.14 (1.48 ï 44.82)

Values are n (%); * VEGFA-2578 A allele/TGFB1+868 CT genotype.

Table 4.24.2f Occurrence of MI stratified by VEGFA-2578 (A allele- vs. A allele+) and TGFB1+868 (CT- vs. CT+) in RA patients who had never smoked and in those who had ever smoked

	Non-smokers			Ever-smokers		
A/CT*	Non-MI	MI	OR (95% CI)	Non-MI	MI	OR (95% CI)
-/-	22	0 (0.0)	1.0 referent	35	1 (2.8)	1.90 (0.03 ï 5.08)
-/+	18	0 (0.0)	1.22 (0.02 ï 64.31)	29	1 (3.3)	2.29 (0.04 ï 6.12)
+/-	55	0 (0.0)	0.41 (0.01 ï 21.06)	101	13 (11.4)	5.99 (0.51 ï 30.23)
+/+	40	4 (9.1)	5.0 (0.32 ï 22.45)	70	27 (27.8)	17.55 (1.59 ï 91.35)

Values are n (%); * VEGFA-2578 A allele/TGFB1+868 CT genotype.

4.25 SUMMARY OF FINDINGS FOR 4.22 – 4.24

- Strong LD was detected across the region marked by TGFB1 polymorphism named TGFB1-509, +868 and +913. C-T-G and T-C-G were the major haplotypes across this region.

- Serum TGF-ß1 level was positively correlated with levels of several MMPs (-1, -8 and -9), TIMPs (-1 and -2) and angiogenic proteins (VEGF-A, HGF and PDGF-BB).

- No association was found between TGFB1 polymorphisms (-509, +868 and +913) and serum levels of TGF-ß1 in RA.

- A decreased serum TGF-ß1 level was observed in patients with nodular disease, compared to those without this extra-articular manifestation.

- In the subset of RF+ patients, an increase of serum TGF-ϐ1 was associated with erosive disease. Such relationship did not exist in RF- patients.

- There was an association between TGFB1+913 polymorphism and serum levels of MMP-1 and -2 (insignificant trend), independent of MMP polymorphisms.

- Lower serum levels of TGF-ϐ1 were found in patients with diabetes mellitus, especially in those with diabetes type 2.

- Lower serum levels of TGF-ϐ1 were observed in patients who were taking medications for heart-related diseases, compared to the levels in the remaining subjects.

- There was evidence suggesting that polymorphisms in the TGFB1 gene may be associated with IHD and in particular with MI, with TGFB1+868 showing the strongest effect. This evidence remained after adjustment for other known factors related to IHD and/or MI in this population.

- The above effect was largely due to an interaction with smoking, and was independent from other risk factors including the VEGFA-2578 A-smoking interaction.

PART G: PTPN22 POLYMORPHISM, SEVERITY AND CO-MORBIDITY IN RA (4.26 – 4.29)

4.26 PTPN22 POLYMORPHISM

4.26.1 Brief introduction

Phosphatases are known to play a role in immune-cell homeostasis. PTPN22 appears to set thresholds for T cell receptor signalling. There is a missense SNP (rs2476601) in the PTPN22 gene. Carriage of the T allele (known as R620W allele) of this polymorphism is associated with increased risk for the development of human auto-immunity, including RA. The disease-associated polymorphism encodes an amino acid change in one of four SRC homology 3 domain (SH3) binding site in the PTPN22 molecule, which would potentially alter the normal function of this protein as a negative regulator of T cell activation (Gregersen 2005).

4.26.2 PTPN22 polymorphism

Studied SNP

Polymorphism rs2476601 (C/T) is located at +1858 according to the transcriptional start site of the PTPN22 gene, and +620 relative to the translational start site.

Allelic and genotypic distribution

Genotypes were determined in 428 patients. The allelic frequencies were 85.3% (n = 730) and 14.7% (n = 126) for C and T alleles respectively. The genotypic distributions were 71.7% (n = 307), 27.1% (n = 116) and 1.2% (n = 5) for CC, CT and TT respectively. Genotype distribution was distributed in accordance with a close fit to HWE.

4.27 PTPN22 POLYMORPHISM AND DISEASE SEVERITY

4.27.1 Brief introduction

Several studies have suggested that R620W allele in the PTPN22 gene has early onset and disease severity effects in Caucasians (Steer et al, 2005; Karlson et al, 2008). An association between the PTPN22 risk variant and increased progression rate for structural damage has been observed (Lie et al, 2007; Karlson et al, 2008). These indicated that the PTPN22 may not only be associated with disease susceptibility, but also with disease progression. In this study, we looked at whether this genetic variant was associated with disease activity/severity in our patients of RA.

4.27.2 PTPN22 polymorphism and RA clinical measures

No association of the R620W allele in the PTPN22 gene with any disease measures was observed, expect for a borderline association between the T allele (TC + TT genotypes) and increased MJS (8.5 (IQR 4.0 ï 17.0) vs. 7.0 (IQR 3.0 ï 13.0), p = 0.063). However,

this relationship disappeared after adjustment for age and RA duration in multivariable analysis.

4.28 PTPN22 POLYMORPHISM AND CO-MORBIDITY

4.28.1 Brief introduction

Since the PTPN22 R620W allele is thought to be a general risk factor for the development of human auto-immunity, in the present study we looked at whether this SNP was associated with co-morbid conditions in RA, with particular interest in diabetes type 1 and 2.

4.28.2 PTPN22 polymorphism and co-morbidity

No association of the R620W allele in the PTPN22 gene with any co-morbid condition in RA was found, except for an association between TT genotype and increased risk of hypercholesterolemia (3/5 (60.0%) vs. 68/417 (16.3%), $p = 0.034$). Since the frequency of TT genotype was extremely low ($n = 5$), we did not carry out further investigation.

4.29 SUMMARY OF FINDINGS FOR 4.26 – 2.28

- PTPN22 R620W polymorphism was not associated with disease activity and functional outcome in this population of RA.

PART H: RESULTS SUMMARY

4.30 SUMMARY OF THE MAJOR FINDINGS

- Cigarette smoking is closely associated with autoantibodies, especially with RF, in this RA population. In autoantibody negative patients, it shows significant effect on disease outcome.

- An association between polymorphism in the VEGFA gene and serum level of VEGF-A is observed. VEGFA polymorphism is not only associated with RA activity but also associated with the occurrence of IHD and MI in these patients. A strong interaction with smoking is involved in the latter association.

- Polymorphism in the MMP1-3 loci influences serum levels of MMP-1, -2, -3 and -9, and ratios of MMP-1/TIMP-1, -2, -3 and -4. It also influences RA activity, and erosive development especially in earlier disease.

- Polymorphism in the TGFB1 gene influences the occurrence of IHD and MI in RA, which may be due to an interaction with cigarette smoking.

CHAPTER 5 DISCUSSION

5.1 OVERVIEW

The main objective of this study was to identify some of the main determinants of poor outcome in RA, both in terms of disease severity and co-morbidity. A number of candidate genes were selected based on the findings of earlier studies and their likely importance in the immune response, angiogenesis and athereosclerosis, tissue destruction and detoxification of ROS. A detailed study of the influence of smoking and its interaction with various genetic factors has also been carried out. The results have confirmed and extended a number of previous findings from this laboratory, and have identified several, important new associations and interactions in relation to RA disease activity, severity and co-morbidity. The study has highlighted the complexity of factors associated with severity and co-morbidity in RA, and has shown the importance of smoking in exacerbating various aspects of disease outcome. The discussion is split into a number of sections which follow the same general layout as the results section.

5.2 SMOKING, AUTOANTIBODY, SEVERITY AND CO-MORBIDITY IN RA

5.2.1 Smoking characteristics

Much of the data was presented as a comparison between patients who had never smoked and those who had ever smoked. Preliminary results showed that there was no significant difference in outcome variables (with regard to disease severity and co-morbidity) between past and current smokers, so these 2 categories were combined for most analyses. The lack of a differential effect of past and current smoking is consistent with previous studies. For example, a large longitudinal study found that past and current smokers were associated

with similarly elevated risks of RA, in particular seropositive disease, and that a substantial effect of past smoking remained up to 20 years after smoking cessation (Costenbader et al, 2006). Furthermore, in a study evaluating the association of smoking with release of TNF-Ŭsoluble tumour necrosis factor receptors (sTNFRs), it was suggested that there was no differential effect between past and current smokers, and that the influence of smoking appeared to be a chronic response (Glossop et al, 2006). Taking these together, we assumed that, in many aspects such as disease susceptibility and autoantibody/cytokines release, the effect of smoking on RA was prolonged. On the other hand, there was evidence showing that altered levels of some biomarkers (e.g. MMP-2, MMP-3, MMP-9, G-CSF, etc) were particularly associated with current smoking (MMP data presented in Section 4.17.3; G-CSF data not shown).

Ever-smoked status was also considered to be a more appropriate category to investigate the relationship with outcome variables related to health conditions. Separate analysis of past and current smoking may cause a selection bias since patients associated with poorer health conditions, including worse RA and more co-morbidities, were more likely to have given up smoking under advice from health professionals.

Nicotine is an important component of cigarette smoke, affecting cytokine production and cellular response. Recently, using a rat model of autoimmune arthritis (AA), a study found that treatment with nicotine before the onset of AA led to a worse disease, whereas interestingly the same treatment starting after the onset of AA suppressed the disease (Yu et al, 2011). This observation implied that smoking may have differential impact on disease severity depending on its first-exposure time relative to the onset of disease. In the present study, the age of RA onset and the age of starting smoking were carefully recorded on each

patient. Descriptive statistics demonstrated smoking as an early-exposure factor many years prior to the onset of disease. Furthermore, we scanned the whole database and found no patient who started smoking after the onset of disease. Thus, the potential confounding effect noted by Yu et al did not apply in this study.

It appears that smoking has a stronger influence in males than in females on the development of RA. In the most recent meta-analysis, it was indicated that the risk of developing RA was about 2-times higher for male smokers than non-smokers, whereas the ratio dropped to 1.3-times for females (Sugiyama et al, 2010). Although we were unable to directly test this difference in our case-only study, we found an extremely high rate (82.5%) of smokers in males, which showed a significant difference compared to that in females (58.4%). This was supportive of the conclusion made by Sugiyama et al. RA has been inherently more common in females, suggesting that certain female host factors, such as female sex hormones, may be involved in the development of RA, and the mechanism here does not need the involvement of smoking. For males, however, the occurrence of disease relies more upon the smoking-related pathways, since male host factors are thought to be protective. The proportion of disease triggered by smoking is higher in men, while the higher risk female group is influenced by more risk factors apart from smoking. In addition, based on the records of smoking in our RA patients, it seemed that males smoked more compared with females, in terms of cigarettes per day, years of smoking (insignificant trend) and total pack-years. These quantitative parameters certainly play a role, and therefore could be confounding factors in studies where only qualitative status is available.

5.2.2 Smoking, RF and anti-CCP

Smoking has been associated with the presence of both RF and anti-CCP. In this study, we used multivariate logistic regression model to evaluate which association was stronger. Our results indicated that smoking was more closely associated with the presence of RF in this particular group of RA patients. However, this analysis did not consider the effects from certain genetic polymorphisms. In smokers, a correlation between the number of years of smoking and the levels of RF was seen, showing a dose response effect. In contrast, the linear relationship between pack-years and the levels of RF was less significant. Accordingly, the results suggested that smoking duration was more important in relation to RF release, compared with smoking intensity. This was comparable with previous studies indicating that smoking duration was more important compared with intensity relative to the development of RA (Stolt et al, 2003; Karlson et al, 1999). Such correlation was also seen with the levels of anti-CCP, but it was considered likely to be due to the strong correlation of anti-CCP with RF, since the significance was dramatically reduced after adjustment for RF levels. We also showed that the risk of developing RF+ RA influenced by smoking was modified by gender, with a stronger association in males (OR: 4.35 in males vs. 1.95 in females). Our data was comparable with the latest meta-analysis (Sugiyama et al, 2010). The reason for the modification may be associated with a complex of other factors which are different between genders, such as hormonal effect and metabolic rate. However, interestingly it was found that the overall frequency of RF+ in males and females was the same (male 56.5% vs. female 56.9%).

Several genetic factors have been shown to be involved in the association of smoking with RF and anti-CCP. According to the literature, it was suggested that HLA-DRB1 SE alleles

were involved in the association of smoking with RF, demonstrating an interaction effect (Mattey et al, 2002; Padyukov et al, 2004). However, more recent studies indicated that smoking was primarily associated with anti-CCP in the presence of HLA-DRB1 SE alleles, and that PTPN22 gene may be also involved (Klareskog et al, 2006; Linn-Rasker et al, 2006; Kallberg et al, 2007). Most recently, a report which recruited 5020 RA patients from 6 centres across the UK summarised that both HLA-DRB1 SE and smoking were independently associated with RF and anti-CCP, and that interaction between smoking and HLA-DRB1 SE may occur resulting in anti-CCP+ RA (Morgan et al, 2010). In the present study, we were unable to test such proposed interaction, due to lack of HLA-DRB1 genotyping. We investigated the potential interaction with PTPN22, but obtained negative results. However, the frequency of homozygosity for the PTPN22 T allele was very low (the major risk genotype), so a larger population size would be required to provide sufficient power to test this.

We successfully confirmed another gene-environmental interaction between smoking and GSTM1-null genotype in relation to the production of RF (Mattey et al, 2002). Given the known role of the GSTM1 enzyme in detoxifying chemicals in cigarette smoke (Rebbeck 1997; Seidergard et al, 1990; Chenevix-Trench et al, 1995), it has been speculated that lack of such enzyme (GSTM1-null type) in smokers may promote increased RF production through a failure to detoxify smoke-derived chemicals (and/or their byproducts) which have the potential to damage IgG (Mattey et al, 2002). Previous studies have suggested that free radical-mediated modification of IgG may stimulate the formation of immune complexes with RF antibody, thereby promoting tissue damage during rheumatoid inflammation (Lunec et al, 1988; Swaak et al, 1989).

5.2.3 RF, anti-CCP and RA clinical measures

The associations of RF and anti-CCP with a more severe disease course have been described before. Previous studies showed that both autoantibodies were associated with increased disease activity and worse joint radiographic progression (Berglin et al, 2006; Lindqvist et al, 2005; van der Helm-van Mil et al 2005; Miriovsky et al, 2010). In particular, RF was a better predictor of the extra-articular manifestations of RA, whereas anti-CCP had superiority in predicting an erosive course. However, associations with the disease severity measures which include an element of functional outcome have been less studied. In the present study, functional outcome was assessed using the validated measures including MJS and HAQ, and the associations were assessed by multivariate regression models containing both autoantibodies as independent variables in order to remove the confounding effects between them. Interestingly, we could not find an association of anti-CCP with functional outcome measures in this group of patients, but we confirmed the association with erosive disease. RF was significantly associated with MJS and HAQ as well as markers of disease activity and nodular disease. Our results suggested that the presence of RF and anti-CCP may reflect different disease processes/pathways which have independent effects on disease outcome. A combination of these 2 may increase the ability to predict the long term outcome in RA.

RF includes several isotypes, while ACPA contains many subsets. These different subtypes may have individual roles in influencing disease outcome. However, in this study, they were not individually tested. Our results were based on diagnostic assays detecting IgM RF and anti-CCP2.

5.2.4 Smoking and RA clinical measures

There was a large proportion of RA patients characterized by autoantibody positivity (RF+ 57.6%; anti-CCP+ 75.5%). Since these autoantibodies had great impact on disease severity, any smaller effect (e.g. influence from smoking) could therefore be masked in the whole population. However, using analysis stratified by RF/anti-CCP status, we found evidence of effect of smoking on disease severity (HAQ and erosive course) in autoantibody negative patients who displayed less severity compared to autoantibody positive patients. In contrast, no effect was observed in autoantibody positive subjects. This suggested that smoking may play differential roles in different subsets of RA. The total effect on RA outcome attributed to cigarette smoking was considerable, since it contributed to the production of autoantibodies prior to the onset and/or in the early stage of disease, and in those without autoantibodies it showed a direct impact leading to a worse outcome. A radiographic damage measure was not available, thus we were unable to assess its relationship with smoking, in particular among seronegative patients. We were therefore unable to compare our data with recent studies indicating that there was no obvious association between smoking and radiological progression and structural damage restricted to the same serological group (Manfredsdttir et al, 2006; Westhoff et al, 2008; Finckh et al, 2007). The radiographic score does not fully reflect the biological outcome of the disease, being primarily a measure of cartilage and bone damage and not of damage to other tissue and organs. MJS was not associated with smoking in this study, which may be due to it also being a measure concentrating on the joints. Smoking may be more associated with measures that describe an overall health and functional status in RA.

5.2.5 Smoking and co-morbidity

For the first time, the current thesis described interactions of smoking with polymorphisms in the VEGFA and TGFB1 genes in relation to IHD/MI in RA. A detailed discussion is provided in Sections 5.3 and 5.6 respectively. For other co-morbidities recorded, there was no significant association with smoking. However, this may be due to the low incidence of the many conditions and the moderate effect from smoking. There was a trend that patients who had ever smoked had more co-morbid conditions, compared with those who had never smoked.

5.3 GSTM1, T1 POLYMORPHISM, SEVERITY AND CO-MORBIDITY IN RA

5.3.1 Genotypic characteristics

The frequency of GSTM1 and GSTT1 genotypes in our population was comparable with the data in other Caucasian studies (Mikuls et al, 2010; Keenan et al, 2010; Mattey et al, 2002; Ghelani et al, 2010; Criswell et al, 2006). According to the literature, in Caucasian populations, GST genes are not likely to be significant susceptibility genes. However, with the combination of smoking (in particular heavy smoking), both GSTM1 and T1 polymorphisms were shown to be associated with the risk for RA (Criswell et al, 2006; Keenan et al, 2010). In eastern Asian populations, mutations in the GSTM1 gene may have a stronger effect, since there are 2 studies independently indicating that the GSTM1-null genotype is associated with RA susceptibility without seeking an interaction with smoking (Morinobu et al, 2006; Yun et al, 2005).

5.3.2 GSTM1, T1 genotype and RA clinical measures

We confirmed a previous finding that the association of smoking with RF occurred only in GSTM1-null patients, suggesting a gene-environment interaction relative to RF production (as discussed). In the original report, it was also indicated that the combination of GSTM1-null genotype with smoking was associated with more severe RA measured by Larsen score and HAQ score (Mattey et al, 2002). In the present study, Larsen score was not available. Results based on HAQ, however, did not show a significant result in the whole population. Since the population used in the previous study was a female group, we also carried out an analysis using the female patients only. Interestingly, it was found that females who had ever smoked and were carrying GSTM1-null were associated with the highest HAQ, compared with the remaining combinations (Appendix 8.6). We therefore suggest that the effect of smoking-GSTM1-null interaction may be more influential in females.

Apart from an interaction with smoking, GSTM1 was found to show interaction with the major susceptibility gene, HLA-DRB1, in terms of the ACPA status (Miluls et al, 2010). However, we did not see any association of GSTM1-null genotype with anti-CCP+ in this population, although HLA-DRB1 genotyping was not available to assess any interaction.

For the first time, we found an association of GSTM1-null genotype with erosive disease, restricted to RF+ patients. Similar relationships were also found when patients were stratified by anti-CCP and ever-smoked status respectively. Multiple logistic regression analysis suggested that RF+ was not associated with erosive disease in patients with GSTM1-null genotype after adjustment for anti-CCP+ and ever-smoking. In the same

model, the results indicated that ever-smoking and anti-CCP+ were both independently associated with erosive disease in patients with GSTM1-null genotype. However, neither association was seen in patients with GSTM1-expressing genotype. In the present study therefore, we have added a novel contribution to the literature that the association of anti-CCP+ with erosive disease may, at least partly, depend on the non-functional polymorphism of the GSTM1 gene. The mechanism of such interaction is unknown and replication of such a relationship in another independent cohort is required.

A recent report suggested that GSTT1-null genotype was associated with higher disease activity, and such association was enhanced by the combination with smoking (Bohanec Grabar et al, 2009). However, we did not find any association of GSTT1 polymorphism with RA measures, either before or after the combination with smoking.

5.3.3 GSTM1, T1 genotype and co-morbidity

Although in the general population, GSTM1-null and GSTT1-null have been proposed as risk factors for the development of coronary artery disease, little has been done in RA subjects. We looked at the relationship between GSTM1-null/T1-null genotype and IHD/MI, and considered any interaction with smoking. No association or trend was found. However, this may be due to the relatively small number of patients with IHD/MI in this population. Since these polymorphisms have also been associated with hypertension in the general population, we also investigated this in our RA population. Our results suggest that GSTM1-null or T1-null is not associated with hypertension in RA.

5.4 VEGFA POLYMORPHISM, SERUM LEVEL OF VEGF-A, SEVERITY AND CO-MORBIDITY IN RA

5.4.1 VEGFA polymorphism

In the present study, we included 4 of the most common SNPs in the VEGFA gene which were -2578, -460, +405 and +936. VEGFA-2578 has a novel insertion/deletion of 18 nucleotides with the sequence 5ô-TCCCACTCTTCCCACAGG-3ô (accession number AF098331) starting from -2549 relative to the translation start site (Brogan et al, 1999). All these polymorphisms may possibly influence the regulation of gene expression at the transcriptional and/or translational level. In the promoter region, there is another common polymorphism named VEGFA-1154 (after its position relative to the translation start site, rs1570360). However, we did not include it in this study since it is close to and is flanked by VEGFA-460 and +405. Also, we did not include the polymorphisms within the coding region of the gene, since all of the common ones are intronic, and therefore are not likely to have a large impact on alteration of protein structure/activity (Hapmap August 2010 phase II+III database).

A high degree of LD was detected across the 5ô-flanking region marked by VEGFA-2578, -460 and +405 in our population, which was in close agreement with previous observations in other populations (Yang et al, 2010; Attar et al, 2010). The 3ô-flanking region, marked by VEGFA+936, was not linked to the 5ô-flanking region.

Allelic and/or genotypic frequencies of VEGFA-2578, -460 and +405 in healthy volunteers of UK origin from a nearby region (Manchester) were reported before (Brogan et al, 1999;

Watson et al, 2000). We compared the figures to those in our RA population and found no significant difference, which implied that polymorphisms in the promoter region and the 5ô-UTR are not associated with RA susceptibility in UK population. It was consistent with the data in 2 previous studies based on a Korean and a Spanish population respectively (Han et al, 2004; Rueda et al, 2005).

In the Korean study, it was indicated that the 3ô-UTR SNP, VEGFA+936, was associated with susceptibility to RA (Han et al, 2004). We were unable to test this on our UK population. In contrast, recent large GWAS revealed no association between SNPs within the VEGFA gene and susceptibility to RA, either in Caucasians or eastern Asians.

5.4.2 Serum level of VEGF-A and other angiogenic factors

The levels of VEGF-A in the circulation are primarily dependent on the cellular release of VEGF-A which then enters the peripheral blood. Circulating VEGF-A binds to VEGF Receptors (mainly VEGFR2) on vascular endothelial cells, triggering a tyrosine kinase pathway leading to angiogenesis. Since this action takes place in the blood vessel, the levels in the circulation may be more representative than those restricted to particular cell type. In this study, we investigated the concentration of VEGF-A in serum. To determine the levels, we used the Luminex suspension array system which has higher sensitivity compared to older methods for quantitative detection (e.g. ELISA).

It has been suggested that angiogenesis is more active during the early stage of RA compared to that in the established disease since the expansion of abnormal tissue mainly occurs in the initial pathologic progress and therefore requires new vessels to deliver

oxygen, nutrients, different kinds of molecules (e.g. growth factors, cytokines, etc) and blood-derived inflammatory cells (e.g. macrophage, B, CD4+ T lymphocytes, etc). In the present study, we did not find an association of VEGF-A levels with RA duration, suggesting that the above difference was not directly affected by the levels of VEGF-A in the circulation. However, angiogenesis is a complex mechanism that involves several pathways and is also dependent on other angiogenic factors and the local micro-environment.

The serum levels of VEGF-A have been included as a marker in RA studies. However, there have been few studies concerning the serum levels of other angiogenic proteins. Furthermore, there has been no research on simultaneous measurement of the group of angiogenic proteins, with evaluation of their relationships. In the present study, we also measured other angiogenic factors simultaneously. Interestingly, it was found that PDGF-BB was negatively correlated with RA duration, with a very strong relationship ($r = -0.27$, $p < 0.0001$, adjusted for sex and age). In addition, angiopoietin-2 and leptin also had negative relationships with RA duration ($r = -0.12$, $p = 0.054$ and $r = -0.14$, $p = 0.079$ respectively, adjusted for sex and age), although they were not significant. These negative relationships suggested that the levels of PDGF-BB, angiopoietin-2 and leptin were the highest in the early stage of RA, and therefore suggested that these factors, in particular PDGF-BB, may be responsible for the higher activity of angiogenesis in earlier RA. PDGF-BB is derived from platelets. We observed a very strong association of platelet counts with PDGF-BB levels ($p < 0.0001$, adjusted for age and sex) in our patients. Further, we found that platelet counts also had a negative relationship with RA duration ($p = 0.054$, adjusted for age and sex) although it was not significant. These results suggest that platelets may be important in early disease.

Angiogenic factors, which were measured in this study, were highly correlated with each other, although there were exceptions. It appeared that these factors were all involved in a single pathway or pathways depending on each other. However, several independent pathways for individual angiogenic factors have already been determined. Abnormality in a certain pathway may lead to increased release of a particular angiogenic factor, which may induce angiogenesis and therefore play a role in promoting RA. However, the situation in which several abnormal pathways occur at the same time and in the same direction is rare. Our observation may be better explained by RA-induced hypoxia stimulating several angiogenesis pathways at the same time, leading to strong correlations between angiogenic factors. Thus, it is more likely that angiogenesis is a consequence of the disease rather than a cause/promoter.

5.4.3 VEGFA polymorphism and serum VEGF-A level

The regulatory region of the VEGFA gene contains a number of transcription factor binding sites, and the transcriptional regulation of this gene appears to be extremely complex, with levels of control at the transcriptional and translational level (Stein et al, 1998; Akiri et al, 1998). Therefore, polymorphisms within the regulatory region may lead to differences in VEGF-A expression between individuals and could potentially contribute to a variety of pathological processes where VEGF-A is important.

According to the literature based on non-RA subjects, several common SNPs including VEGFA-2578, -460, +405 and +936 have been associated with differential expression of VEGF-A protein (Renner et al, 2000; Awata et al, 2002; Stevens et al, 2003; Koukourakis et al, 2004; Watson et al, 2000; Prior et al, 2006; Petrovic et al, 2008; Steffensen et al,

2010; Chen et al, 2011; Zhai et al, 2007). In the present study, for the first time in a RA population, we showed that VEGFA-2578 SNP was associated with the serum levels of VEGF-A, with AA associated with the lowest levels. Although haplotype analysis did not increase the significance of association, it was suggested that patients carrying A-C-G (-2578/-460/+405) tended to have lower VEGF-A levels. Our finding thus was in agreement with the majority of previous studies which included polymorphisms within the 5ô-flanking region of the VEGFA gene (Petrovic et al, 2008; Steffensen et al, 2010; Watson et al, 2000; Prior et al, 2006; Awata et al, 2002; Stevens et al, 2003; Zhai et al, 2007). However, there were 2 reports demonstrating an opposite trend (Koukourakis et al, 2004; Chen et al, 2011). For the 3ô-flanking SNP, our data was in disagreement with the reports which indicated that VEGFA+936 was associated with the levels of VEGF-A (Renner et al, 2000; Zhai et al, 2007). However, it is acknowledged that, in this SNP, the minor/major allele proportion was not ideal, with T allele only being 15.7%. To give a better evaluation, a larger population would be required.

The inconsistency between studies may be due to the fact that the studied subjects had different conditions. It is known that VEGF-A expression is influenced by a variety of growth factors, cytokines, hormones and hypoxia (Ferrara et al, 2003; Shweiki et al, 1992; Liu et al, 1995) apart from genetic regulation. *In vitro*, Watson et al found that VEGFA+405 GG genotype was associated with the highest VEGF-A protein production in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) but not in cobalt-stimulated cells (Watson et al, 2000), suggesting that the relation between VEGFA 5ô-flanking variants and VEGF-A expression is conditional. *In vivo*, interestingly, evidence of association between VEGFA 5ô-flanking polymorphisms and VEGF-A levels was only obtained in diseases associated with conditions such as inflammation and hypoxia

(Stevens et al, 2003; Koukourakis et al, 2004; Prior et al, 2006; Petrovic et al, 2008; Steffensen et al, 2010; Chen et al, 2011; Zhai et al, 2007), and no such association was observed in healthy individuals (Lambrechts et al 2003, Prior et al, 2006). These findings raise the possibility that conditions that arise during the disease state (e.g. inflammation, hypoxia) may have a differential effect on gene expression of particular VEGFA alleles.

In the present study, we found evidence that the association of VEGFA-2578 with serum levels of VEGF-A may be depend upon inflammatory status (stratified by CRP status, < 10 vs. Ó 10 mg/l). In patients with acute myocardial infarction (AMI), a study focusing on the time course of release of VEGF-A found that the serum levels of CRP peaked on day 3 after admission and the serum VEGF-A peaked on day 7. The CRP on day 3 was correlated with the VEGF-A on day 7 (Soeki et al, 2000). This delay may imply a responsive expression of VEGF-A to inflammation. CRP is an inflammation marker in blood, reflecting the strength of the underlying inflammation. It is influenced by few known factors (apart from liver failure). Although < 1 mg/l is ideal, clinical diagnosis usually identifies a level < 10 mg/l as normal and a level Ó 10 mg/l as presentation of obvious inflammation. We saw no difference in distribution of VEGFA-2578 genotypes between 2 groups stratified by CRP status, indicating no association of VEGFA-2578 with inflammation. These 2 factors thus were independent of each other, but interestingly they seemed to play roles together as a combination. It was seen that serum VEGF-A was increased only in patients carrying the VEGFA-2578 CC genotype and having obvious inflammation. The correlation analysis further demonstrated that the correlations of serum VEGF-A with inflammatory markers (CRP and ESR levels) only occurred in subjects carrying VEGFA-2578 CC, implying a genotype/allele specific relationship. The promoter sequence containing the C allele at position -2578 may be more sensitive to inflammation

(or related conditions), and thus may up-regulate VEGF-A expression in response to increased inflammation (or related conditions). The VEGFA-2578 C allele is associated with a deletion of 18 nucleotides. Although it has been established that single-base-pair changes within the regulatory DNA sequences of a gene can significantly alter the level of its expression, it is more likely that the 18 nucleotides insertion/deletion plays a functional role. It would be of great interest to investigate the functional role of the 18 nucleotides insertion/delection in response to inflammation (or related conditions). In addition, other SNPs linked with VEGFA-2578 may also be involved, including the rare ones that have not been studied.

It has been known that an element in the 5ô-flanking region of the VEGFA gene is sensitive to hypoxia (Minchenko et al, 1994). Further study indicated that a 28 bp element within the human VEGFA promoter, believed to contain a consensus binding site for HIF-1, was necessary and sufficient to increase transcription of a CAT reporter gene in endothelial cells cultured under hypoxic conditions (Liu et al, 1995). Although we were unable to test the effect of the VEGFA-2578 polymorphism (or other related/linked polymorphisms) on VEGF-A expression under hypoxic conditions, we suggest that increased inflammation is somehow related to elevated hypoxia since swelling caused by inflammation increases the distance between cells/tissues and therefore the burden of oxygen delivery. Abnormal activation or deregulation of hypoxia-induced transcriptional pathways may contribute to the pathogenesis of conditions including inflammation, vascular disease and cancer (Melillo et al, 2004). It seems that there is a regulatory loop between hypoxia and inflammation.

Our results were based on the analysis stratified by CRP status. However, CRP may not be directly involved itself. In RA, VEGF-A release has been known to be positively associated with several important pro-inflammatory cytokines including TNF-Ŭand IL-1Ŭ(Klimiuk et al, 2004). Whether these cytokines are playing the primary roles in signalling remains to be clarified.

5.4.4 Serum VEGF-A, serum angiopoietin-2 and RA clinical measures

Both serum and synovial levels of VEGF-A have been shown to be increased in patients with RA, compared with osteoarthritis patients or healthy controls (Lee et al, 2001; Ozgonenel et al, 2010). Several studies have demonstrated associations between serum VEGF-A levels, and inflammatory markers and disease activity (Lee et al, 2001; Ozgonenel et al, 2010; Kurosaka et al, 2010; Klimiuk et al, 2004). In the present study, the serum VEGF-A was correlated with inflammatory markers including CRP and ESR. Specifically, such correlations were restricted to patients carrying VEGFA-2578 CC genotype, which suggested a genotype/allele specific relationship responding to inflammation (as discussed). This is a novel finding and implies a potential mechanism which may be important in many disorders involving angiogenesis and inflammation.

Serum VEGF-A was correlated with the major destructive enzymes in the MMP family including MMP-1, -3, -8 and -9. In particular, MMP-1 and -3 are believed to play significant roles in joint destruction in RA. The correlation with MMP-3 has been described (Kurosaka et al, 2010), but the correlations with serum MMP-1, MMP-8 and MMP-9 have not been reported.

We confirmed the previous finding that serum VEGF-A was associated with disease activity (baseline data), and further indicated that it was also associated with functional outcome (measured by HAQ and MJS, baseline data). It has been shown that serum levels of VEGF-A at presentation could predict radiographic damage over the subsequent year in early RA (Ballara et al, 2001; Taylor 2005). It would be also interesting to see whether serum VEGF-A at baseline could predict disease activity over time and thus could predict a functional outcome for a longer period of time. Our initial results (based on 296 patients) showed that serum VEGF-A was associated with the mean time-averaged DAS28 over 24 months (baseline, 12^{th} month and 24^{th} month, $p = 0.034$, adjusted for age, sex and RA duration), and was associated with HAQ ($p = 0.0040$, adjusted for age, sex and RA duration) and MJS ($p = 0.0059$, adjusted for age, sex and RA duration) at the 24^{th} month.

The patients recruited to this study were being followed over a 5-year period, so future analysis will be able to better assess the long-term predictive role of serum VEGF-A. We were unable to assess the relationship between serum VEGF-A and joint destruction, since the measures describing radiographic damage were not available. However, we considered that severity indices measuring overall health and functional status were more appropriate than those restricted to joint changes, in terms of quality of life of patients.

In addition to serum VEGF-A, several other angiogenic factors, including angiopoietin-2, HGF, C-CSF, IL-8, PDGF-BB and PECAM-1, were found to be associated with one or more disease measures, adjusted for age, sex, RA duration and serum VEGF-A (data not shown). In particular, serum levels of angiopoietin-2 showed relationships with all the disease indices including ESR ($p = 0.050$), CRP ($p = 0.064$), DAS28 ($p = 0.0053$), MJS ($p = 0.039$) and HAQ ($p = 0.017$), although for CRP it was not significant. In RA, no study

concerning the levels of serum angiopoietin-2 has been reported, except for one that indicated a correlation with CRP level (Kurosaka et al, 2010). In this study, we added the association with disease activity and functional outcome, and therefore provided further evidence showing that angiopoietin-2 appeared to also have a particular clinical significance in RA. Furthermore, serum levels of this protein at baseline showed associations with mean time-averaged DAS28 over 24 months, MJS at 24th month and HAQ at 24th month, suggesting a predictive role in disease severity (data not shown). Further work is required to confirm these relationships.

5.4.5 VEGFA, MMP polymorphism and age at onset of RA

Genetic polymorphisms in the IL8 gene (Emonts et al, 2011), and in the PTPN22 gene (the R620W SNP, Kokkonen et al, 2007) have been associated with age at onset of RA. In females only, a similar association was found with the variant in the estrogen receptor (ER) gene (Ushiyama et al, 1999). Polymorphism in the tumour necrosis factor ligand superfamily member 11 (TNFSF11) gene has also been found to be associated with age at onset of RA among 3 independent cohorts, although the association was restricted to seropositive patients (Tan et al, 2010). In an earlier report from the same research group, it was suggested that the above association was possibly due to an interaction with HLA-DRB1 (Wu et al, 2004). In the present study, we observed another 2 polymorphisms (VEGFA+936 and MMP1-3 intergenic rs495366) that were associated with age at onset of RA in Caucasians. The T allele at VEGFA+936 and the AA genotype at rs495366 were associated with earlier age at onset. The mechanism behind these associations remains to be clarified. VEGFA+936 has been associated with RA susceptibility in a Korean case-control study (Han et al, 2004). In a GWAS, rs495366 has been shown as the strongest

genetic marker for serum levels of MMP-1 in healthy subjects (Cheng et al, 2009). In disagreement with Kokkonen et al 2007, we did not find any association between PTPN22 R620W and age at onset of RA.

In addition, we also showed that sex and smoking status had an effect. Interestingly, our result showed that ever-smoking is associated with a later onset of disease, in line with a previous study by Hutchinson et al. In their report, the results showed that current-smoking was associated with a later onset of disease in sporadic patients with RA, but not in familial patients (Hutchinson et al, 2001). Diaz et al has shown that the presence of anti-CCP is associated with earlier age at onset of RA (Diaz et al, 2011). Our preliminary data showed similar results (anti-CCP+ vs. anti-CCP-, 48.3 ± 12.3 vs. 52.7 ± 14.8, $p < 0.0001$). However, the development of anti-CCP antibodies in some patients may have occurred after the onset of disease. Thus, we further adjusted for RA duration, after which the association disappeared.

5.4.6 VEGFA polymorphism and smoking addiction

In 2 independent cohorts of RA, we observed an interesting association between a higher proportion of smokers and the G allele of VEGFA+405. This suggests that genetic variants in the VEGFA gene may be somehow related to tobacco addiction. However, our finding was only seen in populations with RA, with no data being available on the general population. This observation might be explained by smoking and VEGFA+405 G being risk factors for the development of RA. There is no doubt about smoking, but the G allele of VEGFA+405 has never been reported as a risk factor. However, it is possible that only

the combination of smoking with the VEGFA+405 G allele provides a risk for RA. Comparison with a control population is needed to test this.

Recently, a nicotine receptor gene called neuronal acetylcholine receptor subunit alpha-5 (CHRNA5) was found to be associated with tobacco addiction (Weiss et al, 2008). This gene is located on chromosome 15, whereas the VEGFA gene is located on chromosome 6. Thus, our observation cannot be explained by linkage between the CHRNA5 and the VEGFA genes. However, it is possible that VEGFA+405 SNP is linked with some gene which has an impact on tobacco addiction. In this regard, Füst et al found a highly significant association between ever-smoking and a specific haplotype (RCCX modules and TNFA promoter SNPs) containing genes within the HLA class III region on chromosome 6 (Füst et al, 2004). Otherwise, the VEGFA gene could be a novel candidate gene associated with tobacco addiction.

5.4.7 VEGFA polymorphism and RF production

No studies concerning the relationship between VEGFA polymorphism and the production of RF have been reported. In the present study, we found that the G allele of VEGFA+405 may play a protective role against RF production in patients who had never smoked, whereas this effect was largely reduced in those who had ever smoked. It is possible that smoking disturbs the normal regulatory pathway. It was noted that in the absence of smoking, the difference in RF+ frequency between patients carrying at least 1 copy of the G allele and those carrying the CC genotype was dramatic (39.7% vs. 73.7%). We thus hypothesized that the VEGFA gene was also involved in inflammation and/or auto-immunity in relation to RF release. Since the majority of RA patients were smokers

(66.6%) and smoking reduced the effect significantly, this relationship would be easily masked in the whole population. Furthermore, the finding that the G allele of VEGFA+405 was associated with higher frequency of smokers (as discussed above) would also reduce the impact of this relationship in the whole population.

5.4.8 VEGFA polymorphism and RA clinical measures

In the present study, we described a relationship between VEGFA polymorphism and disease activity in RA, which has not been reported previously. The results indicated that the VEGFA-2578 polymorphism was significantly associated with disease activity measured by DAS28, with the AA genotype associated with the lowest score. Other polymorphisms within the 5ô-flanking region (-460 and +405) also showed relationships (insignificant trends), which may be due to the linkage with the -2578 SNP, and therefore may not have a direct functional effect. Alternatively, a number of polymorphisms within the promoter and 5ô-UTR may play roles based on the complexity of transcriptional regulation of the VEGFA gene (Stein et al, 1998; Akiri et al, 1998). Haplotype analysis indicated that the most frequent haplotype (48.3%), A-C-G (-2578/-460/+405), was associated with lower disease activity. In these patients, we showed that the AA agenotype of VEGFA-2578 (or the A-C-G haplotype) was associated with lower VEGF-A in the circulation. Thus, the protective role provided by the AA genotype of VEGFA-2578 (or the A-C-G haplotype) may be due to the impaired release of VEGF-A protein. Interestingly, a multivariate multiple regression containing VEGFA-2578 SNP and serum levels of VEGF-A as independent variables together demonstrated that their associations with disease activity were independent of each other. This implied that polymorphism in the 5ô-flanking region of the VEGFA gene may play some additional role leading to a differential disease

activity apart from its role in regulation of VEGF-A expression. However, this observation may possibly be explained by the regulatory effect being non-linear.

More interestingly, we further determined that the above association occurred only in patients who had never smoked, whereas in those who had ever smoked the DAS28 was no different amongst AA, AC and CC at VEGFA-2578. We therefore speculate that the AA genotype at -2578 has a protective role against high disease activity under normal conditions (absence of smoking), while in the abnormal environment (presence of smoking) such a protective mechanism is blocked. In non-smokers only, significant association of the AA genotype with DAS28 over a 2-year period was found, independent of serum levels of VEGF-A, MMP-1 and -3. Thus, the VEGFA-2578 polymorphism may be useful as a marker to predict future overall disease activity in non-smokers. In addition, it was interesting to see that the associations of serum levels of VEGF-A, MMP-1 and -3 with disease activity were much more significant in patients who had never smoked, compared to those who had ever smoked. Again, the reason behind these observations is not clear.

In the present study we also showed that VEGFA-2578 SNP had a relationship with HAQ, although it was insignificant after adjustment for RA duration. In haplotype analysis, patients with A-C-G (-2578/-460/+405) tended to have lower HAQ (without adjustment). Our results thus demonstrated some initial but interesting observations that polymorphism in the VEGFA gene may influence RA severity including disease activity and functional outcome.

5.4.9 VEGFA polymorphism, smoking and IHD/MI

We found that SNPs in the VEGFA promoter region (-2578 and -460) were associated with an increased risk of IHD, and particularly MI, in patients with RA. Data also suggested that another SNP (+936), in the 3ô-UTR of the VEGFA gene, was not involved in this association. The VEGFA-2578 polymorphism showed the strongest association, suggesting that it may provide the primary association where the A allele increased the risk and/or the C allele played a protective role. These results were consistent with the findings of Howell et al, who showed that the -2578 AA genotype was a risk factor, while the CC genotype was protective in the development of atherosclerosis (Howell et al, 2005). Similarly, an association between the -2578 AA genotype and the severity of coronary artery disease has been shown in a Brazilian population (Biselli et al, 2007). It has been shown in this study that the -2578 CC genotype is associated with higher levels of serum VEGF-A compared to the AA/AC genotypes, which adds weight to the suggestion that this genotype is associated with a protective effect due to greater production of VEGF-A (Howell et al, 2005). Comparison of the serum levels of VEGF-A between patients with and without IHD/MI indicated that there was no significant difference, although there was a slight reduction in MI patients. However, it is understandable that serum levels at presentation may be not associated with previous cardiac events. A detailed time course of VEGF-A release may help to better evaluate the relationship between VEGF-A levels and cardiac events. In addition, treatment for IHD/MI may also have an effect.

As mentioned before, the VEGFA-2578 has an unusual insertion/delection of 18 nucleotides, with the A allele associated with the insertion. This insertion could be responsible for this increased risk. Thus, it would be of great interest to investigate the

functional importance of this insertion further. Again, a number of polymorphisms within the 5ô-flanking region may be involved including -460, +405 and other polymorphisms that have not been studied.

We further observed a strong interaction between VEGFA SNPs (-2578 and -460) and smoking relative to IHD and especially MI. Our data suggested that the strongest interaction was between the VEGFA-2578 A allele and smoking, and it was particularly noteworthy that the risk of IHD or MI was increased only in patients carrying this combination. Carriage of 1 factor (either smoking or the -2578 A allele) only was not sufficient to provide an increased risk. Further, > 60% of the ñexcessò risk for IHD and > 75% of the ñexcessò risk for MI were directly attributable to the gene-smoking interaction. In patients carrying the VEGFA-2578 A allele, the amount of smoking quantified in pack-years demonstrated a dose effect with regard to the occurrence of IHD and MI. In contrast, in the absence of the -2578 A allele there was no such relationship between smoking and IHD and MI, suggesting a possible protective effect of the -2578 CC genotype.

The kind of interaction demonstrated here has been described before in relation to the increased risk of esophageal adenocarcinoma (Zhai et al, 2008). There is evidence that the combination of VEGF-A and certain chemical constituents in cigarette smoke may be important in particular biological pathways related to angiogenesis, atherosclerosis and inflammation. Studies on nicotine have demonstrated that it can induce VEGF-A expression via several pathways involving nicotinic acetylcholine receptors (Egleton et al, 2009; Martin et al, 2009), Ův6 integrin (Martin et al, 2009) and angiotensin-converting enzyme (Saijonmaa et al, 2005). However, investigation of nicotine only is not sufficient to understand the pathogenic effect of cigarette smoking, since other components certainly

play a role. In contrast, cigarette smoke exposure, overall, has been shown to inhibit VEGF-A expression through decreased expression of hypoxia-inducible factor-1Ŭ (HIF-1Ŭ) (Michaud et al, 2003). In CVD, smoking has a causal role in the development of atherosclerotic changes in the various vascular bed and effects on acute thrombosis of the narrowed vascular lumen (Burns et al, 2003). Meanwhile, VEGF-A is involved in the repair of damaged vessels by acting on vascular endothelial cells and promoting proliferation and migration of endothelial cells, and lumen formation (Neufeld et al, 1999). Thus, smoking not only directly damages vessels but also has an effect resulting in impaired expression of VEGF-A which inhibits this protein from repairing damaged vessels. Furthermore, it has been hypothesized that the ability of the coronary artery collateral circulation to protect against myocardial ischemia is strongly associated with the ability to induce VEGF-A in response to hypoxia (Schultz et al, 1999). As mentioned, Michaud et al indicated that smoking could reduce the expression of HIF-1Ŭ (Michaud et al, 2003) and therefore weakened the ability to induce VEGF-A, under hypoxic condition.

Studies focusing on the VEGFA gene have indicated that an element in the promoter region of the gene is important in relation to hypoxia response (Liu et al, 1995; Pages et al, 2005). Therefore, variation in DNA sequence could potentially alter the responsive ability. Recently, it has been demonstrated that haplotypes that include the -2578 and +405 SNPs influence VEGF-A expression in human myoblasts under hypoxic condition (Prior et al, 2006). In the present study, although we were unable to test the effect of hypoxia, we observed that the -2578 polymorphism may show differential expression of VEGF-A in response to inflammation. It has also been discussed in this thesis earlier that there may be a regulatory loop between inflammation and hypoxia. Taken together, we speculate that the

association of IHD/MI with the VEGFA gene-smoking interaction in our study might be related to a mechanism involving differential response to hypoxia.

The associations of VEGFA polymorphisms with IHD and MI were examined only in an RA population. We do not know therefore whether similar associations exist in the general population or whether this is somehow related to the disease process in RA. If the mechanism behind this requires conditions such as hypoxia and inflammation, the kind of association would be only easily seen in populations with those conditions and the effect would be expected to be weaker in the normal population. Several publications have suggested an association of VEGFA polymorphisms with certain cardiovascular disorders (Howell et al, 2005; van der Meer et al, 2005; Ripa et al, 2009; Kangas-Kontio et al, 2009; Petrovic et al, 2007; Biselli et al, 2008; Petrovic 2009; Vannay et al, 2006), but as far as we aware none have looked at interaction between smoking and VEGFA polymorphisms. Smoking is a risk factor for RA, thus a greater proportion of smokers in the RA population is expected, which provides an advantage in the investigation of smoking-involved associations/interactions. To better evaluate this interesting finding, further studies in other RA and non-RA patients are needed.

There are some limitations. First, the number of patients with IHD and/or MI was relatively small, so further studies, preferably with a larger number of cases, will be needed to confirm these results. However, the ORs achieved in the present study were at a very high level, especially for risk of MI, and especially when gene-smoking interactions were examined. Based on our results there was more than sufficient power (> 80%, a type I error rate of 0.05) to achieve the sort of effect sizes we were seeing. Another limitation is the possibility that some patients with silent, non-full-thickness or atypical MI were missed.

However, we were able to identify 13 patients with previously unrecognized MI who had ECG evidence of a full-thickness MI. In a small number of patients (n = 8) there was a bundle branch block severe enough to prevent assessment. In the analyses these were classed as not having IHD. Exclusion of these patients made no significant difference to the associations found (data not shown). More tests to try and identify other silent MI, such as the cardiac stress test, would not be appropriate in many patients with RA because of their physical limitations. The incidence of MI reported in our RA population is comparable with other studies in this field (Maradit-Kremers et al, 2005; Panoulas et al, 2008). Finally, the study was cross-sectional in design, so it was possible to assess only patients who had survived previous cardiac events. However, the patients recruited to this study are being followed over a 5-year period. Therefore, future studies will be able to assess both fatal and nonfatal cardiac events, as well as further development of IHD in this population.

5.4.10 VEGFA polymorphism and hypertension

VEGFA-2578 polymorphism appeared to be associated with hypertension in this RA population, especially in RF+ subjects. Further adjustment including IHD/MI indicated that it may be a secondary relationship due to the association of VEGFA polymorphism with IHD/MI. However, the development of hypertension usually occurs prior to cardiovascular events, and is considered as a risk factor for preclinical atherosclerosis. More generally, our results may suggest that the VEGFA-2578 polymorphism is a risk factor for a co-morbid combination consisting of IHD/MI and hypertension. It was noted that several co-morbidities in RA were highly associated with each other, and for some particular pairs they tended to co-exist. The presence of one disease may predispose to

another. Identification of several particular co-morbid combinations may help to understand the pathological mechanism, to improve the patients care and treatment, and to better evaluate the impact from these combinations on disability and early mortality of RA.

5.4.11 Differential effects of VEGFA polymorphism in RA and in CVD

We observed that the A allele at VEGFA-2578 was associated with lower disease activity of RA (and maybe with less severe functional outcome), whereas the same allele was also shown to be associated with higher occurrence of IHD and MI (as RA co-morbid conditions). We suggest that this allele may be associated with reduced VEGF-A-induced angiogenesis which tends to play a protective role against RA and a pathological role promoting IHD/MI. Most interestingly, we observed the protective effect (on RA) only in non-smokers, and the risk effect (on IHD/MI) only in ever-smokers. Based on this single cohort, our results have shown that a genetic polymorphism could have different effects in different diseases and among different subsets stratified by ever-smoking status.

5.5 ENOS POLYMORPHISM, SEVERITY AND CO-MORBIDITY IN RA

5.5.1 ENOS polymorphism

There are several common genetic polymorphisms in the eNOS gene, including -1468 (A/T), -922 (A/G) and -786 (C/T) SNPs in the promoter region, the 4a/b VNTR polymorphism (a 27-bp tandem repeat-based polymorphism) in intron 4, and the +894 (G/T) SNP in exon 7. It has been shown that the former 4 polymorphisms are in LD, with

the former 3 in absolute linkage, while the +894 SNP is not linked with others (Jo et al, 2006). The -786 and 4a/b VNTR polymorphisms have been associated with RA susceptibility in a German population (Melcher et al, 2007) and in a Greek population living in the island of Crete (Vazgiourakis et al, 2007) respectively, while no research regarding the relationship of +894 SNP with RA susceptibility has been published. It would be interesting to know whether the missense mutation is associated with the development of RA.

5.5.2 ENOS polymorphism and RA clinical measures

Previously, a Brazilian report indicated that the -786 SNP was associated with extra-articular manifestations in RA (Brenol et al, 2008). Although it was insignificant, in the present study we found that patients carrying the T allele of the +894 SNP were associated with increased incidence of nodular disease, with the carriage of 2 copies associated with the highest risk (GG: 19/161 (11.8%) vs. GT: 25/178 (14.0%) vs. TT: 10/55 (18.2%), p trend = 0.24). However, since the +894 SNP is not linked with the -786 SNP, these results may be not comparable. Apart from this, we did not find any association of eNOS+894 SNP with RA clinical measures, suggesting that the +894 polymorphism does not have a significant clinical impact on RA progress.

5.5.3 ENOS polymorphism, smoking and IHD/MI

In the general population, the association between polymorphisms in the eNOS gene and cardiovascular disorders has been evaluated. Polymorphisms studied mainly included -786 and +894. Interestingly, in studies based on Caucasian populations, the majority of them

demonstrated that +894 was the important risk variant, while -786 was not relevant to disease phenomena (Gulec et al, 2008; Velloso et al, 2010; Cam et al, 2005; Casas et al, 2004; Aroon et al, 1998; Hibi et al, 1998). In contrast, in Asian populations the trend was opposite, with the -786 SNP being the more important disease-associated polymorphism (Jo et al, 2006; Nakayama et al, 2003). In the present study, we therefore selected +894 to assess the relationship with IHD and MI. We found that the TT genotype was associated with an increased risk of IHD and MI in patients with RA, although this was not significant after further adjustment. Our observation was in agreement with others, showing the T allele as the risk allele. We assume that the insignificant result here may be due to the relatively small number of IHD and MI patients, since the literature suggested that the +894 SNP only provided a moderate risk. Our preliminary data provided information that there was an effect from the eNOS+894 SNP on the development of IHD/MI in RA, which may be similar to the effect in the general population. However, in order to confirm this, further research with sufficient RA-IHD/MI patients is needed.

Several previous reports indicated that the association of eNOS polymorphism with CVD (or related conditions) was, at least partly, due to interaction with cigarette smoking (Leeson et al, 2002; Mayer et al, 2010; Lee et al, 2006; Jo et al, 2006; Nakayama et al, 2003). We looked at the interaction in relation to the incidence of IHD/MI in our RA patients. Although the 95% CI for AP was not statistical significant, the AP itself took a significant part out of the total effect (30% for IHD and 55% for MI), implying possible interactive effects.

The mechanism behind the association remains to be clarified. However, it has been reported that the +894 SNP differentiates the enzymatic activity and protein level (in

postpartum placentas), and subsequently the NO production. Whilst the eNOS protein levels were lower in the T allele, the eNOS enzyme activity was significantly higher in the same allele (Wang et al, 2000). More interestingly, the same study also showed that smokers had lower eNOS protein levels in both alleles, and that smoking reduced the eNOS activities only in the subjects with the T allele (Wang et al, 2000). The latter allele-specific relationship may help to explain the (possible) interaction observed in the present study. It was noteworthy that, as mentioned before, reduction in basal NO is associated with increased risk of atherosclerosis. Since NO is an upstream and downstream regulator of VEGF-A mediated angiogenesis (Suganthalakshmi et al, 2006), and in the present study we observed a much stronger interaction of smoking with VEGFA polymorphism, we hypothesize that the (possible) interaction here may be somehow related to that stronger interaction.

5.6 MMP POLYMORPHISM, SERUM MMP LEVEL, SEVERITY AND CO-MORBIDITY IN RA

5.6.1 MMP polymorphism

The most studied polymorphisms in the MMP1-3 loci are rs3025058 and rs1799750, commonly called MMP3 5A/6A and MMP1 1G/2G respectively. These 2 polymorphisms have been demonstrated to influence the transcriptional activity (Rutter et al, 1998; Ye et al, 1996; Zhu et al, 2006) and therefore the expression of these enzymes. In the present study, apart from the above polymorphisms, we included another 2 SNPs designated rs679620 and rs495366. SNP rs679620, located in exon 2 of the MMP3 gene, results in an

amino acid change (Glu Ÿ Lys) at residue 45. Based on the Hapmap August 2010 phase II+III database, this variant is the only common SNP across the coding region of the MMP3 gene which leads to an amino acid alteration in the CEU population (Utah residents with Northern and Western European ancestry). SNP rs495366 is an intergenic polymorphism of the MMP1 and 3 genes. A recent GWAS on the investigation of the relationship between genetic variants and serum levels of MMP-1 in healthy subjects showed this SNP as the strongest marker (Cheng et al, 2009). Although the mechanism of action remains to be elucidated, there is more and more evidence suggesting the roles of intergenic non-coding DNA sequence in expressional regulation (Lander, 2011). We also searched polymorphisms within the coding region of the MMP1 gene. According to the Hapmap August 2010 phase II+III database however, no missense variants were found and thus no further polymorphisms were included.

Our analysis showed that the MMP3 SNPs rs3025058 and rs679620, and the intergenic SNP rs495366 were in the same LD block. Furthermore, pair-wise correlation of genotypes between rs3025058 and rs679620 was very high, indicating these 2 polymorphisms were almost identical (almost in complete LD). This was comparable with the allelic information presented by Beyzade et al (Beyzade et al, 2003). MMP1 rs1799750 demonstrated a moderate linkage with the LD block (D' = 0.44), which was in agreement with a study which included rs1799750 and rs3025058, and showed a moderate LD between them with D' = 0.45 (Dörr et al, 2004).

Several studies have examined the association of MMP1 rs1799750 and MMP3 rs3025058 with the development of RA (Constantin et al, 2002; Dörr et al, 2004; Rodriguez-Lopez et al, 2006; Scherer et al, 2010). No significant association has been found between these

polymorphisms and susceptibility to RA, apart from one study which found a lower frequency of the 5A allele at rs3025058 in RA patients compared with controls (Scherer et al, 2010). Moreover, there were also no relative associations in the GWAS. These suggest that MMP1 and 3 genes may be not significant susceptibility genes for RA.

5.6.2 Serum level of MMP

Some correlations between MMP levels were found. MMP-8 and MMP-9 were very highly and significantly correlated with each other ($r = 0.70$, $p < 0.0001$), consistent with the results presented by Tchetverikov et al (Tchetverikov et al, 2004). It is known that the genes for these 2 enzymes are located on different chromosomes (MMP8: chromosome 11; MMP9: chromosome 20). Thus, the correlated expression is unlikely to be due to genetic linkage or other effects at the genetic level. It may be a reflection of the biological roles of these 2 proteins. It is possible that they act in a co-operative way and/or they are stimulated by the same factor(s). We also found a moderate ($r = 0.20$) but very significant ($p < 0.0001$) correlation between MMP-1 and MMP-3. We speculate that the content of LD between MMP1 and MMP3 genes may limit the strength of this correlation, since in the present study we showed that common haplotypes generally were associated either with high MMP-1/low MMP-3 or with low MMP-1/high MMP-3 (Table 4.17.4b). We speculate further that this genetic arrangement (selected during evolution) may reduce the chance of over-production of both destructive enzymes and therefore could be protective in humans.

The relationship between serum MMP levels and smoking was evaluated in this study. Interestingly, we found that some MMP levels were changed particularly in current smokers, while there was not much difference between non-smokers and past smokers.

This observation contrasts with several previous findings suggesting that the influence of smoking has a chronic effect on autoantibody and cytokine production (Costenbader et al, 2006; Glossop et al, 2006). We speculate that, on the expression of MMPs, smoking may have an acute and short term effect.

5.6.3 MMP polymorphism and serum MMP level

In the present study, we have clearly demonstrated a relationship between important polymorphisms across the MMP1 and 3 genetic loci and the circulating levels of MMP-1 and -3 in patients with RA. To our knowledge we are the first group to have demonstrated an association of the rs1799750 with serum levels of MMP-1 in patients with RA. Rs1799750 is a deletion/insertion polymorphism where an additional guanine (G) creates an Ets binding site at -1607 bp in the MMP1 promoter. The resulting 2G allele has been shown to be associated with higher transcriptional activity in normal fibroblasts and in melanoma *in vitro* (Rutter et al, 1998). However, in the present study it was the 1G allele, rather than the 2G, that was associated with higher MMP-1 serum levels. Since we also found that the 1G allele of rs1799750 and the G allele of rs495366 were in LD our findings are consistent with the observation of Cheng et al who showed that the G allele of rs495366 is associated with higher serum levels of MMP-1 in healthy subjects (Cheng et al, 2009).

We confirmed the association of the intergenic rs495366 SNP with MMP-1 levels and have shown for the first time that MMP3 SNPs rs679620 and rs3025058, located in the same LD block, are also associated with MMP-1 levels, independent of the rs1799750 association. The G allele of rs495366 has been suggested to be the strongest marker associated with

high MMP-1 levels in a GWAS (Cheng et al, 2009). It is interesting that the rs495366 G allele is associated with either higher MMP-1 or higher MMP-3 levels, depending on which MMP1-MMP3 haplotype it is linked with. This suggests that this region has an important effect on modulating both MMP-1 and MMP-3 levels.

Although rs679620 and rs3025058 are functional polymorphisms in the MMP3 gene and any change of MMP-3 expression/activity may potentially influence MMP-1 release, there is currently no direct evidence to support such a relationship. The LD block identified in this study may extend to the promoter region of the MMP1 gene where there may be other polymorphisms which alter transcriptional activity.

Polymorphism rs679620 leads to an amino acid change (Glu Ÿ Lys) at residue 45 in MMP-3. Residue 45 is located just after the first alpha helix in the propeptide, in a region which is believed to be flexible. It has been suggested that the amino acid change at this residue could potentially alter its interaction with other amino acids in this region, and have an effect on MMP-3 activation. However, the Glu45 residue is not conserved across species, implying that mutations occurring in this residue are unlikely to be significantly vital to the function of enzyme (Beyzade et al, 2003). We mentioned before that the genotypic/allelic distributions of rs3025058 and rs679620 were almost identical and thus almost in complete LD, forming 5A-A and 6A-G as the major haplotypes. RA patients carrying 5A-A were associated with lower levels of MMP-3, whereas those carrying 6A-G were associated with higher levels. However, assessment of the relationship between MMP3 haplotype and level of MMP-3 only is not sufficient. To better understand the relationship between the genetic content of MMP3 and overall activity (amount +

individual activity) of MMP-3, investigation of whether SNP rs679620 indeed alters protein function is required.

The MMP3 promoter polymorphism rs3025058 has been reported to influence the transcriptional activity of MMP3, with the 5A allele being associated with higher transcriptional activity in IL-1ϐ stimulated human acute monocytic leukaemia (THP-1) cells (Zhu et al, 2006). However, in contrast to this *in vitro* study, the results of studies on patients with RA, in the current and 2 other studies (Mattey et al, 2004; Tsukahara et al, 2008), have consistently shown that the 6A allele is associated with higher serum levels of MMP-3. Similar contradictory results have also been shown in studies on patients with coronary heart disease and/or MI (Samnegard et al, 2005; White et al, 2007; Ghaderian et al, 2010). The reason for this discrepancy is still unclear, although in the case of MI patients it has been postulated that increased activation of pro-MMP-3 within the atherosclerotic vessel wall may potentially lead to decreased leakage of pro-MMP-3 and/or inactivated MMP-3 into the blood stream (Samnegard et al, 2005). A similar argument could be put for the inflamed synovial joint.

Serum levels of MMP-1 and -3 have been suggested to mainly reflect levels of inflammation in RA. In the present study, we found that in multivariate multiple regression models the CRP and MMP1-3 genetic polymorphisms were independently associated with MMP-1/MMP-3 levels, suggesting that circulating levels of MMP-1/MMP-3 were influenced by both inflammation and genetic factors in RA.

We also found that the genetic polymorphism at the MMP1-3 loci was associated with serum levels of MMP-2. Since the MMP2 gene is located on a different chromosome, it

appears that this relationship is not due to the LD between genetic loci. However, this association also could not be explained by the influences between serum MMPs, since multivariate multiple regression indicated that the association was independent of the correlations of MMP-2 with MMP-1 and -3. Thus, it is possible that there may be an unknown regulation mechanism linking the MMP1-3 loci and the level of MMP-2.

5.6.4 Serum MMP and RA clinical measures

MMPs degrade the components of the ECM. Specifically, MMP-1 (collagenase-1) is responsible for degradation of type I collagen, a major component of the ECM, as well as other fibrillar collagens such as types II, III, V, IX and X. MMP-3 (stromelysin-1) has a broader substrate specificity with activity against type II, III, IV, IX, X and XI collagens, proteoglycans, fibronectin and laminin. Furthermore, it is able to activate other MMPs such as MMP-1, -2, -9 and -13. In the present study, we showed that serum MMP-1 was weakly correlated with disease activity score and with functional outcome measures, while MMP-3 was only weakly correlated with disease activity score but not with functional outcome. Other MMPs were less associated with RA clinical measures, although a decreased MMP-2 was found in autoantibody positive (RF or anti-CCP)/ever smokers with erosive disease. These results were generally in agreement with the concept that MMP-1 and -3 have the most clinical significance in RA, whereas other members were less involved. Since type I collagen is specifically degraded by MMP-1, joint destruction may be primarily associated with this enzyme. Although data were lacking on the radiographic damage of joints on each patient, the correlation of serum MMP-1 with functional outcome measures provided some indirect evidence. Since the actions of these enzymes are in

synovial joints, we speculate that much stronger correlations may occur between synovial fluid MMPs and clinical measures.

5.6.5 MMP polymorphism and RA clinical measures

We initially showed an association of polymorphism in the MMP3 gene with the disease activity score (DAS28) at baseline. Disease activity can fluctuate over time, so we also looked at the association with the mean time-averaged DAS28 in 301 patients followed up over a 2-year period. We believed that this would be more representative of the longer term level of disease activity in each patient than DAS28 measured at a single time point. Further analysis indicated that polymorphism in the MMP3 gene was associated with the level of disease activity over time, and this was independent of the serum MMP-3 association. Interestingly, the highest level of disease activity appeared to be associated with the rs3025058 5A allele (or the rs679620 A allele) which was associated with lower circulating levels of MMP-3 (although it was also associated with the highest levels of MMP-1). However, this genetic association appeared to be independent of serum levels of MMP-1 and particularly MMP-3. The association of the MMP3 polymorphism with disease activity is therefore not a straightforward reflection of circulating MMP levels, but may somehow reflect the expression and local activity of individual MMPs within the joints. This may depend on the conditions within the joints. Recent work has suggested that the rs3025058 polymorphism is functional only during specific environmental conditions involving inflammation (Zhu et al, 2006). It has also been shown that the transcription factor NF-kB has differential effects on 5A and 6A alleles in macrophages, with the greater transcriptional activity of 5A being augmented by NF-kB activation (Souslova et al, 2010). Differential expression of MMP-1 in response to inflammatory mediators has also been

linked to the MMP1 rs1799750 promoter polymorphism (Cao et al, 2010; Affara et al, 2011), although one study found that strong and repeated inflammatory stimulation of macrophages resulted in higher MMP-1 expression irrespective of MMP1 rs1799750 genotypes (Repeke et al, 2009). The possibility of cell and tissue specific differences in gene expression cannot be ruled out. Further work is therefore needed to investigate the influence of MMP1 and 3 polymorphisms on the expression and activity of MMP-1 and -3 in different cell types of the joint and circulation, and whether this is different under normal and inflammatory conditions.

In the present study, we also demonstrated a relationship between polymorphisms in the MMP1-3 loci and the development of erosive disease in RA. In patients with shorter RA duration (Ò6 years), the MMP1 polymorphism rs1799750 and the MMP3 polymorphism rs3025058 were associated with erosive disease. However, the association of the MMP3 rs3025058 polymorphism with erosive disease may be an indirect association due to LD, suggested by a multivariate regression model that contained both polymorphisms as independent variables. Despite this, analysis using haplotypes including both rs1799750 and rs3025058 revealed a stronger effect compared to that using single polymorphisms (data not shown). The 1G allele, associated with high MMP-1 level, was associated with more erosive disease in patients with RA duration Ò 6 years. Several studies have demonstrated the association of synovial/serum MMP-1 levels with new joint erosion/damage in early RA (Cunnane et al, 2001(1); Cunnane et al, 2001(2); Green et al, 2003). In the present study the genotypic and haplotypic associations with erosive disease were not apparent in patients with longer RA duration. This could suggest that 1) the development of erosive disease is mediated by multiple factors where MMP1 polymorphism plays a more important role in the early period of disease; 2) the eventual

development of erosive disease is not determined by the MMP1 variant, and the impact of the polymorphism is only to modify (advance/delay) the progress of the erosive course; 3) the effect of the MMP1 polymorphism on disease outcome is biphasic where one allele could have a very different impact over different periods of the disease course, as suggested by Dörr et al (Dörr et al, 2004).

In Dörr et al, possession of the MMP1 rs1799750 1G allele or the 1G-5A (rs1799750-rs3025058) haplotype appeared to be protective from radiographic progression over the first 15 years of disease, but was associated with more pronounced radiographic progression later on. In contrast, a small study by Constantin et al failed to find an association between the MMP1 rs1799750 polymorphism and radiographic damage over a 4-year follow-up (Constantin et al, 2002). Previous studies (Mattey et al, 2004; Constantin et al, 2002) found an association between increased radiographic damage and the MMP3 rs3025058 6A allele, although this was not confirmed in the study by Dörr et al (Dörr et al, 2004), nor in a study of Japanese patients (Tsukahara et al, 2008). We do not have an explanation for the disparity between these findings, or between those and the current study. One of the limitations of the current study was the lack of sequential radiographs, and we looked only at the presence or absence of erosive disease rather than the amount of radiographic destruction. However, in this respect our findings are in agreement with those of Nemec et al who demonstrated that RA patients carrying an MMP3 rs3025058 5A allele were more likely to have erosive disease (Nemec et al, 2007).

Although not statistically significant, our results showed increased serum MMP-1 levels in early RA patients with erosive disease. The lack of a significant association may possibly be explained by measurement of MMP-1 at only one time point. The same is probably true

for the lack of association between erosive disease and DAS28, and is consistent with previous studies showing that measurements of inflammation at a single time point are poorly associated with erosive damage, the results of cumulative effects over time. Studies using area under curve measurements have shown associations of MMP-1 levels with the development of new erosions (Cunnane et al, 2001). It is noteworthy that the association of the 5A-A-G-1G haplotype with erosive disease remained significant (in patients with RA duration Ò6 years) in models also containing serum MMP levels or DAS28 as independent variables, and may reflect an association of this haplotype with persistently raised levels of MMP-1 and higher disease activity.

In patients with RA duration Ò6 years, a dramatically increased risk was seen in patients who were smokers and carried 2 copies of 5A-1G, whereas those smokers who carried only 1 copy of 5A-1G were not significantly affected. This increased risk implied a possible interaction between smoking and 2 copies of 5A-1G haplotype since a significant attributable proportion due to this combination was observed. The reason behind the observed interaction is unknown. We speculate that certain component(s) of cigarette smoke may interact with MMP(-)1/MMP(-)3 on different levels and result in alterations of enzyme expression/activity. Since our results were based on a relatively small sample size, further confirmation in a larger early RA population is needed.

5.6.6 MMP polymorphism, serum MMP and co-morbidity

Analysis of the whole cohort did not show any association of MMP polymorphism with co-morbid conditions. However, we observed a trend that the 6A allele at rs3025058, the G allele at rs679620 and the A allele at rs495366 were associated with an increased

occurrence of IHD and MI in patients who were RF positive or who had ever smoked. Some of the relationships were not strong enough to reach significance. This may be due to the relatively small number of IHD and MI cases. It was interesting to see the relationship only in RF+ patients or those who had ever smoked. One suggestion was that RF and/or smoking may be involved in the above association. However, since smoking and RF are risk factors for IHD and/or MI, an increased frequency of IHD and/or MI can be seen in these sub-populations. This may increase the possibility of finding positive relationships.

It was shown in the present thesis that the A allele at rs3025058 and the G allele at rs679620 were significantly associated with higher levels of MMP-3. Therefore, the relationship between SNPs in the MMP1-3 loci and the occurrence of IHD/MI may be due to the alteration of MMP-3 expression. We found that there was elevated serum MMP-3 in patients with previous MI compared to the remaining subjects. However, we also observed that in IHD+/MI- patients the level of serum MMP-3 was the lowest, although it was not significantly lower compared to IHD- patients. Samnegård et al have shown that at MI admission the serum MMP-3 is at a low level, while it increases during recovery (Samnegård et al, 2006). Taken together, we speculate that MMP-3 may be involved in a post-MI mechanism rather than in general atherosclerosis progression. On the other hand, serum MMP-1 did not appear to be involved. This was consistent with the genetic data that MMP1 SNP rs1799750 was not associated with the occurrence of IHD and MI. For the first time, we have provided some preliminary results showing that MMP(-)3 is associated with IHD/MI in patients with RA. However, further evaluation using another independent database is needed.

Our data suggest that polymorphisms in the MMP1-3 loci and in the MMP2 gene, and the levels of serum MMPs (including -1, -2, -3, -8 and -9) are not associated with hypertension in patients with RA.

5.6.7 Serum MMP and TIMP

Most serum levels of TIMPs were found to be positively correlated with each other. This implied that these inhibitors may be, at least partly, influenced by the same factor(s)/condition(s). Also, they may interact with each other, and play co-operative roles.

Previous studies have shown that there is an imbalance between MMPs and TIMPs, which is in favour of the MMPs, in various pathological conditions including inflammatory arthritis (Tchetverikvo et al, 2005). This suggests that MMPs activity may not be sufficiently counteracted in those conditions. The extent of activation of the MMPs (proMMPs Ÿ activated form) is largely unknown *in vivo*. Our preliminary results based on RA synovial fluid suggested that MMP-1 activity was associated with major pro-inflammatory markers including CRP, IL-1b and TNF-Ŭ, whereas these markers were not associated with MMP-1 level (data not shown). Furthermore, there was no strong association of MMP-1 activity with MMP-1 level in joints (data not shown). On the other hand, inactivation of the MMPs involves specific TIMPs and the high molecular weight proteinase inhibitor Ŭ$_2$ macroglobulin (Ŭ$_2$M) (Woessner, 1999). TIMP is thought of as a major inhibitor of MMPs at the tissue level. However, determination of specific MMP/TIMP complex remains a challenge. In the present study, we observed several specific correlations between MMPs and TIMPs. For example, MMP-1 was correlated

with TIMP-1, -2 and -3, while MMP-2 was only correlated with TIMP-2. These correlations may be a reflection of the inhibitive specificity of a particular TIMP.

The detail of the specificity of the TIMPs is unclear. Thus, in the current study we used all the combinations of MMP/TIMP ratios (20 in total) to investigate the possible associations. This certainly increased the number of comparisons, and therefore raised the likelihood of false associations by chance. Therefore, these results needed to be treated with caution.

We observed that the genetic polymorphisms in the MMP1-3 loci (mainly the MMP1 rs1799750 and the MMP3 rs3025058) were specifically associated with MMP-1-related MMP/TIMP ratios, but not with other ratios. Haplotype analysis indicated that this effect was attributed to the 5A-1G (rs3025058-rs1799750) haplotype. We hypothesized that patients carrying this haplotype may be associated with higher MMP-1 activity. However, none of these ratios were found to be associated with RA clinical measures recorded in this study. We therefore speculate that direct use of MMP/TIMP ratio may be not a straightforward way to predict the overall MMP activity.

There were relationships between levels of TIMPs (TIMP-1 and -4) and disease measures. Interestingly, these were positive correlations, although TIMPs play an inhibitive role. Our data was in line with some previous reports. Fiedorczyk et al observed positive correlations of serum TIMP-1 and -2 with markers of the disease activity including Ritchie articular index, ESR and DAS (Fiedorczyk et al, 2006(2)). Murphy et al found a positive correlation between baseline serum TIMP-1 and periarticular bone loss over 1 year in early patients with RA (Murphy et al, 2009). Kawashiri et al observed that serum TIMP-1 was positively correlated with the power Doppler ultrasonography score (PDUS), a new clinical measure

for RA (Kawashiri et al, 2011). Given these data, we speculate that the levels of TIMPs may reflect the activity of disease, but may be less indicative of their overall inhibitive effects. In more active RA, increased release of TIMPs may occur in order to respond to increased levels of activated MMPs. This hypothesis may help to explain another observation in the current study. We found that MMP/TIMP ratios were lower in RF+ patients, which may be due to the fact that RF+ patients were associated with a more active RA.

5.7 TGFB1 POLYMORPHISM, SERUM LEVEL OF TGF-β1, SEVERITY AND CO-MORBIDITY IN RA

5.7.1 TGFB1 polymorphism

Cambien et al screened the coding region and 2181 bp upstream of the TGFB1 gene, and identified several common polymorphisms in Caucasians, including -800, -509, +868, +913 and +11929 (Cambien et al, 1996). The -800 G/A and -509 C/T polymorphisms are located in the promoter region, near to the consensus DR1 or DR5 nuclear hormone receptor binding sites, considered possible modulators of expression of the TGFB1 gene and levels (Crobu et al, 2008). In the present study we only included -509, on the basis of that there was a better minor/major allele proportion in -509 compared to -800 (Crobu et al, 2008; Amirghofran et al, 2009) and that there was a very strong LD between these 2 SNPs (Sie et al, 2006; Amirghofran et al, 2009; Grainger et al, 1999). The +868 (exon 1, codon 10, Leu Ÿ Pro) and +913 (exon 1, codon 25, Arg Ÿ Pro) polymorphisms are located in the signal peptide sequence which is involved in the export of synthesized proteins across

membranes of the endoplasmic reticulum (Cambien et al, 1996). Furthermore, they were also suggested to be located at potentially important positions that influence activation of the TGF-ß1 protein (Syrris et al, 1998). Compared to +868 and +913, another common SNP in the coding region, +11929 C/T (exon 5, codon 263, Thr Ÿ Ile), appeared to be less important, since it was in the region coding for the precursor part of the protein not present in the active form (Cambien et al, 1996). We therefore further included +868 and +913 but not +11929 in the present investigation.

Genotypes of -509, +868 and +913 were determined in our RA patients. LD was detected across these SNPs, which was in line with previous observations (Sie et al, 2009; Cambien et al, 1996; Syrris et al, 1998). Our result on the genotypic distribution of +868 was similar to that in another RA population from a nearby region (Dudley). In that study, a case-control comparison suggested that this SNP was not a risk factor for RA susceptibility (Panoulas et al, 2009). To date, no association of polymorphisms in the TGFB1 gene with the risk for the development of RA in Caucasians has been reported. In contrast, in Asians this gene was associated with RA susceptibility (Kobayashi et al, 2009; Alayli et al, 2009; Sugiura et al, 2002). Most recently, a meta-analysis focusing on +868 suggested that TGFB1 gene may only play roles in RA susceptibility for Asians but not for non-Asians, although other SNPs in the gene were less studied (Chang et al, 2010).

5.7.2 Serum level of TGF-β1 and other investigated serum proteins

We found that the level of TGF-ß1 was positively correlated with several other proteins that were important in RA such as MMP-1 and VEGF-A. Although it was not associated

with the clinical measures of RA, this fundamental cytokine may still have some effects on promotion of RA.

5.7.3 TGFB1 polymorphism and serum TGF-β1 level

The relationship between common polymorphisms in the TGFB1 gene and the expression of TGF-ϐ1 has been described (Grainger et al, 1999; Awad et al, 1998; Yamada et al, 1998; Yokota et al, 2000; Suthanthiran et al, 2000). The associations were mostly seen in healthy subjects. In the present study, we investigated such relationship using TGFB1-509, +868 and +913 SNPs, and serum levels of TGF-ϐ1 in patients with RA for the first time. Based on our results, we did not see any association or trend. It was possible that the regulation of TGF-ϐ1 in disease conditions (especially in auto-immune conditions) is more complicated, compared to that in the normal condition. Genetic polymorphisms may have an effect, but this may be masked by other influences. Also, our result is based on the serum level only, and is unable to provide information on cell-type/location specific expression.

5.7.4 Serum TGF-β1 and RA clinical measures

Muñoz-Valle et al found an association of increased serum levels of TGF-ϐ1 with the functional class evaluated in RA patients (Muñoz-Valle et al, 2010). In contrast, in the present study, based on our functional measures (MJS and HAQ) we did not find such relationship. However, we found an association between increased TGF-ϐ1 and erosive disease, although it achieved significance only in RF+ patients. It was unclear why it was only seen in RF+ subjects. We speculate that there may be specific relationships between TGF-ϐ1, B cell regulation and RF release.

For the first time, we found an association between serum levels of TGF-ϐ1 and nodular disease, with lower levels associated with more incidence of this manifestation. Thus, it is possible that up-regulation of TGF-ϐ1 in RA may have a protective effect against the development of nodular disease. TGF-ϐ1 is a multi-functional cytokine. Our results provided evidence that it may have several independent roles in RA, since an increase was associated with joint erosion in RF+ patients while a reduction was found to be associated with nodular manifestation.

5.7.5 TGFB1 polymorphism and serum MMP-1 and -2

TGFB1+913 polymorphism was found to be associated with serum MMP-1 and -2, although for MMP-2 it was not significant. Since these genes are located on different chromosomes, this was not likely due to the LD between the genes. Instead, this association may suggest that TGF-ϐ1 is involved in the regulation of MMP-1 and -2 in RA. In addition, (as mentioned above) we also found that serum TGF-ϐ1 was correlated with serum MMP-1. However, an association of TGFB1+913 polymorphism with serum levels of TGF-ϐ1 was not seen.

5.7.6 Serum TGF-β1 and diabetes mellitus

Recent data showed that regulatory cells with TGF-ϐ1-dependent activity were able to restore self-tolerance in overtly diabetic non-obese diabetic (NOD) mice. Thus, TGF-ϐ1 seems to have a relevant role in protection from autoimmune diabetes (Olivieri et al, 2010). In this study, we found that RA patients with diabetes were associated with lower serum levels of TGF-ϐ1, compared to those without diabetes. Our observation was in line with the

data from Olivieri et al, and further suggested that down-regulation of TGF-ϐ1 is associated with increased risk of diabetes in humans. However, as this was a cross-sectional study, we were unable to demonstrate any cause-effect relationship. Since this association was found in a RA population, we do not know whether such association also occurs in the general population or whether this is somehow related to the disease process in RA. The results here can only be treated as preliminary data since the number of patients with diabetes was low. Further studies both in RA and in the general population are required.

5.7.7 TGFB1 polymorphism, serum TGF-β1 and cardiovascular disease

In the present study, for the first time in a RA population we observed that TGFB1+868 polymorphism was associated with the occurrence of IHD and MI, and that TGFB1-509 SNP was also associated with MI but the association was weaker. Our results were similar to those of Crobu et al and Koch et al which indicated that both TGFB1-509 and +868 had relationships with MI but the association involving -509 was insignificant or relatively weaker (Crobu et al, 2008; Koch et al, 2006). In contrast, Cambien et al suggested that TGFB1+913 provided the strongest signal in relation to the association with MI (Cambien et al, 1996). Using multivariate logistic regression model, we suggested that the association with -509 may be an indirect one due to LD. However, owing to its location (in the gene promoter) this SNP is likely to play roles in influencing the expression of TGF-ϐ1, and therefore may also have an effect. TGFB1+868 SNP is located at a position that has potential function on effecting activation of the TGF-ϐ1 protein. Overall, the associations may very well be attributable to changes in the expression and/or activity of TGF-ϐ1. As shown, in our RA patients these genetic polymorphisms were not associated with serum

TGFβ1 levels. We therefore speculate that the variants (in particular TGFB1+868 and +913) may be associated with different efficiency of protein activation *in vivo,* although the activity of this protein between genotypes has not been assessed in the current study. Furthermore, the peptide where the 2 missense polymorphisms (+868 and +913) are located is involved in the export of the protein across membranes of the endoplasmic reticulum, so these SNPs may also be associated with the transportation and/or localization of TGF-β1 and therefore with functional alteration.

Interestingly, we observed that heterozygous genotypes of TGFB1-509 and +868 were associated with the increased risks compared to homozygous genotypes. Results in previous studies suggested that the TT genotype at -509 and the CC genotype at +868 were associated with the increased risk of MI (Crobu et al, 2008; Koch et al, 2006). Our data was not in agreement with those. However, our subjects were a specific population of RA patients, and therefore unique association in such a population is possible. TGF-β is a pleiotropic cytokine with a diversity of effects, with both proatherogenic and antiatherogenic effects. It is not known yet what the overall effect of TGF-β1 is. Certain expression and/or activity of this protein (neither the highest nor the lowest) may be required to be associated with the most proatherogenic effects. In RA, the expression of TGF-β1 is elevated compared to the general population. Thus, the association may shift from one genotype to another between populations with different conditions. Despite the controversial data based on genotypes, haplotype analysis in our study provided some evidence that the T-C (-509/+868) haplotype may be associated with increased MI (insignificant trend), which was in agreement with the previous studies (Crobu et al, 2008; Koch et al, 2006).

Our observation that heterozygotes for a genetic polymorphism show a significantly greater or lesser effect for a trait than homozygotes can be explained by so-called ñmolecular heterosisò. This kind of effect has been detected in several genes in relation to particular phenotypes. These genes included ADRA2C, C3 complement, DRD1, DRD2, DRD3, DRD4, ER1, HP, HBB, HLA-DR DQ, HTR2A, properdin B, SLC6A4, PNMT and secretor in humans or animals (Comings and MacMurray, 2000). For the dopamine D3 receptor (DRD3) gene, molecular heterosis was only significant in male schizophrenics, but not in female schizophrenics (Asherson et al, 1996). For smoking, also, a positive effect of molecular heterosis was only observed in male schizophrenics (Lee et al, 2002). These suggested that in some cases molecular heterosis may be gender-specific. In terms of our observation and the results published based on the non-RA population, we hypothesize that the molecular heterosis effect of TGFB1 SNP on CVD is a disease-specific effect, since RA creates a unique environment associated with chronic inflammation which is very different from the normal condition. There are several levels at which molecular heterosis may be operating, such as the levels of gene regulation, protein subunit interaction, lethality for homozygosity of the mutant allele and the whole cell or organ (Comings and MacMurray, 2000). Since in the present study TGFB1+868 causes an amino acid change and therefore (maybe) the structure of peptide, an interaction at the level of protein subunits would be the easiest explanation for our observation. It is not difficult to visualize an allosteric effect where 2 identical subunits would function more (or less) efficiently than structurally different subunits (Comings and MacMurray, 2000). Such an effect has been described for the acid phosphotase enzyme in *Drosophilia* (Trehan and Gill, 1987). However, an inverted ñU-shapedò response curve could also be applied to explain the molecular heterosis effect in our study. High and low expression/activity of TGF-ß1 are associated with protective effect while the medium level is associated with the risk.

Furthermore, in the current study we found a significant interaction between the TGFB1+868 polymorphism and ever-smoking in relation to the occurrence of IHD and MI. Interestingly, the data indicated that the risk effect mainly came from this gene-environemtal interaction while the SNP alone did not confer the risk. It will be worth investigating the reason for this observation in future studies. In addition, the interaction with smoking added to the specificity of the mechanism of molecular heterosis.

IHD and MI are thought to be under the control of many genes and other factors that contribute individual effects. Using multivariate logistic regression models, we found that the association of TGFB1+868-smoking interaction with IHD/MI was independent from other risk factors tested in the present study, including the VEGFA-2578-smoking interaction.

The limitations of study herein were similar to those presented in Section 5.3.8 (discussion for the association of VEGFA with IHD/MI). The TGFB1-IHD/MI data had sufficient power (> 80%, a type I error rate of 0.05) to achieve the sort of effect sizes we were seeing. However, to confirm these observations, other independent populations are required, especially those with more cases of IHD/MI.

Serum TGF-ϐ1 was not significantly associated with IHD or MI. However, it was significantly reduced in patients who took medications for heart-related diseases. We do not know if this is an effect from the medications. Prospective studies are needed to determine whether low levels of TGF-ϐ1 are predictive of development of heart disease.

5.8 PTPN22 POLYMORPHISM, SEVERITY AND CO-MORBIDITY IN RA

5.8.1 PTPN22 polymorphism

The T allele (R620W allele) of PTPN22 has been the first and the most reproducible genetic polymorphism identified as a RA susceptibility gene outside the HLA region in Caucasians. This risk allele has been shown to confer an approximately 2-fold increased risk for RA development (Begovich et al, 2004; Hinks et al, 2006). There is no evidence of association with RA in non-white populations because the PTPN22 risk allele is extremely rare in those ethnic groups.

5.8.2 PTPN22 polymorphism and RA clinical measures

Previously, there was a study suggesting that the T allele of rs2476601 was associated with increased RF+ RA, with 2 copies conferring the highest risk (Lee et al, 2005). Furthermore, as mentioned before, this risk allele was also associated with structural damage in joints (Lie et al, 2007; Karlson et al, 2008). In the present study, we did not find any significant association between the T allele of rs2476601 and RA clinical measures. However, we did not assess the radiographic damage since the data was not available. We found an increased MJS in patients with the T allele, although it was an insignificant association. In contrast, the HAQ scores were equal between patients with and without the T allele of rs2476601. This difference suggests that the PTPN22 risk polymorphism may be involved more in joint events rather than the overall outcome of disease.

5.8.3 PTPN22 polymorphism and co-morbidity

In the present study, no significant association of the PTPN22 polymorphism with any co-morbidity recorded was found. Since the R620W allele is thought to be a general risk for auto-immunity, we were particularly interested in the possible relationship between this allele and diabetes mellitus (including type 1 and 2). No association was found. However, the number of patients with diabetes type 1 or 2 was small, and therefore larger studies are needed to better investigate this relationship in RA patients.

5.9 POSSIBLE LIMITATIONS OF THE RESEARCH

5.9.1 Patient population sample size

A limitation of the work was the number of patients. Although we included over 400 RA patients, the numbers were reduced following patient stratification into various subgroups (genotypes, smoking status, seropositivity, etc) and because certain data were unavailable for some individuals (e.g. serum TIMP levels were only tested among patients with RA duration Ò6 years). The occurrence of certain co-morbid conditions was low, although it may be already elevated in the RA population compared to the general population. As a result, the analyses were only able to identifiy major effects. A larger sample size therefore is needed to reach sufficient power to test some of the preliminary. Assessment of the appropriate population size needed to be made prior to the study using statistical methods to perform power calculations. However, this was sometimes not stratightforward since some variables used for stratification, such as genotype distributions, were unknown before the study.

5.9.2 Drug therapy

All the patients were receving anti-inflammatory and/or anti-rheumatic drugs at recruitment. Although the majority (93%) were being treated with DMARDs (mainly MTX, SSZ and hydroxychloroquine), there was a number of patients (14%) who were receiving a biologic agent (mainly etanercept and infliximab). Whilst individual drug regimens may have differed to some extent, the commonest drug used was MTX. The difference in drug regimens may produce some confounding, especially with regard to the investigation of RA clinical measures. However, in such a large cohort of established RA on different drug combinations, it was difficult to control for such an effect. Another related concern was that there may have been differences in response to drug therapy between patients. However, it was not possible to measure this in a systematic way in this study. Also, the difference in drug regimens may influence the serum levels of the studied proteins and the incidence of certain co-morbid conditions. However, for some of the important observations we have adjusted for the use of MTX.

5.9.3 Experimental problems

Due to the low quality/quantity of some DNA samples, some genotyping failed. This led to a reduction of sample size. Furthermore, between investigations using each polymorphism, the patients included were sometimes slightly different. Also, some serum samples were not available, and therefore the levels of some proteins were not measured in certain patients.

5.9.4 Identification of co-morbid condition

Evidence of co-morbidity was obtained from a structured interview, review of the medical notes and current and cumulative medication, and the results from the physical and medical examination conducted at recruitment. For specific conditions, such as MI, its clinical presentation was suggested to be different in RA compared to that in the normal population, with RA patients more likely to experience unrecognized MI. To identify these silent MI, all patients also underwent resting 12-lead ECG. Based on this, we were able to identify 13 patients with previously unrecognized MI who had evidence of a full-thickness MI. However, it was possible that some patients with silent, non-full-thickness or atypical MI were still missed, as the sensitivity of ECG was not high. This could be a limitation of the current study. However, more tests to try and identify other silent MI, such as the cardiac stress test, would not be appropriate in many patients with RA because of their physical limitations.

5.9.5 Cross-sectional in design

The current study was cross-sectional in design, so any time course relationship was not able to be tested. Also, in a cross-sectional study it was only possible to assess patients who had survived from previous events (e.g. cardiac events, stroke, etc). However, this cohort would be followed up annually for a 5-year period. Any future analysis regarding the further development of co-morbidities, RA progression and fatal events would be prospective in design.

5.9.6 Control group

It was not essential to have a healthy population as control in this particular study. However, addition of a control group may help to investigate whether or not the associations observed in RA were disease-spectific associations. In addition, it would allow us to assess whether the studied genetic variants were risks for RA susceptibility.

5.9.7 Statistical analyses

Multiple comparisons were performed when comparing patients who were stratified into several subgroups according to genotypes, smoking status, etc, and/or further combinations. This increased the likelihood of identifying a significant difference between two groups if in fact the null hypothesis of no difference between them was true. For example, a significant result might be expected in every 20 comparisons performed when using a p value of 0.05 (95% significant level). One method to correct for this is to use the Bonferroniôs correction for multiple testing. This is a conservative method that simply multiplies the p values obtained from each test by the number of comparisons conducted. However, whilst this reduces false-positive results (type I errors), it also increases false-negative results (type II errors) such that true associations might be missed.

The approach used in this thesis was based on the investigation of previously implicated candidate genes, and only certain comparisons between specific groups were made. It was thus decided to include all the significant associations found without correction in the present thesis. The thesis can thus serve as a report of novel but initial findings. Each association will need to be further assessed in terms of biological/clinical plausibility.

Confirmation studies in other independent populations are usually required for most association studies for epidemiology.

5.10 SUGGESTED FURTHER RESEARCH

5.10.1 Follow-up study

The design of this study is a prospective cohort that will be followed up annually for a 5-year period. Further research will focus on the investigation of genetic and environmental factors that are associated with any newly developed co-morbid conditions, the RA progression measured by the change of clinical parameters, and any fatal events, during a certain period. Since the present thesis has shown abundant evidence that smoking and polymorphisms in the VEGFA, GSTM1, MMP1, MMP3, eNOS and TGFB1 genes are influencing disease severity and/or co-morbidity, these factors will be included in any follow-up studies. Serum samples will be collected again from each patient at the final visit, which allows us to measure serum markers (e.g. angiogeneic proteins, MMPs, TIMPs, etc) at the 5^{th} year. Any relationship involving the change of the level of a particular marker during a 5-year peroid can then be assessed.

5.10.2 Repetition of the study

Repeating the studies using larger patient numbers and/or independent RA populations is needed. In the current study, since the case numbers of IHD and MI (and other co-morbid conditions) are not high, we can only identify some major effect, such as the VEGFA-

smoking influence on IHD and MI, with a sufficient statistical power. Some smaller impacts, such as the eNOS-smoking and the MMP1-3-smoking effects on IHD/MI, are either insignificant or lacking in power. Independent populations are always useful to confirm associations, although some of the observations in the present thesis, such as the association of MMP1-3 polymorphism with serum MMP-1 and -3, are evidenced by 2 individual populations. In addition, repetition in the general populations is essential for us to understand whether or not the observations seen in RA are disease-spectific relationships.

5.10.3 Addition of other genes

HLA-DRB1 is the major risk gene both in terms of disease susceptibility and disease severity in RA. In addition, evidence of interaction of this gene with smoking has been found. Recently, HLA-DRB1 SE has been suggested to be related to several signalling pathways involving NO and ROS production, MMP activation, angiogenesis and atherosclerosis, immune dysregulation, and T cell hyporesponsiveness (de Almeida et al, 2011). Addition of the genetic content of HLA-DRB1 would allow us to investigate whether there are interactions with the genes investigated in the current thesis. Some genes, such as VEGFA, have shown a significant role in influencing RA outcome. Therefore, it would be interesting to see whether other genes in the major VEGFA signalling pathway are also important to RA. These genes may include HIFŬ and VEGFR2. To date, there are more than 40 susceptibility genes to RA. Some of them may also influence the disease outcome. It would be interesting to include some in the future studies.

5.10.4 Measurement of the levels of local protein and protein activity

Only serum levels of the proteins have been measured, and the activities of the proteins have not been introduced in the current study. The levels and the activities of the proteins may be different in the circulation compared to that in the local sites such as synovial fluid. For example, we have some evidence showing that there is no significant correlation between MMP-1 in serum and MMP-1 in synovial fluid (data not shown). Further measurement of the levels and the activities of the proteins is needed.

5.10.5 Investigation of molecular and biological mechanisms

Our epidemiological research has provided some very interesting and important associations and interactions. However, the reasons behind these relationships are unknown. To fully understand the mechanisms involved, molecular, cellular and animal based research is needed.

CHAPTER 6 CONCLUDING SUMMARY

The main objective of this study was to identify some of the main determinants of poor outcome in RA, both in terms of disease severity and co-morbidity. The results indicate that smoking (ever-smoking) is associated with seropositive RA, in particular with RF+ RA. The presence of RF and anti-CCP is strongly associated with RA clinical measures, with RF associated more with nodular disease, higher disease activity and worse functional outcome and anti-CCP more with a greater likehihood of erosive disease. In seropositive RA, no association of smoking with measures indicating disease severity was found. We speculate that smoking has some effect but any effect in those patients is masked by a greater effect from autoantibodies. After removing the influence of autoantibodies (in seronegative patients), significant associations of smoking with erosive disease and functional outcome measured by HAQ were observed. We conclude that the total effect on RA outcome attributed to smoking is considerable, because it contributes to the production of autoantibodies prior to the onset and/or in the early stage of disease, and in those without autoantibodies it shows a direct impact leading to a worse outcome.

Smoking was associated with the increased number of co-morbid conditions presented in RA, implying that smoking may be a risk factor for a number of co-morbidities. In particular, smoking was found to be significantly associated with the occurrence of IHD and MI.

The results indicated that the association of smoking with the release of RF only occurs in patients carrying the GSTM1-null genotype. This confirms the smoking-GSTM1-null interaction previously observed by Mattey et al (Mattey et al, 2002). Our results also indicate that the association between anti-CCP and erosive disease only occurs in GSTM1-null patients. Furthermore, this relationship was independent of the association between

smoking and erosive disease in those patients. No association of GSTM1, T1 polymorphisms with any co-morbid condition was found.

VEGFA polymorphisms within the 5ô-flank region of the gene are highly linked with each other, while no LD is detected with the 3ô-flank polymorphism. A SNP in the promoter region, VEGFA-2578, was significantly associated with serum levels of VEGF-A, with the CC genotype associated with the highest levels. There is evidence suggesting that the above association may be depend on the level of inflammation. Furthermore, it appears that only this CC genotype is associated with increased levels of VEGF-A when the inflammation also increases, suggesting a genotype-specific response. Serum VEGF-A level was associated with pro-inflammatory markers and disease clinical measures. VEGFA-2578 SNP was also associated with some of the measures, particularly with disease activity. Interestingly, this association was restricted to patients who had never smoked. The AA allele at VEGFA-2578 seems to play a protective role against high disease activity in patients who had never smoked, and this function disappears in patients who had ever smoked. Such a relationship was consistent when analysing disease activity score over time (a 2-year period). In addition, we find that the genetic association with disease activity is independent from the serum level association. The results also provide some evidence that polymorphisms in the VEGFA gene may be associated with age at onset of RA, tobacco addiction and RF production. A major observation in the current study was that by interacting with smoking the polymorphisms in the VEGFA promoter region (VEGFA-2578 and -460) are associated with the occurrence of IHD and MI in patients with RA. The majority of the effect appears to be from this interaction rather than individual factors. In addition, several traditional and established risk factors for CVD were confirmed in this study, such as hypertension, diabetes type 2 and the inflammatory

markers. Some evidence of an association of the VEGFA-2578 SNP with hypertension in RA was also found.

No association of the eNOS+894 polymorphism with any RA clinical measures was found. A trend of the G allele with decreased occurrence of IHD and MI was observed. However, the results were not significant after adjustment for age, sex and RA duration. Furthermore, a possible interaction with smoking was found, although it was an insignificant trend. Since NO is an upstream and downstream regulator of VEGF-A mediated angiogenesis, we speculate that this possible interaction may be somehow related to the stronger interaction of VEGFA-2578 with smoking.

The polymorphisms studied in the MMP1-3 genetic region are all linked, with the 2 MMP3 (rs679620 and rs3025058) and the intergenic (rs495360) SNPs in the same LD block. MMP1 polymorphism (rs1799750) was associated with serum levels of MMP-1. Furthermore, the results indicated that the other 3 SNPs were all associated, and that the associations were independent of the rs1799750 association. Overall, the haplotype analysis indicated that 5A-A-G-1G (rs3025058-rs679620-rs495366-rs1799750) was associated with the higest levels of MMP-1, whereas 6A-G-A-2G was correlated with the lowest. Serum MMP-3 levels were mainly associated with the MMP3 polymorphisms. However, the intergenic and the MMP1 polymorphisms may also have some effect. In haplotype analysis containing all the 4 SNPs, 6A-G-G-2G was associated with the highest serum levels of MMP-3, while 5A-A-G-1G was associated with the lowest. In addition, data suggested that the polymorphisms located within the MMP1-3 gene region may be associated with serum levels of MMP-2 (rs495366) and -9 (rs1799750).

The results confirm that serum levels of MMP-1 and -3 are positively correlated with some of the clinical measures. More interestingly, the genetic polymorphisms in the MMP1-3 region were found to be associated with disease activity, independent of inflammatory markers, and MMP-1 and -3 levels. This impact mainly comes from the MMP3 variants, with 5A-A (rs3025058-rs679620) associated with the highest disease activity. Moreover, this association was also significant when analysing disease activity over time (a 2-year period).

In the current study it was found that the 5A-A-G-1G haplotype may have an effect on earlier development of erosive disease, although the major influence appeared to come from the MMP1 SNP (rs1799750). Further, this effect was enhanced by the combination with cigarette smoking, suggesting a possible interaction.

We found some tentative evidence that polymorphism in the MMP1-3 loci may have a relatiponship with the occurrence of IHD and MI. An interaction with smoking may occur regarding this relationship. However, more solid evidence is needed. Apart from the above findings, other interesting results included an association of the intergenic SNP (rs495366) with age at onset of RA and elevation of serum MMP-3 levels in patients with previous MI. Information with regard to the relationship between MMP1-3 genetic polymorphisms, serum MMPs, serum TIMPs, and outcome variables including disease severity and co-morbidity is also provided.

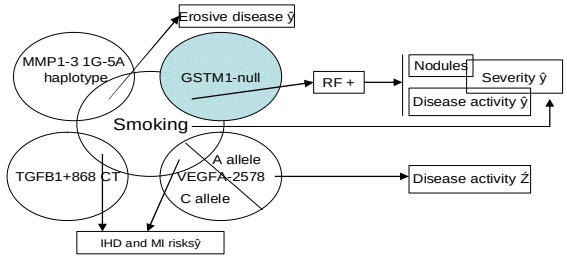
Analysis of TGFB1 polymorphisms showed that those studied, including a SNP in the promoter region and SNPs in the coding region, are in the same LD block. There was no association of serum TGF-ß1 levels with these genetic polymorphisms. However, serum

TGF-ϐ1 levels were correlated with other serum markers such as MMP-1 and VEGF-A. No significant association of polymorphisms in the TGFB1 gene with RA activity/severity was found. In contrast, serum TGF-ϐ1 levels were found to be negatively associated with nodular disease in the whole population, and positively associated with erosive disease in RF+ patients.

The results regarding the relationship with CVD indicated that TGFB1-509 and TGFB1+868 SNPs are associated with the occurrence of IHD and MI, with the latter SNP demonstrating the strongest effect. Interestingly, the heterozygous genotypes were associated with the highest occurrence. We speculate that this may be explained by a disease-specific moleculer heterosis. More interestingly, the results indicated a strong interaction with cigarette smoking in relation to the above association. This interaction appears to be independent of the VEGFA-2579-smoking interaction and other risk factors observed in this study.

Although the association of the PTPN22 gene with RA susceptibility is well established, the results of this study suggest that this gene is not involved in the determination of RA severity or co-morbidity.

A composite review of the major findings in the present study, highlighting the possible smoking-gene interactions



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CHAPTER 8 APPENDICES

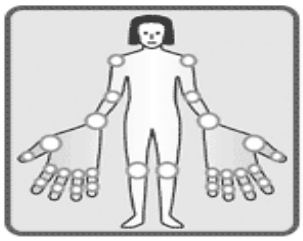
APPENDIX 8.1 CLINICAL INDICES FOR RA

Appendix 8.1.1 Disease activity score based on 28 joints

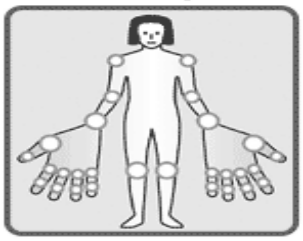
Disease Activity Score (DAS 28)

Joint Status - 28 Joint Count

Tenderness



Swelling



❶ **Joint Count TEN28** ____|____ ❷ **Joint Count SW28** ____|____

❸ **ESR** (after 1 hour in mm) ____|____|____

❹ **General Health** or patient's global assessment of disease activity
How active has your rheumatoid arthritis been during the last 7 days?*

no activity — highest activity possible

0 10 20 30 40 50 60 70 80 90 100

*Please let patient assess this by drawing a vertical line.

Patient's assessment in mm ____|____|____

Formulas for DAS 28 calculation

$$0{,}56 \times \sqrt{\text{❶ TEN28}} + 0{,}28 \times \sqrt{\text{❷ SW28}} + 0{,}70 \times \ln(\text{❸ ESR (after 1 hour in mm)}) + 0{,}014 \times (\text{❹ Patient's assessment in mm*})$$

= ____.____ **DAS 28**

Evaluation DAS 28

Current DAS 28		DAS 28: Difference to initial value		
		> 1,2	> 0,6 and ≤ 1,2	≤ 0,6
≤ 3,2	Inactive	Good improvement	Moderate Improvement	No Improvement
> 3,2 ≤ 5,1	Moderate	Moderate Improvement	Moderate Improvement	No Improvement
> 5,1	Very active	Moderate Improvement	No Improvement	No Improvement

(Copied from the Rheumatology Information Service Europe (RISE) website, http://www.kineret-eu.com/img/en/prodas_das28.gif)

Appendix 8.1.2 Health assessment questionnaire

Dateé é é é é é é .. Patient study numberé é é é é é .

We are interested in learning your illness affects your ability to function in daily life.

Please feel free to add any comments at the end of this form.

PLEASE TICK THE ONE RESPONSE WHICH BEST DESCRIBES YOUR USUAL ABILITIES OVER THE **PAST WEEK**:

	Without ANY Difficulty	With SOME Difficulty	With MUCH Difficulty	UNABLE TO DO
1. DRESSING AND GROOMING				
Are you able to:				
- Dress yourself, including tying shoelaces and doing buttons?	Ĭ	Ĭ	Ĭ	Ĭ
- Shampoo your hair?	Ĭ	Ĭ	Ĭ	Ĭ
2. RISING				
Are you able to:				
- Stand up from an armless straight chair?	Ĭ	Ĭ	Ĭ	Ĭ
- Get in and out of bed?	Ĭ	Ĭ	Ĭ	Ĭ
3. EATING				
Are you able to:				
- Cut up your meat?	Ĭ	Ĭ	Ĭ	Ĭ
- Lift a full glass of milk to your mouth?	Ĭ	Ĭ	Ĭ	Ĭ
- Open a new carton of milk (or soap powder)?	Ĭ	Ĭ	Ĭ	Ĭ
4. WALKING				
Are you able to:				
- Walk outdoors on flat ground?	Ĭ	Ĭ	Ĭ	Ĭ
- Climb up five steps?	Ĭ	Ĭ	Ĭ	Ĭ

PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES:

é é Caneé é Devices used for dressing (button hook, zipper pull, long handed shoe-horn etc)

é é Walking frameé é Built up special utensilsé é Crutchesé é Special or built-up chair

é é Wheelchair Other (specify)é .

PLEASE TICK THE ONE RESPONSE WHICH BEST DESCRIBES YOUR USUAL ABILITIES OVER THE **PAST WEEK**:

	Without ANY Difficulty	With SOME Difficulty	With MUCH Difficulty	UNABLE TO DO
5. HYGIENE				
Are you able to:				
- Wash and dry your entire body?	Ĭ	Ĭ	Ĭ	Ĭ
- Take a bath?	Ĭ	Ĭ	Ĭ	Ĭ
- Get on and off the toilet?	Ĭ	Ĭ	Ĭ	Ĭ
6. REACH				
Are you able to:				
- Reach and get down a 5lb object (e.g. a bag of potatoes) from just above your head?	Ĭ	Ĭ	Ĭ	Ĭ
- Bend down to pick clothing from the floor?	Ĭ	Ĭ	Ĭ	Ĭ
7. GRIP				
Are you able to:				
- Open car doors?	Ĭ	Ĭ	Ĭ	Ĭ
- Open jars which have been previously opened?	Ĭ	Ĭ	Ĭ	Ĭ
- Turn taps on and off?	Ĭ	Ĭ	Ĭ	Ĭ
8. ACTIVITIES				
Are you able to:				
- Run errands and shop?	Ĭ	Ĭ	Ĭ	Ĭ
- Get in and out of a car?	Ĭ	Ĭ	Ĭ	Ĭ
- Do chores such as vacuuming, housework or light gardening?	Ĭ	Ĭ	Ĭ	Ĭ

PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES:

é é Raised toilet seaté é Bath railé é Bath seaté é Long handled appliances for reach

é é Jar opener (for jars previously opened) Other (specify)é é é é é é é é é é é é é é é é ..

PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON:é Hygieneé Gripping and opening thingsé Reaché Errands and housework

Appendix 8.1.3 Mechanical joint score

Examination

ñIn each hand the PIPs and MCPs are examined by observing the patient making a full fist and then fully extending the fingers (Figure 8.1.3a). Wrists are examined using the óprayerô and óinverse prayerômanoeuvres (Figure 8.1.3b). Each elbow is examined by bending and straightening it (Figure 8.1.3c).ò

ñThe shoulder is examined by asking the patient to place their hand behind their head with their arms pointing laterally (Figure 8.1.3d); internal rotation of the shoulder is tested by asking the patient to put their hand behind their back and tough their shoulder blade (Figure 8.1.3e). Hip abduction is examined by using the examinerôs hand to fix the pelvis (Figure 8.1.3f); hip rotation is examined with the patientôs legs held extended (Figure 8.1.3g). Flexion and extension of the knee are examined with the examinerôs hand on the knee to feel for joint instability (Figure 8.1.3h).ò

ñDorsiflexion, plantar flexion, inversion and eversion of the ankle joint are examined. Finally, in examining the MTPs the feet are inspected for gross deformity and palpated for subluxation and callus formation (Figure 8.1.3i).ò

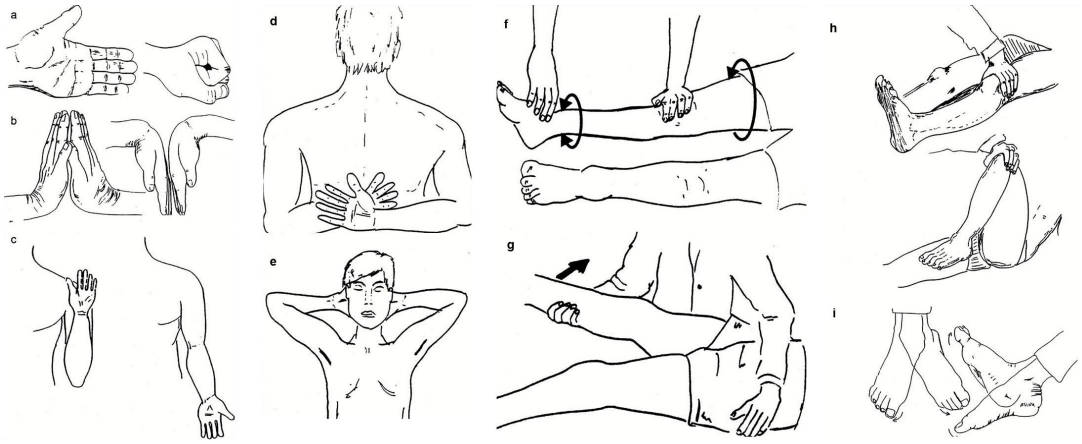
Scoring

ñWhen scoring each joint or set of joints, 0 is taken to mean no abnormality but is also the score given if any joint is absent for any reason or if the joint deformity is congenital in origin.ò

ñA score of 1 represents possible or minor abnormality; it is the score given if there is a slight resting deformity or if the reduction in the range of joint movement is less than 20%. A score of 2 represents definite or moderate abnormality; i.e. a definite resting deformity or a moderate reduction in the range of joint movement (20 ï 40%). A score of 3 indicates severe abnormality or bony surgery. Total PIPs, MCPs and MTPs of each hand/foot are scored as one joint.ò

ñThe final score is calculated by summing the scores for the individual joints or sets of joints, giving a minimum score of 0 and a maximum of 54.ò

Figure 8.1.3a-i Examination of mechanical joint score



(Reproduced from Johnson et al, 2002)

APPENDIX 8.2 ETHICS APPROVAL

Study title: Prevalence of co-morbidity in rheumatoid arthritis

REC reference: 1360

Ethics approval for this study was originally granted to Dr. Julie Barber as the chief investigator, with Dr. Derek Mattey as one of the research team. Dr. Barber was a specialist registrar in Rheumatology who finished her training shortly after commencement of the study, and subsequently left the region. Due to this, an application was made for a substantial amendment to allow Dr. Mattey to take over as chief investigator, and for an extension of the recruitment period. This amendment was granted in April 2005. The letter of approval from the ethics committee for the amendment to the study is enclosed.

APPENDIX 8.3 SMOKING QUESTIONNAIRE

To complete the survey we would like to ask some general questions about you, and also about your health.

Please tick the box, which applies to you, or write on the dotted line.

Qu. 1 Are you Male Ǐ

Female Ǐ

Qu. 2 Have you ever smoked? Yes Ǐ **Go to Qu. 3**

No Ǐ **Go to Qu. 8**

Qu. 3 How old were you when you started smoking?.....

Qu. 4 If you no longer smoke at what age did you stop smoking?.....

Qu. 5 Did or do you smoke cigarette Ǐ

cigars Ǐ

a pipe Ǐ

Qu. 6 How many cigarettes or cigars did or do you smoke per day on average?.....

Qu. 7 If you smoke or smoked a pipe, how many ounces of tobacco per day?.....

Qu. 8 Dose anyone else you live with smoke? Yes Ǐ No Ǐ

Qu. 9 At the moment do you have a paid occupation? Yes Ǐ No Ǐ

Qu. 10 Are you retired? Yes Ǐ No Ǐ

Qu. 11 What was your last paid employment?.....

Qu. 12 Do you live with a partner Ǐ in residential care Ǐ alone Ǐ

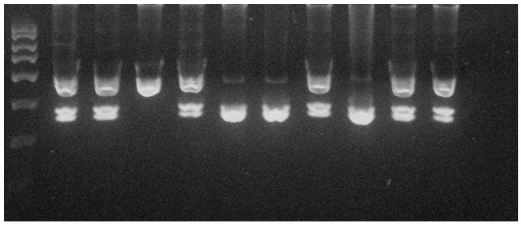
with relatives Ǐ with friends Ǐ

Qu. 13 If you have a partner what is, or was, their employmenté é é é é é é é ..

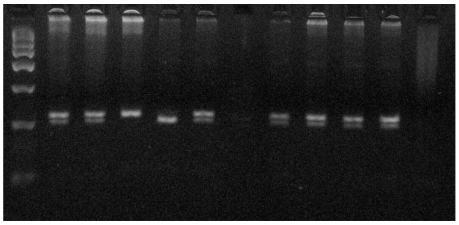
Qu. 14 If you have a partner have they retired? Yes Ǐ No Ǐ

Patient study numberé é é .

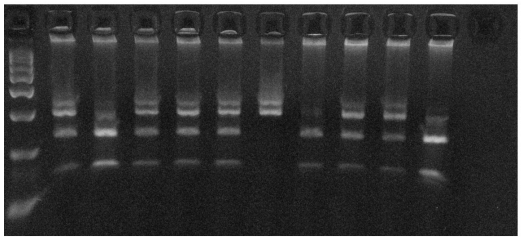
APPENDIX 8.4 DIAGRAMS OF DIGESTION OUTCOME FOR RFLP



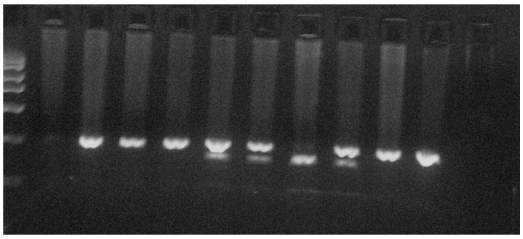
VEGFA-2578(A/C): A allele cuttable



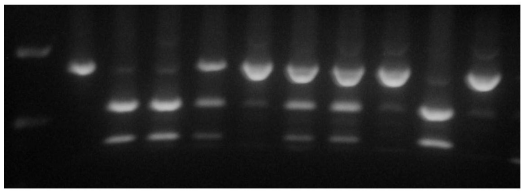
VEGFA-460(T/C): C allele cuttable



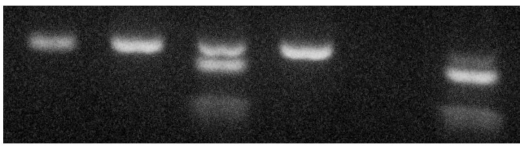
VEGFA+405(C/G): G allele cuttable



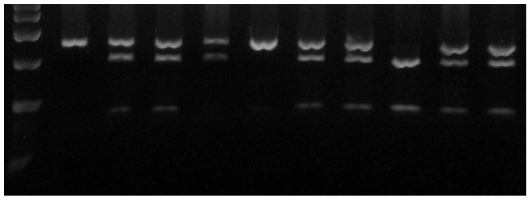
VEGFA+936(C/T): T allele cuttable



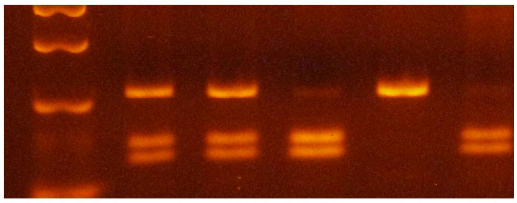
eNOS+894(G/T): T allele cuttable



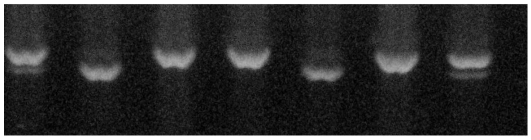
MMP3 5A6A: 5A allele cuttable



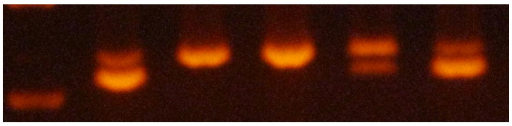
MMP3 rs679620(A/G): G allele cuttable



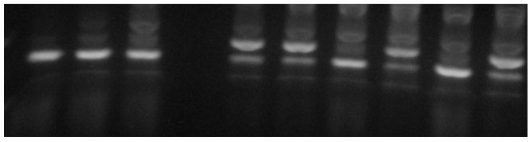
MMP1-3 intergenic rs495366(A/G): G allele cuttabl



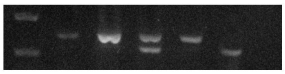
MMP1 1G2G: 1G allele cuttable



MMP2-1306(C/T): T allele cuttable



TGFB1-509(C/T): C allele cuttable



PTPN22 R620W(C/T): T allele cuttable

APPENDIX 8.5 ESTIMATION OF PERCENTAGE OF THE 5A-1G (RS3025058-RS1799750) HAPLOTYPE IN PATIENTS CARRYING 5A6A AND 1G2G GENOTYPES

Appendix 8.5.1 Relationship between genotypic combinations and the 5A-1G haplotype

Genotypic combination (n)	Number of copies of 5A-1G
5A5A/1G1G (65)	2
5A6A/1G1G (50)	1
5A5A/1G2G (31)	1
5A6A/1G2G (123)	1*
6A6A/1G1G (8)	0
6A6A/1G2G (37)	0
5A5A/2G2G (10)	0
5A6A/2G2G (46)	0
6A6A/2G2G (39)	0

* The majority (84%) of the patients carrying 5A6A/1G2G had a copy of 5A-1G due to the LD between these 2 polymorphisms.

Appendix 8.5.2 Calculation method

Among the rs3025058-rs1799750 haplotypes, the total percentage of 5A-1G was 38.6% (estimated by Haploview). If all 5A6A/1G2G patients contained a copy of 5A-1G, the total 5A-1G haplotype would be (65 × 2 + 50 +31 +123)/[(65 + 50 + 31 + 123 + 8 + 37 + 10 + 46 + 39) × 2] = 41%. The determined 5A-1G was (65 × 2 + 50 + 31)/[(65 + 50 + 31 + 123 + 8 + 37 + 10 + 46 + 39) × 2] = 26%. Therefore, the percentage of 5A6A/1G2G patients who carrying a copy of 5A-1G was (38.6% - 26%)/(41% - 26%) = 84%.

APPENDIX 8.6 HAQ SCORE IN FEMALE RA PATIENTS STRATIFIED BY THE COMBINATION OF GSTM1 GENOTYPE (NULL VS. EXPRESSING) WITH EVER-SMOKED STATUS

Combination, n	HAQ
Never-smoked, GSTM1-expressing, 53	1.49 ± 0.73
Never-smoked, GSTM1-null, 65	1.45 ± 0.76
Ever-smoked, GSTM1-expressing, 76	1.54 ± 0.73
Ever-smoked, GSTM1-null, 90	1.68 ± 0.69*

Values are mean ± SD; * p = 0.029, ever-smoked, GSTM1-null vs. the remaining combinations as a whole, adjustment for age and DD.