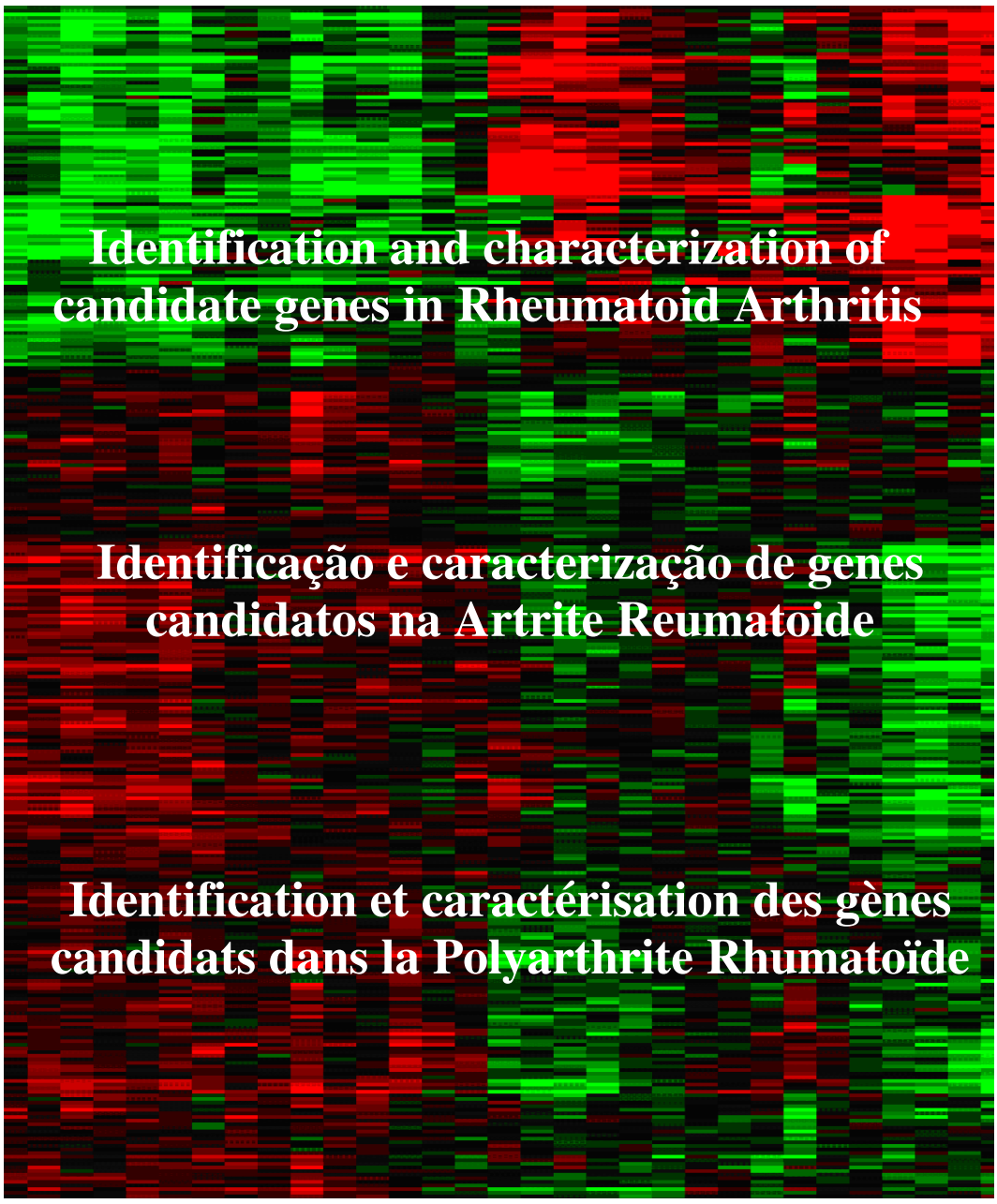

Identification and characterization of candidate genes in Rheumatoid Arthritis

Identificação e caracterização de genes candidatos na Artrite Reumatoide

Identification et caractérisation des gènes candidats dans la Polyarthrite Rhumatoïde

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Universidade de Coimbra
Université d'Evry Val d'Essonne
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Identification and characterization of candidate genes in Rheumatoid Arthritis

Vitor Hugo Sousa Teixeira

The research work presented in this thesis was performed at the GenHotel Laboratory - EA3886 - Research Laboratory in Rheumatoid Arthritis (Evry and Paris-VII Universities), Evry, France under supervision of Dr Elisabeth Petit-Teixeira and Prof. Catarina Resende de Oliveira.

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Front cover:

The figure shows a cluster diagram of the expression of significantly expressed genes in 18 RA patients and 15 controls. Genes are organized by hierarchical clustering based on overall similarity in expression patterns. Red represents relative expression greater than the median expression level across all samples, and green represents an expression level lower than the median. Black indicates intermediate expression.

University of Coimbra

2009

To my Parents,
Grand-Parents
and Rheumatoid Arthritis Patients

Aos meus Pais,
Avós
e Pacientes com Artrite Reumatóide

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I remember it well
The first time that I saw
Your head around the door
'Cause mine stopped working
I remember it well
There was wet in your hair
You were stood in the stairs
And time stopped moving
I want you here tonight
I want you here
'Cause I can't believe what I found
I want you here tonight
I want you here
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Abbreviations

ACPA	Antibodies to citrullinated protein antigen
ACR	American College of Rheumatology
ACTB	Actin beta
ADAMs	Disintegrin and metalloproteases domain
AFBAC	Affected Family Based-Controls
AhR	Hydrocarbone receptor
AIF1	Allograft inflammatory factor 1
AKA	Anti-keratin antibody
APCs	Antigen presenting cells
APF	Anti-perinuclear factor
APRIL	A proliferation-inducing ligand
ASPs	Affected sibling pairs
B2M	Beta-2-microglobulin
BCR	B cell receptor
BLyS	B lymphocyte stimulator
BiP	Immunoglobulin binding protein
C5	Complement Component 5
CAMP	Cathelicidin antimicrobial peptide
CASP7	Caspase 7
CCL21	Chemokine (C-C motif) ligand 21
CD	Crohn's disease
CD4+	Helper T cell
CD8+	Cytotoxic T cell
CD4+CD25	Regulatory T cells
CDK6	Cyclin-dependent kinase 6
cFMS	Colony stimulating factor 1 receptor
CIA	Collagen-induced arthritis
CRP	C-reactive protein
Csk	C -terminal Src kinase
CTLA4	Cytotoxic T lymphocyte–associated antigen 4
DAP12	TYRO protein tyrosine kinase binding protein

DAS	Disease activity score
DCs	Dendritic cells
DMARDs	Disease modifying antirheumatic drugs
ECM	Extracellular matrix
ECRAF	European Consortium of Rheumatoid Arthritis Families
EIRA	Swedish Epidemiological Investigation of Rheumatoid Arthritis
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
F5	Coagulation factor V
FBAT	Family-Based Association Test
FcRγ	Fc receptor common gamma subunit
FCRL3	Fc receptor-like 3
fDCs	Follicular dendritic cells
FLS	Fibroblast-like synoviocytes
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GD	Graves' disease
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GRB2	Growth factor receptor-bound protein 2
GRR	Genotype Relative Risk
GSMA	Genome search meta-analysis
GWA	Genome-wide association
HAQ	Health Assessment Questionnaire
HLA	Human leucocyte antigen
HRR	Haplotype Relative Risk
HWE	Hardy–Weinberg equilibrium
IBD	Inflammatory bowel diseases
IFN	Interferon
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgG2a	Gamma-2a immunoglobulin heavy chain
IKKα	IκB kinase alpha
IKKβ	IκB kinase beta

IL	Interleukin
IL-1α	Interleukin 1, alpha
IL-1β	Interleukin 1, beta
IL-1ra	Interleukin 1 receptor antagonist
IL-6R	Interleukin 6 receptor
IL-32γ	Interleukin 32, gamma
IRF5	Interferon regulatory factor 5
ITAM	immunoreceptor tyrosine-based activation motif
IWHS	Iowa Woman's Health Study
JIA	Juvenile idiopathic arthritis
JUN	Jun oncogene
KIF5A	Kinesin family member 5A
KLF12	Kruppel-like factor 12
LCK	Lymphocyte-specific protein tyrosine kinase
LD	Linkage disequilibrium
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LTβ	Lymphotoxin beta
LYP	Lymphoid tyrosine phosphatase
LY96	Lymphocyte antigen 96
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MLC	Mixed lymphocyte culture
MMPs	Metalloproteinases
MMEL1	Membrane metallo-endopeptidase-like 1
MTX	Methotrexate
NARAC	North American Rheumatoid Arthritis Consortium
NFAT5	Nuclear factor of activated T cells 5
NFATC1	Nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	Nuclear factor kappa-B

NK	Natural killer cells
NIMA	Non-inherited maternal antigen
OA	Osteoarthritis
OCPs	Osteoclast precursors
OLIG3	Oligodendrocyte transcription factor 3
OPG	Osteoprotegerin
ORs	Odds ratios
OSM	Oncostatin M
ORM1	Orosomucoid 1
ORM2	Orosomucoid 2
PADs	Peptidylarginine deiminases
PADI4	Peptidyl arginine deiminase, type IV
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
PGs	Peptidoglycans
PGE_2	Prostaglandin E2
PGLYRP1	Peptidoglycan recognition protein 1
PHF19	Plant homeodomain-finger protein 19
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PLC-γ	Phospholipase C, gamma
PRRs	Pattern recognition receptors
PRKCH	Protein kinase C, eta
PRKCQ	Protein kinase C, theta
PTPN22	Protein tyrosine phosphatase non-receptor 22
Ps	Psoriasis
PsA	Psoriatic arthritis
RA	Rheumatoid Arthritis
RA33	Nuclear ribonucleoprotein complex
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor- kappa B ligand
RANTES	Regulated upon activation, normally T-expressed, and secreted
RA-SFs	Rheumatoid arthritis-synovial fibroblasts

RF	Rheumatoid Factor
RhD	Rhesus D
RR	Relative risk
S100A8	S100 calcium-binding protein A8
S1000A9	S100 calcium-binding protein A9
S100A12	S100 calcium-binding protein A12
SCID	Severe combined immunodeficient mouse
SDF-1	Stromal cell-derived factor 1
SE	Shared epitope
SH3	Src homology 3
SLC11A1	Solute carrier family 11, member 1
SLC22A4	Solute carrier family 22, member 4
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Sjogren's syndrome
STAT1	Signal transducer and activator of transcription 1
STAT4	Signal transducer and activator of transcription 4
T1D	Type 1 diabetes
TCDD	2,3,7,8-tetrachlorodibenzop-dioxin
TCR	T cell receptor
TDT	Transmission Disequilibrium Test
Tenr	Testis nuclear RNA-binding protein
TGF	Transforming growth factor
TGF-β	Transforming growth factor beta
Th	T helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor, alpha
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TRAFs	Tumor necrosis factor receptor-associated factors
TRAF1	Tumor necrosis factor receptor-associated factor 1
Treg	Regulatory T-cells
TXN	Thioredoxin
UC	Ulcerative colitis

VCAM-1 Vascular cell adhesion molecule 1

xMHC Extended MHC

WTCCC Wellcome Trust Case Control Consortium

Summary

Rheumatoid Arthritis (RA), one of the most common autoimmune diseases, affecting ~1% of the population worldwide is characterized by progressive articular damage leading to joint deformities and disability. The multifactorial nature of RA provides high disease heterogeneity with specific combinations of a genetic background and environmental factors that influence the susceptibility, severity and outcome of the disease. RA heterogeneity is demonstrated by the presence of distinct autoantibody specificities, such as rheumatoid factor (RF) and antibodies to citrullinated protein antigen (ACPA) in the serum, the differential responsiveness to treatment and the variability in clinical presentation.

Although the etiology of RA remains unsolved, genetic contribution has been estimated to be 50–60%. The human leukocyte antigen (*HLA*) locus, the first identified genetic risk factor, accounts for 30% to 50% of overall genetic RA susceptibility. Outside the *HLA* locus, the strongest association identified to date (2006) is with a polymorphism in the protein tyrosine phosphatase non-receptor 22 (*PTPN22*-1858T) gene. Therefore, the identification of genes contributing to the disease is important for the understanding of underlying biologic mechanisms. In addition, several gene expression profiling studies of peripheral blood mononuclear cells (PBMCs) from RA patients showed marked variation in gene expression profiles that allowed the identification of distinct molecular disease mechanisms involved in RA pathology.

Numerous environmental risk factors have been studied in RA, but tobacco smoking is to date the only well-established environmental risk factor. Tobacco smoking was shown in several studies to be a risk factor for the RF-positive or ACPA-positive subset of RA patients and to have no or a very minor effect on the autoantibody-negative subset. Furthermore, a major environment interaction was noted between *HLA-DR* risk alleles and tobacco smoking in patients who were positive for RF or ACPA. Consequently, these data from genetic epidemiological studies need biological explanations for the combined effects of genetic and environmental risk factors.

The aim of this thesis was the identification and characterization of candidate genes in Rheumatoid Arthritis using both different genetics and molecular approaches.

In the first part of this work (chapters 3 and 4) we have performed several family-based association and linkage studies in order to identify and/or confirm new RA susceptibility genes in French and European population. Furthermore, expression studies on two candidate

genes were carried out to complete genetic analysis. The genetic approach was based on candidate gene family-based association and linkage studies using RA trio families (each composed of one patient and both healthy parents) from French and West European origin. We have confirmed the association and linkage of tumor necrosis factor receptor-associated factor 1-complement component 5 (*TRAF1-C5*) and 6q23 gene regions, and demonstrated a trend for the association and linkage of the 4q27 gene region with RA in European descent populations. Furthermore, we provided evidence against the involvement of the protein kinase C, eta (*PRKCH*) gene and the *PTPN22*–1123G allele in RA genetic susceptibility in the French Caucasian population. In addition, we also failed to identify an association and linkage of caspase 7 (*CASP7*), receptor activator-kappa B (*RANK*) and receptor activator of nuclear factor-kappa B ligand (*RANKL*) genes with RA in European Caucasian population. We have also evaluated the expression level of *PRKCH* and *CASP7* genes to complete the genetic analysis performed. We observed that *PRKCH* mRNA was expressed in RA patients at lower level than in healthy controls in PBMCs. Moreover, we demonstrated that *CASP7* functional isoform and non functional isoform mRNAs were expressed in RA patients at lower level than in healthy controls in peripheral blood cells. These results lead to support the interest of expression studies in RA.

In the chapter 5, we performed a large-scale gene expression profiling study using 48.701 cDNAs in PBMCs of RA patients and healthy controls. A differentially expression of 339 Reference Sequence genes (238 down-regulated and 101 up-regulated) between the two groups was observed. We identified a remarkably elevated expression of a spectrum of new genes involved in different functional immunity and defense related mechanisms as pro-inflammation, anti-microbial activity, cellular stress and immunomodulatory functions in RA. Thus, we contributed to gain insights into RA molecular mechanisms.

In chapter 6, we have evaluated the interaction between genetic, biological and environmental factors in RA patients. We identified an association between *HLA SE* alleles and tobacco smoking for anti-CCP positivity and a tendency toward an interaction between the *HLA-DRB1**0401 allele and smoking in the development of ACPA positivity in French population with familial and sporadic RA. We also observed a correlation between the cumulative dose of cigarette smoking and ACPA titres and a lack of association between *PTPN22*-1858T allele and tobacco smoking for autoantibody positivity. Therefore, we have confirmed the role of tobacco smoking in RA etiology.

Finally, several collaboration studies were performed using familial and case-control approaches, on RA susceptibility genes in different ethnic populations (Annexes).

All these complementary approaches that allowed the identification of new genes and gene-autoantibodies-environment interactions contribute to a better understanding of RA disease mechanisms and could lead to the identification of innovative clinical biomarkers for diagnostic procedures and therapeutic interventions.

Resumo

A Artrite Reumatóide (AR), uma das doenças auto-imunes mais comuns afectando 1% da população mundial, é caracterizada por uma progressiva lesão articular originando deformações articulares e incapacidade motora. A natureza multifactorial da AR origina uma elevada heterogeneidade com combinações específicas entre um perfil genético e factores ambientais que influenciam a susceptibilidade, a gravidade e o desenvolvimento da doença. A heterogeneidade desta doença é demonstrada por diferentes especificidades de auto-anticorpos séricos, como o factor reumatóide (RF) e os anticorpos contra os antigénios das proteínas citrulinadas (ACPA), a resposta diferenciada ao tratamento e a variabilidade do quadro clínico.

Mesmo se a etiologia da AR permanece desconhecida, a contribuição genética está estimada entre 50 a 60%. O locus do complexo maior de histocompatibilidade (locus *HLA*), o primeiro factor genético identificado como estando associado à doença, contribui entre 30 a 50% para a susceptibilidade genética global da AR. Externamente ao locus *HLA*, a mais forte associação genética descrita até ao ano de 2006 é um polimorfismo do gene proteína tirosina fosfatase, não receptor tipo 22 (*PTPN22*-1858T). Como tal, a identificação de genes que contribuem para a patologia desta doença é importante para a compreensão dos mecanismos biológicos subjacentes. Adicionalmente, vários estudos envolvendo a análise do nível de expressão génica a partir de células mononucleadas do sangue periférico (PBMCs) de pacientes de AR revelaram uma clara variação entre os perfis de expressão investigados, permitindo a identificação de mecanismos moleculares distintos implicados na patologia da AR.

Numerosos factores ambientais de risco para a AR foram investigados, no entanto, o tabaco é o único factor que foi claramente confirmado. Em diferentes estudos, o tabaco foi descrito como factor de risco para pacientes que possuem RF ou ACPA exercendo aparentemente um efeito mínimo em pacientes que não possuem estes auto-anticorpos. Adicionalmente, uma elevada interacção ambiental foi observada entre os alelos de risco (SE, epítopo partilhado) do locus *HLA-DR* para a AR e o tabaco nos pacientes com RF ou ACPA. Consequentemente, estes resultados obtidos através de estudos genéticos epidemiológicos necessitam de explicações biológicas para os efeitos conjuntos dos factores genéticos e ambientais de risco para a AR.

O objectivo deste trabalho é a identificação e caracterização de genes candidatos na AR utilizando diferentes abordagens genéticas e moleculares.

Na primeira parte deste trabalho (capítulos 3 e 4), foram realizados vários estudos de associação e ligação genética com o intuito de identificar e/ou confirmar novos genes de susceptibilidade para a AR na população francesa e/ou europeia. Adicionalmente, o estudo de expressão de dois genes candidatos foi efectuado para completar a análise genética.

A abordagem genética foi baseada em estudos de associação e de ligação de genes candidatos utilizando famílias trio (cada uma constituída por um paciente AR e pais saudáveis) de origem francesa e/ou europeia. Nestes estudos, confirmamos a associação e a ligação das regiões genéticas *TRAF1-C5* e 6q23 com a AR, e demonstramos uma tendência para a associação e ligação da região genética 4q27 com a AR na população europeia. Adicionalmente, obtivemos resultados conclusivos que excluem uma implicação do gene *PRKCH* e do alelo *PTPN22*-1123G na susceptibilidade genética da AR na população caucasiana francesa. Similarmente, a associação ou ligação dos genes *CASP7* (caspase 7), *RANK* e *RANKL* (receptor activador do factor nuclear - kappaB e o seu ligando) com a AR foi excluída na população caucasiana europeia. Para completar a análise genética, avaliamos igualmente os níveis de expressão dos genes *PRKCH* e *CASP7*. Os resultados obtidos demonstram que o ARNm do gene *PRKCH* é mais expresso nas PBMCs dos pacientes com AR comparativamente aos controlos saudáveis e que o ARNm da isoforma funcional ou não funcional do gene *CASP7* é menos expresso nas PBMCs dos pacientes com AR comparativamente aos controlos saudáveis. Estes resultados confirmam o interesse dos estudos de expressão génica nesta doença.

No capítulo 5, realizamos um estudo de expressão génica de grande amplitude utilizando 48.701 ADNc em PBMCs de pacientes e controlos saudáveis. Uma expressão diferencial de 339 genes referenciados (238 sub-expressos e 101 sobre-expressos) entre os dois grupos foi observada. Neste estudo, identificamos uma expressão excepcionalmente elevada dum conjunto de novos genes implicados em diferentes mecanismos imunitários na AR, tais como, processos pró-inflamatórios, actividade anti-microbiana, stress celular e funções imunno-moduladoras, contribuindo assim para a descoberta de novos dados relativamente aos mecanismos moleculares da AR.

No capítulo 6, avaliamos a interacção entre factores genéticos, biológicos e ambientais em pacientes franceses com AR. Neste trabalho, observamos uma associação entre os alelos *HLA* SE e o tabaco nos pacientes com ACPA e uma tendência positiva na interacção entre o alelo *HLA-DRB1**0401 e o tabaco nos pacientes com ACPA. Adicionalmente, observamos uma correlação entre a dose cumulativa de cigarros fumados e os níveis de ACPA detectados. Nenhuma associação entre o alelo *PTPN22*-1858T e o tabaco foi observada nos pacientes com ACPA. Com este estudo confirmamos o papel do tabaco na etiologia da AR.

Finalmente, várias colaborações foram efectuadas com outros laboratórios. Estes estudos familiares ou caso-controlo foram realizados em genes de susceptibilidade para a AR em populações de diferentes origens étnicas (Anexos).

Todas estas abordagens complementares permitiram a identificação de novos genes e a constatação de interacções gene-autoanticorpos-ambiente, contribuindo deste modo a uma melhor compreensão dos mecanismos patológicos da AR que podem conduzir a uma identificação de bio-marcadores inovadores no quadro duma melhoria dos métodos de diagnostico e terapia.

Résumé

La Polyarthrite Rhumatoïde (PR), une des maladies auto-immunes les plus répandues et qui affecte environ 1% de la population mondiale, est caractérisée par une destruction progressive des articulations, conduisant à des déformations et handicaps. La nature multifactorielle de la PR fournit une hétérogénéité élevée avec des combinaisons spécifiques entre un profil génétique et des facteurs environnementaux qui influencent la susceptibilité, la gravité et le développement de la maladie. L'hétérogénéité de cette maladie est démontrée par différentes spécificités d'auto-anticorps sériques, tels que le facteur rhumatoïde (RF) et les anticorps contre les antigènes des protéines citrullinés (ACPA), la réponse différentielle au traitement et la variabilité du cadre clinique.

Bien que l'étiologie de la PR demeure non résolue, la contribution génétique a été estimée à 50-60%. Le locus du complexe majeur d'histocompatibilité (locus *HLA*), le premier facteur génétique à risque identifié, contribue de 30 à 50% à la susceptibilité génétique globale de la PR. En dehors du locus *HLA*, la plus forte association décrite jusqu'à ce jour (2006) est celle d'un polymorphisme du gène codant la protéine tyrosine phosphatase 22 (*PTPN22*-1858T). De ce fait, l'identification de gènes qui contribuent à la pathologie est importante à la compréhension des mécanismes biologiques sous-jacents. De plus, plusieurs études impliquant l'analyse du taux d'expression à partir des cellules mononucléées du sang périphérique (PBMC) de patients atteints de PR, montrent une variation marquée entre les profils d'expression établis, permettant l'identification de mécanismes moléculaires distincts impliqués dans la pathologie de la PR.

De nombreux facteurs de risque environnementaux ont été étudiés dans la PR; néanmoins, il semblerait que le tabac soit le seul à avoir été clairement établi. Le tabac a été décrit dans différentes études comme étant un facteur de risque chez le sous-groupe de patients RF ou ACPA positifs, n'exerçant apparemment qu'un effet minimal chez le sous-groupe négatif pour les auto-anticorps. De plus, une interaction environnementale majeure a été notée entre les allèles à risque EP (epitope partagé) du locus *HLA-DR* et le tabac chez les patients RF ou ACPA positifs. Par conséquent, ces résultats obtenus lors d'études génétiques épidémiologiques requièrent des explications biologiques concernant les effets combinés des facteurs de risque génétiques et environnementaux.

L'objectif de cette thèse est l'identification et la caractérisation de gènes candidats dans la PR en utilisant différentes approches génétiques et moléculaires.

Dans la première partie de ce travail (chapitres 3 et 4), nous avons réalisé plusieurs études d'association et liaison de façon à pouvoir identifier et/ou confirmer de nouveaux gènes de susceptibilité à la PR au sein de la population française et/ou européenne. De plus, l'étude d'expression de deux gènes candidats a été effectuée afin de compléter l'analyse génétique.

L'approche génétique s'est fondée sur des études d'association de gènes candidats de familles et sur des études de liaison, en utilisant des familles trios (un patient et ses deux parents sains) d'origine française et/ou européenne. Nous avons confirmé l'association ainsi que la liaison des régions *TRAF1-C5* et 6q23, et démontré une tendance pour une association et liaison entre la région génique 4q27 et la PR dans la population européenne. De plus, nous avons fourni des résultats concluants qui s'opposent à une implication du gène *PRKCH* (codant pour la protéine kinase C) et l'allèle *PTPN22*-1123G dans la susceptibilité génétique à la PR caucasienne française. Nous avons également écarté une association ou liaison des gènes *CASP7* (caspase 7), *RANK* et *RANKL* (récepteur activateur-kappaB et son ligand) avec la PR dans la population caucasienne européenne. Nous avons également évalué les niveaux d'expression des gènes *PRKCH* et *CASP7*, afin de compléter l'analyse génétique. Nous avons observé que l'ARNm codant *PRKCH* était plus exprimé dans les PBMCs chez les patients PR que chez les contrôles sains. Nous avons également démontré que l'ARNm codant l'isoforme fonctionnelle ou non fonctionnelle de *CASP7* était moins exprimé dans les PBMC des patients PR que chez les contrôles sains. Ces résultats viennent confirmer l'intérêt des études d'expression pour cette maladie.

Dans le chapitre 5, nous avons effectué une étude d'expression génique à grande échelle utilisant 48.701 ADNc des PBMC de patients et contrôles sains. Une expression différentielle de 339 gènes référencés (238 sous exprimés et 101 surexprimés) entre les deux groupes a été observée. Nous avons identifié une expression remarquablement élevée d'un spectre de nouveaux gènes impliqués dans différents mécanismes immunitaires de la PR, tels que: les processus pro-inflammatoires, l'activité antimicrobienne, le stress cellulaire et les fonctions immuno-modulatrices. Nous avons ainsi contribué à de nouvelles connaissances concernant les mécanismes moléculaires de la maladie.

Dans le chapitre 6, nous avons évalué l'interaction entre les facteurs génétiques, biologique et environnementaux chez des patients français atteints de PR. Nous avons identifié une association entre les allèles *HLA* EP et le tabac au sein des patients ACPA positifs, et une tendance positive en ce qui concerne une interaction entre l'allèle *HLA-DRB1**0401 et le tabac dans les patients ACPA positifs avec une PR familiale ou sporadique. Nous avons de plus observé une corrélation entre la dose cumulative de cigarettes fumées et les titres

d'ACPA détectés. Aucune association entre l'allèle *PTPN22*-1858T et le tabac n'a été démontrée dans les patients ACPA positifs. Nous avons ainsi confirmé le rôle du tabac dans l'étiologie de la PR.

Différentes collaborations ont été menées, en réalisant des études de familles ou des études cas-contrôles, sur des gènes de susceptibilité à la PR pour des populations d'origine ethnique différente (Annexes).

Ces différentes approches complémentaires ont permis l'identification de nouveaux gènes et la mise en évidence d'interactions gène-autoanticorps-environnement, contribuant ainsi à une meilleure compréhension des mécanismes pathologiques de la PR et pourra ainsi mener à l'identification de biomarqueurs innovants dans le cadre de l'amélioration des méthodes de diagnostic et de thérapie.

Chapter 1

Introduction

1. Rheumatoid Arthritis brief history

Over 100 years ago Paul Ehrlich first proposed the concept of *horror autotoxicus*, the unthinkable possibility that an organism's immune system would mount a response against itself. Today, we know that breakdown of self-tolerance is not uncommon and can result in serious immune-mediated damage and destruction of an individual's own cells and tissues (Silverstein, 2005). There are currently over 80 diseases, including Rheumatoid Arthritis (RA), classified as autoimmune affecting approximately 5% of the human population. Moreover, autoimmune disorders are among the top 10 leading causes of death worldwide. Evidence of RA in Europe first appeared in early 17th century art, especially by the Dutch Masters and Sydenham published the first case report in 1676. Although intermittent case series were subsequently reported, the disease was not fully recognized until it was defined by Garrod in 1859. He named it 'rheumatoid' arthritis to distinguish it from the two well-known forms of arthritis, rheumatic fever and gout. By the early 20th century, RA was viewed as separate from osteoarthritis (OA) ('arthritis deformans'). In 1957, Charles Short described RA definitively and clearly set it apart as a defined clinical entity distinct from the seronegative spondyloarthropathies, crystal-induced disease, OA, systemic lupus erythematosus (SLE), and many other conditions (Firestein, 2003).

2. Rheumatoid Arthritis clinical aspects

RA is a chronic inflammatory polyarthritis, affecting multiple diarthroidial joints in a characteristic distribution, and leading to pain, joint deformities and a reduced quality of life (Firestein, 2003). Patients commonly present with pain and stiffness in multiple joints, although one third of patients initially experience symptoms at just one location or a few scattered sites. In most patients, symptoms emerge over weeks to months, starting with one joint and often accompanied by prodromal symptoms of anorexia, weakness, or fatigue. In approximately 15 percent of patients, onset occurs more rapidly over days to weeks. In 8 to 15 percent of patients, symptoms begin within a few days of a specific inciting event, such as an infectious illness. Joints most commonly affected are those with the highest ratio of synovium to articular cartilage. The wrists are nearly always involved, as are the proximal interphalangeal and metacarpophalangeal joints. The distal interphalangeal joints and sacroiliac joints tend not to be affected (Harris, 2005). Rheumatoid joints typically are boggy, tender to the touch, and warm, but they usually are not erythematous. Some patients complain of "puffy" hands secondary to increased blood flow to inflamed areas. Prominent epitrochlear, axillary, and cervical lymph nodes may be noted. Low-grade fever, fatigue, malaise, and other

systemic complaints may arise, especially in an acute presentation (Akil and Amos, 1995). RA preferentially affects women with a sex ratio of 2 to 4. Its mean age of onset is between 45 and 50 years of age and the prevalence is about 1% of the population worldwide (Firestein, 2003). From Garrod's initial definition of RA as a disease in 1859, current classification criteria were developed by the American College of Rheumatology (ACR) in the mid 1980s (Arnett et al., 1988) which replaced the earlier existing New York classification criteria (Bennett and Burch, 1967). The ACR 1987 revised criteria, represented in Table 1.1, which have served so well in selecting patients for clinical trials, are now becoming less relevant, partly because of the success of these same trials.

Table 1.1. ACR 1987 revised criteria for classification of RA (Arnett et al., 1988).

A patient is said to have RA if he or she meets at least four criteria
1. Morning stiffness lasting at least 1 h, present for at least 6 weeks.
2. At least three joint areas simultaneously with soft-tissue swelling or fluid, for at least 6 weeks
3. At least one area swollen in a wrist, metacarpaophalangeal, or proximal interphalangeal joint, for at least 6 weeks
4. Simultaneous involvement of the same joint areas on both sides of the body, for at least 6 weeks.
5. Subcutaneous nodules seen by a doctor.
6. Positive rheumatoid factor.
7. Radiographic changes on hand and wrist radiographs (erosions or unequivocal bony decalcification)

This classification has some limitations, represented in Table 1.2, because some criteria are generally not present and/or not specific and sensitive enough for diagnosis in the absence of other markers at the best time for early diagnosis and initiation of treatment. Furthermore, the

enhanced understanding of RA molecular pathogenesis reveal that the presence of rheumatoid factor (RF) is not specific for RA but is rather a general consequence of immune activation in the context of immune complex formation (Nemazee, 1985; Tarkowski et al., 1985). In addition, no experimental studies have demonstrated any proarthritogenic effects of RF.

Table 1.2. Limitations and consequences of ACR criteria (Klareskog et al., 2009).

Criteria	Limitations	Consequences
Polyarthritis (>3 joint areas) with hand involvement, symmetric distribution, and morning stiffness	Clinical variables that are not specific and sensitive enough for diagnosis in the absence of other markers	Criterion will still be valid but will most probably include fewer affected joints and a less typical distribution because development of new diagnostic methods will enable earlier diagnosis
Rheumatoid nodules	Better and earlier disease control reduces the likelihood of seeing rheumatoid nodules	Criterion will still be valid but will be relevant for only a few patients
Radiographic changes on plain radiographs	Diagnostic value diminishes because diagnosis and treatment should ideally be started before erosions arise	Development of more sensitive joint imaging methods will probably lead to earlier recognition and new definitions for joint destruction
Serum positive RF	Other serum markers with equal or better diagnostic power have been described	Other serum markers must be added to the criterion. Antibodies to citrullinated protein antigen presence have similar sensitivity to and better specificity than rheumatoid factor for diagnosis.

Thus, based on enhanced understanding of disease pathogenesis, new definitions were needed for RA and its subsets that can be used for early diagnosis and treatment decisions. The European League Against Rheumatism (EULAR) and ACR are currently collaborating to produce such classification criteria (Klareskog et al., 2009).

3. Descriptive epidemiology in Rheumatoid Arthritis

The study of the epidemiological profile of a specific disease includes its frequency, severity, and distribution among different populations and human groups (descriptive epidemiology), as well as the influence of genetic and environmental factors on the occurrence and variation of the disease (analytical epidemiology risk factors) (Alamanos and Drosos, 2005).

Several incidence and prevalence studies of RA have been reported during the last decades, suggesting a considerable variation of the disease frequency among different populations (Gabriel et al., 1999; Simonsson et al., 1999; Saraux et al., 1999; Symmons et al., 2002). Studies of the descriptive epidemiology of RA have methodological differences which include the methods of case identification and case recording, as well as the type of incidence and prevalence rates. Some studies do not present age-adjusted rates. In addition, adjusting methods for age differ among studies or are not described in the manuscripts. However, the methodological differences of the studies could only partly explain the differences in RA occurrence observed across areas. These differences could also be related to medical practice, access to care, and variability in prevalence of environmental and genetic risk factors. These methodological issues may affect the results of studies comparing the occurrence of the disease among different countries and areas, or investigating the time trends of the disease. However, it is likely that RA descriptive epidemiology presents some characteristic trends, which could be considered as independent from methodological issues (MacGregor and Silman, 2003).

3.1. Geographic variation

3.1.1. Prevalence

RA affects about 1% of the adult population worldwide. The median prevalence estimate for the total population in south European countries is 3.3 (range 3.1 to 5.0) cases per 10^3, for north European countries 5.0 (range 4.4 to 8.0), and for developing countries 3.5 (range 2.4 to 3.6). A study from North America showed a prevalence estimate of 10.7 cases per 10^3. The overall distribution of prevalence for the male population differs significantly among different areas of the world. The difference observed is marginally significant for the female population. The few prevalence studies performed in developing countries indicate a significantly lower frequency of the disease in these countries. This lower frequency observed could partly be related to lower occurrence of the disease in developing countries, but may also reflect differences in the age distribution between the populations studied. In addition,

cases with mild RA may be less likely to be ascertained, depending on access to medical care. This may lead to a relative underestimation of RA occurrence for the studies based on medical records (Alamanos et al., 2006). The lack of studies performed in other parts of the world such as Africa, Russia, and large parts of Asia may also represent a limitation. However, there are some interesting exceptions. Native American-Indian populations have the highest recorded occurrence of RA, with prevalence of 5.3% noted for the Pima Indians (del Puente et al., 1989) and of 6.8% for the Chippewa Indians (Harvey et al., 1981). By contrast, there are a number of groups with a very low occurrence. Studies in rural African populations, both in South Africa (Brighton et al., 1988) and in Nigeria (Silman et al., 1993a), failed to find any RA cases in studies of 500 and 2000 adults, respectively.

3.1.2. Incidence

The overall distribution of incidence rates does not differ significantly among different areas of the world. The median annual RA incidence for the total population observed in south European countries is 16.5 (range 9 to 24) cases per 10^5. For North European countries the median annual incidence observed was 29 (range 24 to 36), and for North American countries 38 (range 31 to 45). There is a statistically significant difference observed between North and South European countries for the male population only. South European countries have lower median incidence and prevalence rates than North European and North American countries, although these differences were not statistically significant for the female population. However, the lack of statistical significance could be related to the small number of studies from southern Europe. In addition, some of these studies are based on relatively small sample sizes (Alamanos et al., 2006).

3.2. Time trends

Concerning the changes of RA occurrence over time, the existing data are limited. Only the study by Doran and colleagues (2002) examined the evolution of disease occurrence for a long period (4 decades), applying the ACR criteria retrospectively, based on medical records for a defined area of the USA. This study demonstrates a significant decline of RA incidence over the past decades. The study of Kaipiainen-Seppanen and colleagues (2001) also suggests a slight decline of RA incidence in Finland between 1980 and 1990. However, it is difficult to suggest a general trend of decreasing RA incidence for all countries based on the results of these studies. More data from several countries are needed, investigating the occurrence of the

disease over time in defined populations, based on ACR criteria and using the same methods and sources of case ascertainment for each time period (Uhlig and Kvien, 2005).

3.3. Mortality and survival

Mortality rates are higher among RA patients than in the general population. The mortality rates reported vary widely among studies. They are higher in hospital-based studies and relatively lower (but still higher than in the general population) in population based studies. The expected survival of RA patients is likely to decrease 3-10 years according to the severity of the disease and the age of disease onset. The causes of death do not differ significantly among RA patients and the general population they come from. It can be said that the majority of affected individuals die from the same causes as the general population, but at a younger age (Doran et al., 2002; Gabriel et al., 2003).

4. Rheumatoid Arthritis Immunopathology

As previously stated, RA is a chronic autoimmune disease which comprises a syndrome of pain, stiffness, and symmetrical synovitis (inflammation of the synovial membrane) of diarthrodial joints (freely moveable joints such as the knee) that leads to articular destruction, functional decline, and substantial comorbidity in the cardiovascular, neurologic, and metabolic systems. Extensive genetic and pathogenetic studies indicate dysregulation in both innate and adaptive immune compartments. These lead to an elaboration of autoantibody responses and dyslipidemia, which might predate clinical disease onset by up to a decade. Transition occurs thereafter to articular localization *via* mechanisms as yet unknown, and this leads to chronic synovitis (Brennan and McInnes, 2008).

4.1. Innate immunity

Innate immunity is the first line of defense against pathogenic microorganisms (bacteria, viruses, fungi, and parasites). After a long period of neglect, innate immunity is again recognized as a key mechanism not only in preventing invasion of the body by microorganisms, but also in contributing to the pathogenesis of autoimmune and inflammatory diseases by deviating the immune response or promoting the emergence of a regulatory response. The many factors involved in innate immunity often act in parallel or in alternation to generate adaptive immune responses (Falgarone et al., 2005). These factors include antibacterial peptides, the alternate pathway of complement activation, mannose-

binding lectin, cytokines macrophages, dendritic cells (DCs), neutrophils, natural killer (NK) cells and T γδ lymphocytes (Medzhitov and Janeway, 2000). The cellular and protein effectors of innate immunity are found in the rheumatoid synovium, and an increasing body of evidence indicates that they are directly involved in joint inflammation and in destruction of the joint cartilage and bone (Arend, 2001).

4.1.1. Interaction between Microorganisms, Macrophages and Dendritic cells

Microorganisms (bacteria, virus and mycobacteria) have long been incriminated in the pathophysiology of non infectious chronic arthritis, although proof of a direct role has not been obtained (van der Heijden et al., 2000). Macrophages and DCs are stimulated by pattern recognition receptors (PRRs) present on their surface. PRRs recognize pathogen associated molecular patterns (PAMPs), which are common to many microorganisms. The innate response triggered by PRRs is immediately maximal, whereas adaptive responses build up gradually and reach their maximum intensity only after re-exposure to the antigen (Falgarone et al., 2005). One of the PRRs present in these cells contributes to signal transduction and include the toll-like receptors (TLRs). TLRs cellular recognition of PAMPs activates an innate immune response characterized by the production of polyreactive antibodies, cytokines and chemokines (Klinman, 2003). TLR-2 mediates recognition of bacterial lipoproteins and peptidoglycans (PGs) (Schwandner et al., 1999), TLR-3 binds to viral double-stranded RNA, TLR-4 recognizes the bacterial cell membrane components lipopolysaccharide (LPS) and lipoteichoic acid, TLR-5 recognizes flagellin (Hemmi et al., 2000), and TLR-9 interacts with unmethylated CpG motifs present in bacterial DNA inducing activation of macrophages, DCs and B cells (Takeshita et al., 2001). Several studies identified bacterial components in synovial membrane from patients with active or inactive inflammatory joint disease, including RA (van der Heijden et al., 2000; Schrijver et al., 2000). Generally, the component was DNA, RNA, or a PG. PGs are found in many bacteria that normally inhabit the mucous membranes. They were typically observed within macrophages or DCs, suggesting that they could have been transported actively to the synovial membrane (Arend, 2001). Evidence that some macrophages have a selective tropism for the synovial membrane provides support to this possibility (Salmi et al., 1997). Furthermore, histological studies of synovial membrane samples have revealed large numbers of mature DCs, probably derived from immature circulating cells (Thomas et al., 1994). These mature cells express membrane markers for activation (Thomas and Quinn, 1996) and cell markers, such as RelB, which is a transcription activating factor (Pettit et al., 2000). Although a direct role remains unproven, these cells

exhibit two characteristics that make them key factors in synovial inflammation: one is plasticity, with the ability to promote either a T helper (Th) 1 or a Th2 response according to the cytokine environment, and the other is the ability to present antigens derived from apoptotic cells. Mature DCs may indirectly modulate synovial inflammation in response to stimulation by a virus since some viruses can produce cytokine receptors that, in turn, modulate cytokines involved in inflammation (Falgarone et al., 2005). Thus, DCs along with macrophages produce innate immune inflammatory mediators, and these mediators drive inflammatory pathology in RA (Lutzky et al., 2007). The hypothetical interaction between innate and adaptive immunity is represented in Figure 1.1.

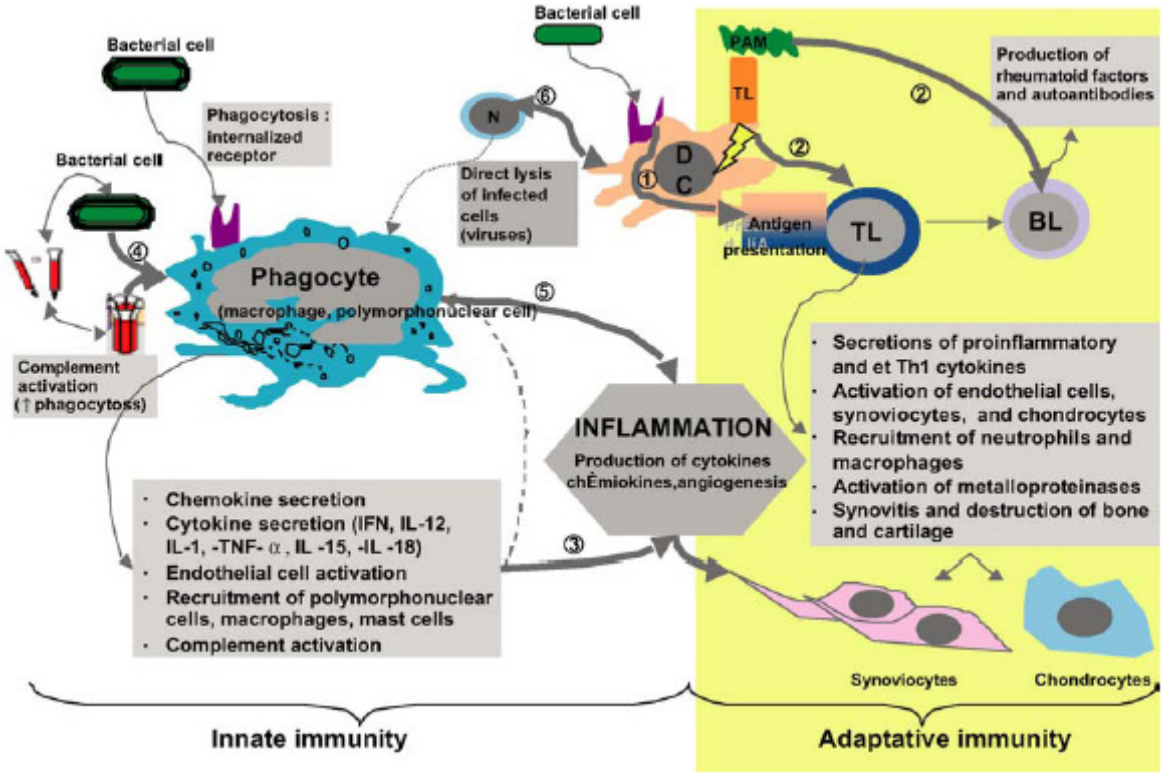

Figure 1.1. Hypothesis of the interaction between innate immunity and adaptive immunity in RA patients (Falgarone et al., 2005).

4.2. Cytokines

RA synovial membrane have activated B and T cells, sometimes organized into germinal center–like structures, plasma cells, mast cells and activated macrophages. These cells are recruited *via* an intense neovascularization process with associated lymphangiogenesis. Moreover, host tissue cells (activated synovial fibroblasts, chondrocytes and osteoclasts) are involved, mediating cartilage and bone destruction as well as feeding back to promote

perpetuation of inflammation. The cellular interactions among macrophages, T cells, B cells, and nonhematopoietic cells (fibroblasts, connective tissue cells and bone) thought to be of importance in the pathogenesis of RA are facilitated by the actions of cytokines released from the activated cells that then, through both autocrine (feedback on same cell) and paracrine (*via* other cell types) mechanisms, induce the production of other proinflammatory cytokines, which together contribute to the pathogenesis of this disease (Figure 1.2) (Brennan and McInnes, 2008). The cytokines pathogenic potential has been identified based on *ex vivo* studies from ill tissue and *in vivo* studies on animal models (Feldmann et al., 1996; Arend et al, 1998; Kishimoto, 2006). These low molecular weight proteins comprise four big families: interleukins (IL), interferons (IFN), tumor necrosis family and chemokines. Several cytokines are therefore involved in RA pathogenesis like tumor necrosis factor (TNF), IL-1, IL-6, and IL-23 superfamilies, cytokines that bind a receptor containing the common γ-chain (γc) and chemokines (Brennan and McInnes, 2008).

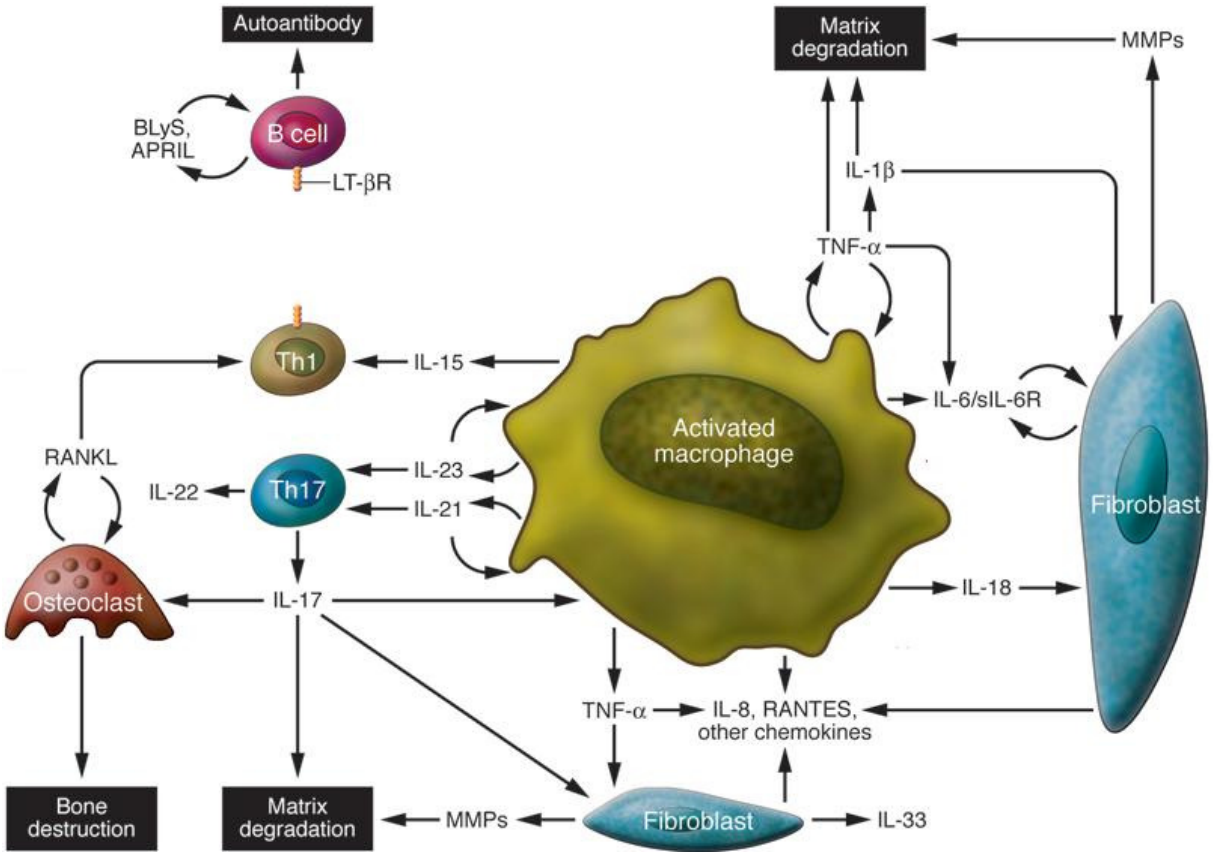

Figure 1.2..Cytokines network in RA synovium pathogenesis (adapted from Brennan and McInnes, 2008).

4.2.1. Tumor Necrosis Factor superfamily

The demonstration that tumor necrosis factor, alpha (TNF-α) play a key role in RA was observed *in vitro* by its potential to degrade cartilage (Dayer et al., 1985) and bone (Bertolini et al., 1986). TNF-α is produced mainly by activated macrophages in the inflamed synovial membrane tissue of RA patients. The actions of TNF-α believed to be important in the pathogenesis of RA were represented in Figure 1.3. Recently, interest has turned to the usefulness of additional members of the TNF superfamily in RA pathogenesis like lymphotoxin beta (LTβ) (Takemura et al., 2001), B lymphocyte stimulator (BLyS), proliferation-inducing ligand (APRIL) (Seyler et al., 2005) and receptor activator of nuclear factor-kappa B ligand/receptor activator of nuclear factor-kappa B/osteoprotegerin (RANKL/RANK/OPG) pathway (Schett et al. 2005).

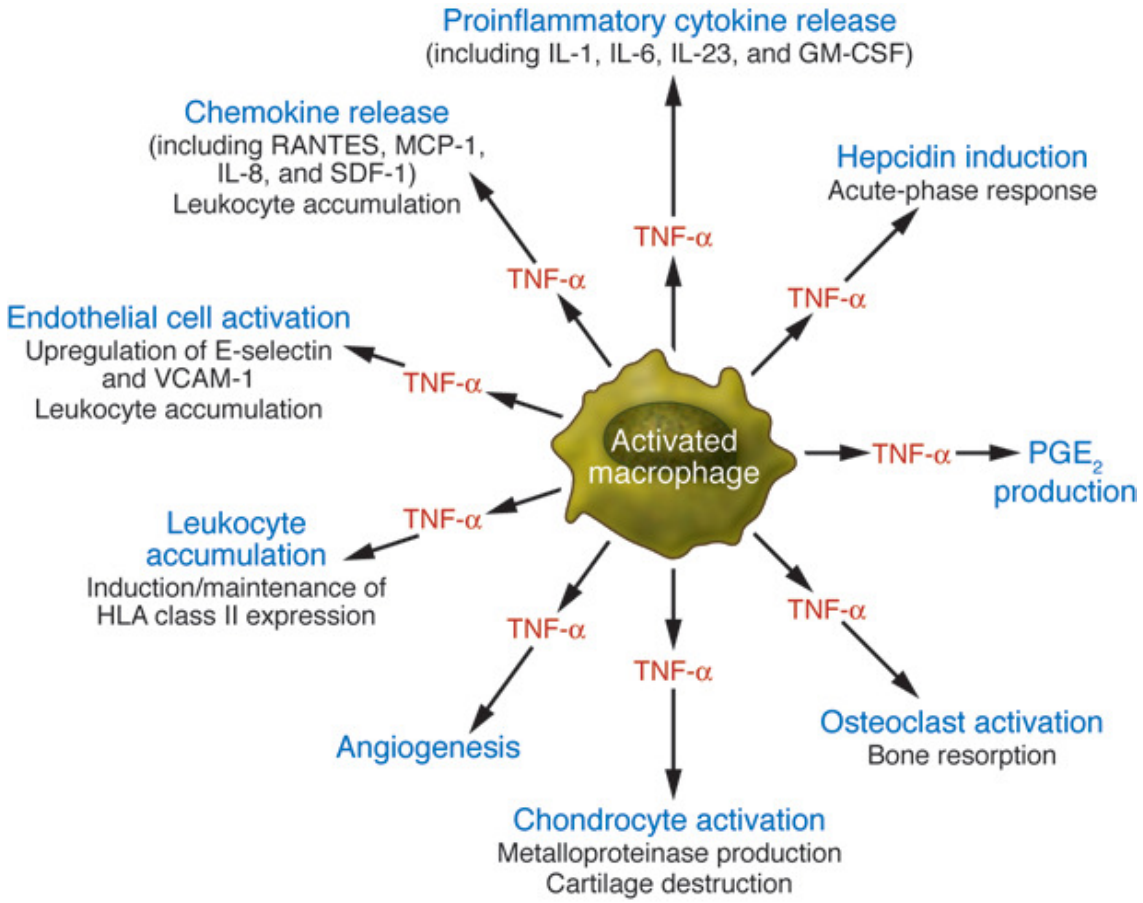

Figure 1.3. Actions of TNF-α relevant to RA pathogenesis. Granulocyte-macrophage colony-stimulating factor (GM-CSF); Regulated upon activation, normally T-expressed, and secreted (RANTES); Monocyte chemoattractant protein-1 (MCP-1); Stromal cell-derived factor 1 (SDF-1); Prostaglandin E2 (PGE_2); vascular cell adhesion molecule 1 (VCAM-1) (Brennan and McInnes, 2008).

4.2.2. Interleukin 1 superfamily

Several members of the IL-1 superfamily such interleukin 1, alpha (IL-1α), interleukin 1, beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-18, IL-32, interleukin 32, gamma (IL-32γ) and IL-33 have been implicated in the pathogenesis of RA. IL-1α and IL-1β as well as the natural IL-1ra are expressed in abundance in the synovial membrane. IL-1α and IL-1β *in vitro* induce cytokine production by synovial mononuclear cells, catabolism and cytokine production by chondrocytes, prostanoid and metalloproteinases (MMPs) release by fibroblasts and bone erosion by osteoclasts (Dayer, 2003). In several rodent models of arthritis, targeting IL-1 and components of the receptor for IL-1 is effective in reducing inflammation and articular damage (van den Berg et al., 1999; Joosten et al., 1999), and when mice created to express human TNF-α were crossed with IL-1-deficient mice, although synovial inflammation still occurred, significantly reduced bone erosion and osteoclasts formation was observed (Zwerina et al., 2007). IL-18 is detected in RA synovium and its blockade in rodent models of RA using neutralizing antibody is effective (Plater-Zyberk et al., 2001). IL-18-deficient mice exhibit ameliorated collagen-induced arthritis (CIA) (Wei et al., 2001). CIA, a rodent model of the human disease RA, is induced by immunization with heterologous type II collagen emulsified in complete Freund's adjuvant (Courtenay et al., 1980). Development of CIA is dependent on both cell-mediated and humoral responses and is characterized by cellular infiltration and synovitis of the joints, resulting in severe swelling of the paws and progressive destruction of bone and cartilage (Seki et al., 1988). IL-32 was revealed as an IL-18-inducible inflammatory cytokine in epithelial tissues (Kim et al., 2005). IL-32γ is expressed by synovial macrophages and promotes PGE_2 and cytokine synthesis in vitro (Joosten et al., 2006). *In vivo*, IL-32 induces TNF-α-dependent articular inflammation but TNF-α-independent matrix degradation (Shoda et al., 2006). IL-33 has been recently identified as a novel IL-1-like cytokine based in its structural and functional similarities to other IL-1 family members (Schmitz et al., 2005). IL-33 expression was detected in RA synovial membrane, predominantly in fibroblast-like synoviocytes (FLS). IL-33 administration exacerbates CIA in mice, and mice lacking IL-33R as well as mice administered a natural antagonist of IL-33 show reduced disease (Xu et al., 2008)

4.2.3. Interleukin 6 superfamily

IL-6 has pleiotropic functions including effects on the maturation and activation of B and T cells, macrophages, osteoclasts, chondrocytes and endothelial cells and effects on hematopoiesis in the bone marrow. *Il6* deletion protects DBA/1 mice from CIA (Kishimoto

2005), and neutralization of IL-6 using antibodies specific for either the cytokine or the α-chain of its receptor (IL-6R) ameliorates disease (Alonzi et al., 1998). Nishimoto and colleagues (2004) demonstrated that targeting IL-6R suppresses significantly the inflammation and clinical disease activity. The role in RA pathogenesis of other members of the IL-6 family, including leukemia inhibitory factor (LIF) and oncostatin M (OSM), is under ongoing investigation (Katoh et al., 2007).

4.2.4. Interleukin 23 superfamily

IL-12 is released by antigen presenting cells (APCs), DCs, and monocytes/macrophages in response to bacterial products and immune signals inducing the development of Th1 cells that secrete interferon gamma (IFN-γ) (Trinchieri, 2003). The role of this cytokine in RA pathogenesis presents conflicting results. Whereas some studies showed a direct correlation between disease activity and elevated IL-12 in the serum and synovial fluid (Kim et al., 2000) other surveys did not find increased levels of IL-12 protein in individuals with RA (Brennan and McInnes, 2008). However in a rodent model, IL-12 administration results in severe arthritis that is associated with improved IFN-γ production (Germann et al., 1995). Further, the severity of arthritis in this model is attenuated with antibodies specific for the mouse IL-12 (Malfait et al., 1998), although continued treatment in established CIA exacerbates disease. Thus, IL-12 has a regulatory role in mouse CIA, with an early proinflammatory and late antiinflammatory effect (Joosten et al., 1997). Subsequently, it was hypothesized that the autoimmune actions of IL-12 were in fact attributable to the newly discovered cytokine IL-23 (Oppmann et al., 2000). IL-23 is secreted by activated DCs and macrophages and binds to memory T cells, NK cells, macrophages, and DCs (Verreck et al., 2004). Mice lacking IL-23 were protected from CIA (Becher et al., 2002). IL-23 induces the proliferation of a newly described subset of memory T cells, known as Th17 cells, that produce IL-17A, IL-17F, IL-6, TNF-α, GM-CSF and IL-22 (Aggarwal et al., 2003; Liang et al., 2006). IL-17 - the signature proinflammatory cytokine produced by Th17 cells - acts on multiple cell types found in inflamed rheumatoid joints: monocytes, macrophages, fibroblasts, osteoclasts and chondrocytes. IL-17 induces a wide range of effector molecules that have been implicated in joint damage, including proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α), multiple chemokines, cyclooxygenase-2, PGE_2, and matrix MMPs. IL-17 upregulates RANKL on chondrocytes and osteoblasts, thus promoting the development of osteoclasts, which are the destructive element in the bone erosions of RA (Kolls and Linden, 2004). Moreover, the neutralization of IL-17A in mice decreased the severity of antigen-induced arthritis (Koenders

et al., 2005) and severity of CIA is reduced in IL-17-deficient mice and mice administered IL-17-neutralizing antibodies (Lubberts et al., 2005).

4.2.5. Cytokines that bind a receptor containing γc superfamily

This family of cytokines includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Particular attention has focused in the role of IL-15, IL-7 and IL-21 in RA pathogenesis. IL-15 is present in the RA synovial membrane and induces release of IFN-γ, IL-17, TNF-α and chemokines (McInnes et al., 1997). Moreover, IL-15 blockade in a rodent model reduces CIA severity, whereas recombinant IL-15 can amplify disease in the absence of complete adjuvant at immunization (Ruchatz et al., 1998). Preclinical data are rising for an inflammatory role for IL-7 in RA synovitis. IL-7 is detected in low concentrations in blood (Ponchel et al., 2005) and synovial membrane, and addition of IL-7 to synovial cultures is proinflammatory (Harada et al., 1999). IL-7 amplifies the effects of interactions between T cells and macrophages (van Roon et al., 2005), and has been shown to modify chondrocyte function (Long et al., 2008). Moreover, it promotes osteoclastogenesis *via* RANKL-dependent pathway (Weitzmann et al., 2000). Additionally, persistent IL-7 expression has been detected in patients with RA following TNF-α blockade, suggesting some autonomy from TNF-α-dependent regulation (van Roon et al., 2001). IL-21 is present in the synovial fluid of individuals with RA. IL-21 mRNA and protein are detected in synovial membrane, and addition of IL-21 to synovial T cell cultures induces activation and cytokine release (Li et al., 2006). Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in RA animal models (Young et al., 2007). Furthermore, neutralization of IL-21 in synovial cultures suppresses TNF-α, IL-1, and IL-6 release (Andersson et al., 2008).

4.2.6. Chemokines

Migration of leukocytes into the synovium is a regulated multi-step process, involving interactions between leukocytes and endothelial cells, cellular adhesion molecules, as well as chemokines and chemokine receptors (Szekanecz et al., 2006). Chemokines are chemoattractant cytokines which play key roles in the accumulation of inflammatory cells at the site of inflammation. Synovial tissue and synovial fluid from RA patients contain increased concentrations of several chemokines such as MCP-1, RANTES, Macrophage inflammatory protein, monocyte chemoattractant protein-4, pulmonary and activation-regulated chemokine, monokine induced by IFN-γ, SDF-1 and Fractalkine (Haringman et al., 2004). These cytokines have an important role in the pathogenesis of RA by recruiting

leukocytes and by controlling other important processes, such as release of mediators of inflammation, cell proliferation and angiogenesis (Iwamoto et al., 2008). Moreover, animal and human studies have shown the biological efficacy of specific antagonism of chemokines ligands and receptors in RA (Haringman et al., 2004; Okamoto and Kamatani, 2006).

4.3. Adaptive immunity

In contrast with innate immunity, adaptive immunity involves B and T cells that specifically recognize non-self antigens and can distinguish between self and non-self. Although innate immunity and adaptive immunity have opposite characteristics, they are linked by a rich network of interactions, most notably in the rheumatoid synovium. In a reversal of conventional immunology in which the innate immune system activates the adaptive one, it is believed that in RA and likely in other entities this relationship can operate in the opposite direction, activating macrophages and leading to inflammatory damage induced by an activated adaptive immune system (Firestein and Zvaifler, 1990; Smolen and Steiner, 2003).

4.3.1. B cells

B cells play an essential role in regulating immune responses, and dysregulation of B cells responses may consequently lead to an attenuated immune response and potentially to the development of autoimmune disease (Golovkina et al., 1999). B cells present in RA synovial tissue express CD20, a surface antigen expressed only on pre-B cells and mature B cells and lost before differentiation of B cells into plasma cells (Edwards et al., 2004a). B cells are precursors of autoantibodies-secreting plasma cells. Several specific autoantibodies are present in RA like RF, antibodies to citrullinated protein antigen (ACPA), anti-keratin (AKA), anti-perinuclear factor (APF) providing indirect evidence for humoral disturbances in RA patients, especially in those with high disease activity (Waaler, 1940; Nienhuis et al., 1964; Young et al., 1979; Schellekens et al., 1998). Autoantibodies form and increase immune complexes leading to activation of B cells and follicular dendritic cells (fDCs) *via* Fc receptors and complement receptors 1 and 2 (CD21 and CD35) expressed on their surfaces, contributing to activation of the immune system. B cells also express a specific B cell receptor (BCR) and all these receptors, similar to the toll like receptors, combine the innate and adaptive immune system on the surface of B cells (Carroll, 2004). Immune complexes can be processed by B and other cells for antigen presentation, thus enhancing local inflammatory processes. B cells are able to internalize and process antigens into antigenic peptides acting like APCs (Martinez-Gamboa et al., 2006). These antigenic peptides are then efficiently

presented by B cells *via* major histocompatibility complex (MHC) class II molecules (Stastny, 1976). Through antigen presentation by B cells, T cells become activated, leading to further TNF-α production, which consequently activates macrophages (Weyand et al., 2000). One of the central effector mechanisms of activated immune cells is the production of cytokines. This is extensively known for T cells and macrophages, and it has also been demonstrated for B cells (Lund et al. 2005). Stimulated B cells secrete TNF-α, LTβ and IL-6, which can act not only as autocrine growth and differentiation factors, but can also amplify immune responses (Dorner and Burmester, 2003). Additionally, they produce IL-10, which activates fDCs and also stimulates B cell function *via* a feedback loop, spreading chronic inflammation (Fillatreau et al., 2002). Although cytokine production by B cells in RA synovitis appears to be of pathogenic importance, this has not been studied in larger detail yet. Enhanced local B cell activation has also been reported by Ohata and colleagues (2005), who showed that BlyS is produced by FLS in RA synovium and contributes to improved B cell survival. The production of BlyS was shown to be increased under the influence of TNF-α or IFN-γ, indicating a close collaboration of different cell types in the inflamed synovium.

Moreover, recent data provide evidence that B cell activation can also occur in the absence of direct T cell help by using CD32, TLRs and transmembrane activator and calcium-modulator and cyclophilin ligand interactor dependent activation in addition to BCR-ligation (Boackle, 2003).

4.3.2. T cells

RA pathogenesis was typically viewed as involving two hierarchical systems, governing inflammation and autoimmunity, respectively. The key players in this concept were the pro-inflammatory cytokine TNF-α and the Th1 subset of Th cells (Falgarone et al., 2007). The identification of Th17 T cells and regulatory T cells (Treg) has challenged this concept for the first time in two decades (Steinman, 2007). CD4+ T cells, or Th cells, are at the centre of this concept, which was developed in the 1980s. Many studies, most of which relied on animal experiments, led to the belief that RA was a Th1-driven disease (Feldmann et al., 1996). The Th1 phenotype is associated with inflammation, whereas the Th2 phenotype combats inflammation to some extent. IFN-γ is the characteristic Th1 cytokine. Differentiation of naive T cells into Th1 cells is accompanied with the production of inflammatory cytokines and chemokines, including IFN-γ, LTβ, and TNF-α. According to the classical paradigm, Th1 cytokines both prevent naive T cells from differentiating into Th2 cells and prevent Th2 cells from producing cytokines including not only anti-inflammatory cytokines (e.g., IL-4 and IL-

13) but also IL-5. The excess of pro-inflammatory cytokines and relative deficiency in anti-inflammatory cytokines defines the Th1/Th2 imbalance, which was believed to drive several autoimmune diseases (known as Th1 diseases) such as RA, type 1 diabetes (T1D), and Crohn's disease (CD) (Boissier et al., 2008). Despite this evidence, the Th1/Th2 imbalance concept fails to explain a number of incontestable facts, such as the absence of IFN-γ within the rheumatoid synovium; the lack of efficacy in most patients of monoclonal antibodies to IFN-γ; and the opposing effects of IFN-γ, which alleviates established arthritis in mice but promotes arthritis in healthy mice (Boissier et al., 1995; Manoury-Schwartz et al., 1997). The identification of the Th17 pathway has renewed interest in a proinflammatory role for T cells in RA (Miossec et al., 2003). Three effector T cells phenotypes have been now identified (Figure 1.4).

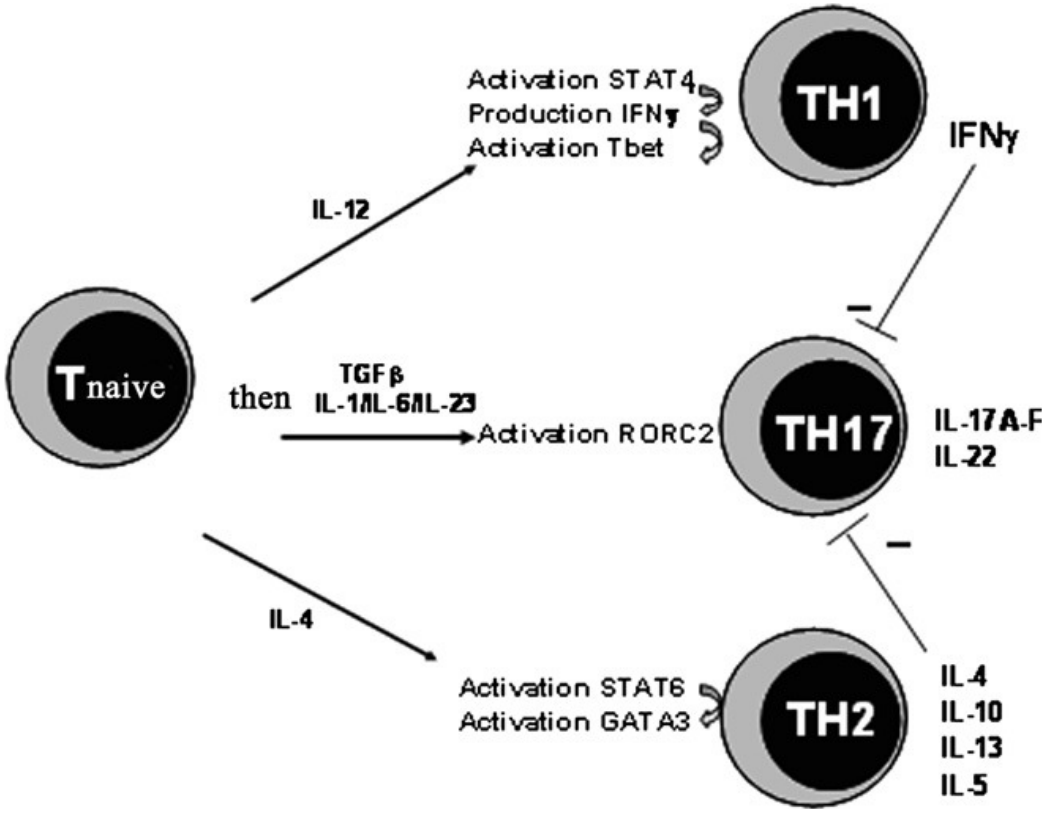

Figure 1.4. Differentiation of the three types of effector T cells in humans. Signal transducer and activator of transcription 4 (STAT4), T-box transcription factor (Tbet), Retinoic acid-related orphan receptor variant 2 (RORC2), Signal transducer and activator of transcription 6 (STAT6), GATA binding protein 3 (GATA3) (Boissier et al., 2008)

Th17 differentiation, which is coordinated by the nuclear receptor ROR-gammat, is initiated by IL-6 acting in concert with transforming growth factor beta (TGF-β) and is improved by TNF-α and IL-1β. IL-23 plays an important role in Th17-mediated tissue damage, probably

by promoting Th17 expansion and survival. Moreover, the effects of IFN-γ on Th17 cells are inhibitory (Annunziato et al., 2007). The already cited cellular targets and biological effects of IL-17 are consistent with the hypothesis that Th17 cells have a key role in mediating synovitis and articular damage in at least a subset of RA patients.

Treg cells are a unique population of CD4+CD25+ T cells whose development needs the transcription factor FoxP3. Treg cells suppress CD4 and CD8 T cell responses though cell-cell contact and play an essential role in maintaining peripheral tolerance to self. They produce IL-35, which synergizes the cell-to-cell contact that leads to induced suppression. Because of the importance of Treg cells in suppressing organ specific and systemic autoimmunity in rodent models, an attractive hypothesis is that quantitative or qualitative abnormalities in Treg cells contribute to inflammation in RA (Liepe et al., 2005). Moreover, Treg cells are enriched in RA patients' synovial fluid relative to peripheral blood (van Amelsfort et al., 2004). The phenomenon of synovial enhancement needs to be clarified to understand the persistence of synovial inflammation still in the presence of Treg cells. Several studies shows that natural Treg cells in the peripheral blood of RA patients with active disease appear to be functionally abnormal suggesting a model in which high levels of TNF-α within inflamed rheumatoid synovium limit the activity of Treg cells. (Ehrenstein et al., 2004; Valencia et al., 2006; Nadkarni et al., 2007)

In RA, the site of T cell differentiation to Th17 or Th1 cells remains uncertain and the starting point of the activation cascade is unknown. There is a certain evidence for involvement of environmental factors, genetic factors and synovial vascular factors. Lymphocyte activation resulting from stimulation of T cell adaptive immunity and innate immunity leads to loss of homeostasis. Early on in the course of inflammatory processes, the production of pro-inflammatory cytokines exerts long-lasting effects: the balance tips toward Th17, which perpetuates the inflammatory and destructive processes. However the treatment of inflammatory autoimmune diseases by the expanding (or transferring) of Treg population should be considered carefully and take in consideration that pro-inflammatory effects of IL-17 can persist despite the presence of Treg. According to the new possible hypothesis, RA is characterized by a Th1/Th17 imbalance (with predominance of Th17). An attractive possibility is that a Th17/Treg imbalance plays an essential role. Although changing the Th1/Th2 concept for RA appears significantly appealing, the new Th1/Th17 imbalance hypothesis needs to be confirmed *via* application to the development of new treatment targets (Boissier et al., 2008).

4.3.3. Rheumatoid Arthritis-Synovial Fibroblasts

Rheumatoid Arthritis-Synovial Fibroblasts (RA-SFs) are mainly found in the synovial sublining of RA joints. The most characteristic functional feature is the ability of RA-SFs to adhere to cartilage and to initiate the degradation of extracellular matrix (ECM) (Fassbender, 1983). Some studies supporting that the activated phenotype of RA-SFs is an intrinsic property of these cells. The best evidence concerns the severe combined immunodeficient mice (SCID) in which synovial fibroblasts are implanted with human cartilage under the renal capsule for 60 days. During this period, RA-SFs attach and invade into the cartilage in a similar way as observed in the human joint. Most interestingly is the fact that the invasion of RA-SFs occurs without any support from the immune system (Muller-Ladner et al., 1996). Synovial hyperplasia could be due to an increased rate of proliferation of the RA-SFs. This concept is supported by the increased expression of transcription factors, markers of proliferation and growth factors, including platelet derived growth factor, basic fibroblast growth factor and TGF-β (Butler et al., 1989; Melnyk et al., 1990). RA-SFs could contribute to synovial hyperplasia decreasing the rate of apoptosis (Baier et al., 2003). Although Fas ligands are present in synovial fluid, the cells appear resistant to apoptosis (Asahara et al., 1996). Several studies examined the expression of anti-apoptotic molecules in RA-SFs demonstrated that sentrin and caspase 8 have not only been shown to interact with the death domain of the receptor and inhibit downstream signalling, but also to be expressed by RA-SFs especially at sites of invasion into cartilage and bone (Franz et al., 2000; Schedel et al., 2002). Moreover, RA-SFs stimulation with macrophage migration inhibitory factor (MIF) reduces the rate of apoptosis in RA-SFs. Antigen induced mouse MIF knock-out has reduced arthritis severity due to high expression of p53 and apoptosis in synovium (Leech et al., 2003). Additionally, activated RA-SFs maintain the ongoing local inflammatory immune response in the joint. The production of IL-15, 16, 17 and SDF-1 from RA-SFs appear responsible for T cell activation (Franz et al., 1998). B cell chemoattractant has been shown to promote the migration of B cells into the synovium (Shi et al., 2001). The expression of members of the IL-6 family such as LIF and OSM are expressed by RA-SFs at the site of invasion (Okamoto et al., 1997). Another critical mediator of inflammation is the production of PGE_2 and RA-SFs secrete high amounts of PGE_2 in the inflammatory synovium (Kojima et al., 2003).

The destructive potential of RA-SFs is revealed by their ability to attach to certain cartilage components. RA-SFs preferentially attach to fibronectin, type VI collagen and cartilage oligomeric matrix protein (Neidhart et al., 2005). The degradation of the ECM is caused by the action of MMPs and cathepsins (Keyszer et al., 1998). RA-SFs secrete MMP-1, MMP-3

and MMP-13 and produce MMP-14 and MMP-15 which activate MMP-2 and MMP-13 (Konttinen et al., 1999). Cathepsins are produced by RA-SFs at sites of invasion and thought to contribute significantly to the joint damage (Keyszer et al., 1998).

4.3.4.. Osteoclasts and intra-cellular signalling pathways

Osteoclasts are multinucleated giant cells with bone-resorbing capacity. These effector cells are essential for focal erosion of bone and cartilage in inflammatory arthritis. Blockade of osteoclastogenesis prevents bone erosion in affected joints in animal models of arthritis or in patients with arthritis, regardless of the cause of the inflammation (Pettit et al., 2001; Redlich et al., 2002). Under normal conditions, osteoclastogenesis and bone modelling and remodelling essentially occur within the bone marrow cavity. However, in some bone disorders characterized with local bone loss, such as RA, accelerated osteoclastogenesis takes place at diseased sites outside of the marrow cavity, resulting in abnormal bone resorption, irreversible joint damage and bone pain (Goldring, 2003). Localized bone loss around joints represents a combination of a focal inflammatory immune reaction and localized osteoclastogenesis. Activated RA-SFs and T cells produce a large amount of RANKL and macrophage colony-stimulating factor (M-CSF), two cytokines essential for osteoclast formation, promoting the differentiation of osteoclast precursors (OCPs) into mature osteoclasts (Yoshida et al., 1990; Kong et al., 1999). The RANKL/RANK system is crucial for mature osteoclasts formation and is an essential player in T-lymphocyte-mediated osteoclastogenesis (Dougal et al., 1999). Some studies revealed that the RANKL/RANK system induces OCPs to produce pro-inflammatory cytokines and chemokines (Li et al., 2004; Seshasayee et al., 2004) and that RANKL blockade reduces the severity of disease in several animal models of inflammation, indicating that this system may affect inflammatory immune responses by regulating OCPs function (De Klerck et al., 2004). Expression of RANKL is regulated by TNF-α as well as other inflammatory cytokines and non cytokine mediators such as PGE_2 (Walsh et al. 2005). RANKL effector function is modulated by OPG, a soluble decoy receptor also expressed by mesenchymal cells that is present at increased levels in RA synovium. Although inhibition of RANKL through OPG did not influence the severity of inflammation, OPG treatment nonetheless abolished the loss of mineral bone in inflamed joints of arthritic rats in a dose-dependent manner (Tanaka et al., 2005).

Interaction between RANKL to its cognate receptor RANK results in the activation of signalling cascades controlling lineage commitment and activation of osteoclasts. However, given the broad expression pattern of both the ligand and the receptor and the pleiotropic

functions of RANKL and RANK *in vivo*, it is obviously of great interest to study RANK(L) signalling pathways (described in Figure 1.5), to identify pathways specific for osteoclast development and to understand their crosstalk with other receptor/ligand systems in different cell types. Either membrane-bound RANKL or soluble RANKL produced by alternative splicing or cleavage by MMPs or disintegrin and metalloproteases domain (ADAMs) family, bind and activate the receptor RANK. RANK stimulation activates, consecutively, several different signalling pathways, such as the mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), or nuclear factor-kappa B (NF-κB) pathway, to control osteoclastogenesis. TNF receptor-associated factors (TRAFs) and other adaptors, such as GRB2-associated binding protein 2 (Gab2), bound to the cytoplasmic tail of RANK are key mediators of RANKL–RANK signalling. Both IκB kinase beta (IKKβ) and IκB kinase alpha (IKKα) NF-κB pathways are activated by RANK stimulation and contribute to transcriptional regulation of target genes. The MAPK pathway leads to activation of jun oncogene (JUN) family members which are also critical for osteoclastogenesis. PI3K signaling links RANK stimulation to activation of protein kinase B (Akt/PKB), and Ca2+ signalling *via* phospholipase C, gamma (PLC-γ), which is essential for calcineurin-mediated nuclear factor of activated T cells, cytoplasmic, 1 (NFATC1) activation. Besides RANK activation, additional costimulatory signals from osteoblasts are required for osteoclast differentiation. M-CSF - colony stimulating factor 1 receptor (cFMS) signaling is crucial for the proliferation and survival of osteoclastic precursor cells. For instance, the immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptors TYRO protein tyrosine kinase binding protein (DAP12) or Fc receptor common γ subunit (FcRγ) are essential for RANK-mediated Ca2+ induction *via* PLCγ. There is also positive and negative crosstalk with other signalling pathways and molecules, such as the TNF-R, which acts positively on NF-κB, or IFNs, which negatively influence RANK signalling by promoting accelerated TRAF6 degradation in the case of IFN-γ or by interfering with RANKL-induced cFos expression in the case of IFN-β (Leibbrandt and Penninger, 2008).

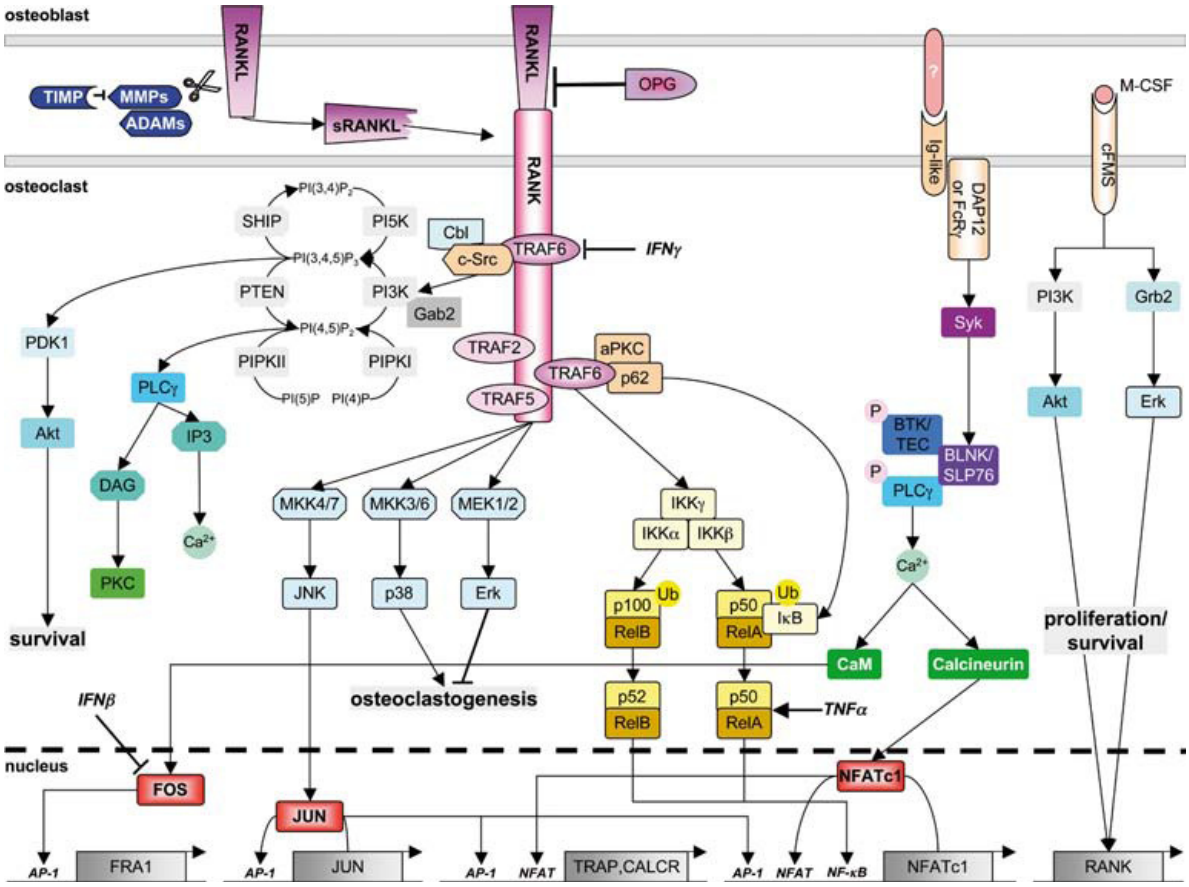

Figure 1.5. RANK-RANKL signalling pathways (Leibbrandt and Penninger, 2008).

4.4. Immune pathways in Rheumatoid Arthritis joint

The current understanding of inflammation in joints during RA is described in Figure 1.6. To summarize, synovial inflammation is characterised by the presence of many different interacting immune cells. Antigen-presenting cells binding with T cells through the T cell receptor (TCR)-MHC interaction, and T-cell activation occurs only in the presence of co-stimulatory signals mediated *via* the CD28-B7 receptor family (CD80/86). B cells have two main functions: antigen-presenting cells and antibody producing cells, which deliver antibodies involved in immune complex formation. Macrophages activated by signals from T cells and by immune complexes produce many proinflammatory cytokines, such as TNF, IL-1 and IL-6, which can enhance expression of cell-adhesion molecules and cytokine production. Dependent on the cytokine environment, activated T cells show distinct phenotypes, such as Th17 cells, which are dependent on IL-6 stimulation and produce IL-17. This molecule enhances cytokine release, production of cartilage-destructive enzymes, and expression of bone destruction-related molecules, such as RANKL (Klareskog et al., 2009).

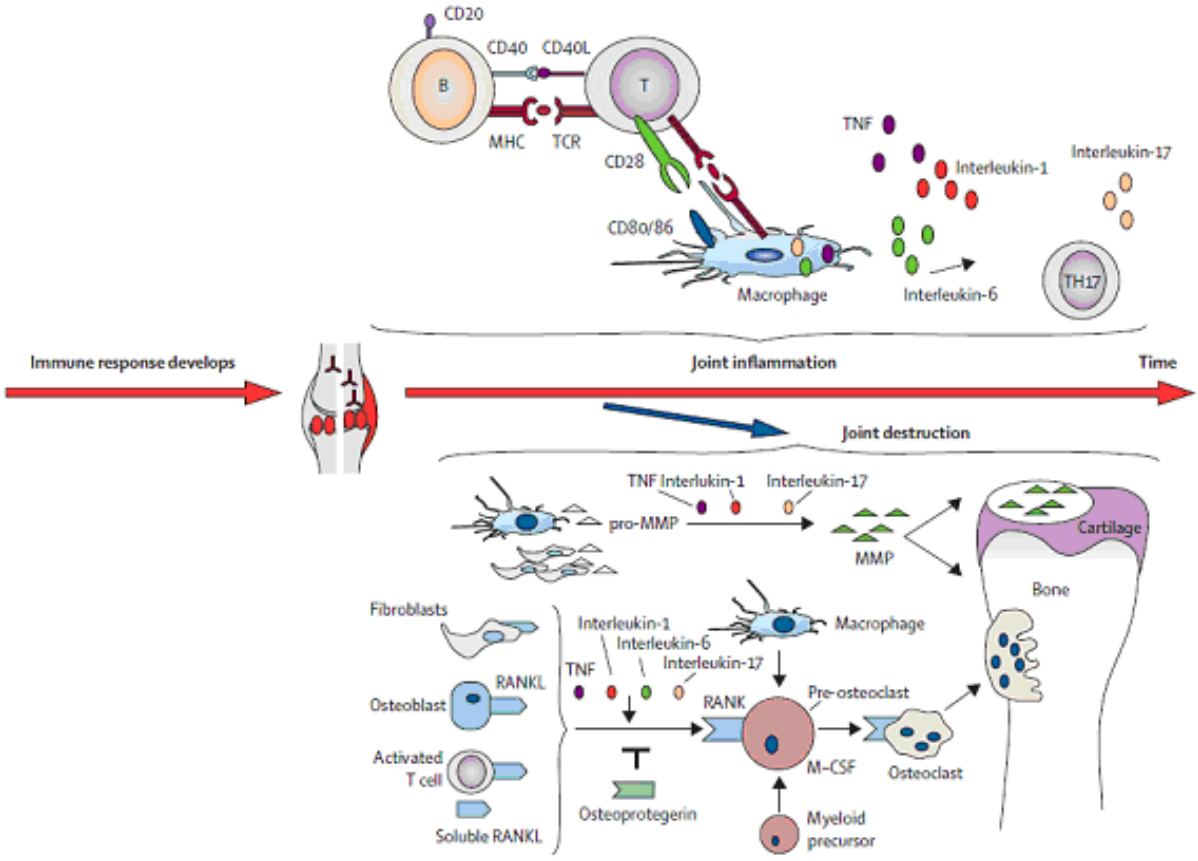

Figure 1.6. Immune pathways in RA joint (Klareskog et al., 2009).

5. Autoantibody Systems in Rheumatoid Arthritis

A number of autoantibody systems have been described in RA and their clinical associations and role as markers of disease examined. The precise mechanisms responsible for the formation of these antibodies have not been well defined; their presence must reflect the interaction between T and B cells believed to be relevant to the pathogenesis of RA (Mimori, 2005). Since the initial description of RF, a large number of both disease-specific and non-specific autoantibodies have been described in patients with RA including ACPA, sometimes referred to as anti-CCP (synthetic cyclic citrullinated peptide), and antibodies to immunoglobulin binding protein (BiP), to nuclear ribonucleoprotein complex (RA33) and to calpastatin (Mewar and Wilson, 2006). Simplicity of measurement and stability make autoantibodies attractive diagnostic and prognostic markers particularly in early disease when it may be difficult to distinguish self-limiting synovitis from persistent disease (Kim and Weisman, 2000). However only two (RFs and ACPA antibodies) are used in clinical practice.

5.1. Rheumatoid Factor

Since the pivotal studies of Waaler (1940), serum RF estimation has been a basic diagnostic aid, varying in popularity with changes in scientific trends and with considerable controversy over its significance. Until now it is still recommended as a prognostic indicator of disease activity and progression in the adult and juvenile forms of arthritis (Vallbracht et al., 2004). RF is directed at the Fc region of immunoglobulin (Ig) G and is usually of IgM isotype and is detectable in up to 10% of normal individuals, 70-80% of patients with RA and in other systemic diseases such as Sjogren's syndrome (SS) and many systemic infections (Tighe and Carson, 1997). RF and the B cells that synthesise and secrete them are thought to have a physiological role as part of the immune system. However RF from healthy individuals and patients with RA differs considerably. RF in healthy individuals is synthesised by CD5+ B cells, exhibit low affinity for IgG, polyreactivity and a low ratio of replacement to silent mutations in their CD receptors (CDRs) (Mantovani et al., 1993; Borretzen et al., 1997) suggesting the presence of a mechanism to suppress the affinity maturation of RF. This is supported by the observation that high affinity RF is deleted in mice (Tighe et al., 1995). In rheumatoid synovium, B cells produce high affinity RF with multiple replacement mutations in their CDRs indicating a process dependent on T cell help (Borretzen et al., 1997). An essential role for CD40 signalling on the production of RF in a transgenic mouse model has been showed suggesting that bystander T cell help could drive RF synthesis (Kyburz et al., 1999). As stated before, RA-SFs can support the survival and differentiation of B cells and consequently in RA synovium RF-producing B cells could meet an environment that would support their survival and provide the T cell help necessary for the production of high affinity RF (Edwards and Cambridge, 1995). There is evidence that RF could contribute to disease by the formation of immune complexes and complement fixation and also by functioning as highly efficient APCs (Brown et al., 1982; Tighe et al., 1993; Edwards and Cambridge, 1998). The correlation of RF status and progression of joint damage in RA support a role in pathogenesis (Masi et al., 1976). However, the presence of RF is not specific for RA but is rather a general consequence of immune activation in the context of immune complex activation (Nemazee, 1985; Tarkowski et al., 1985).

5.2. Antibodies to citrullinated protein antigen

ACPA such as APF (Nienhuis et al., 1964), AKA (Young et al., 1979), anti-Sa/vimentin (Despres et al., 1994) and anti-CCP (Schellekens et al., 1998) are an overlapping group of antibodies with a remarkable specificity completely dependent on the citrullination of arginine

residues. Citrullination of proteins is a post translational modification in which peptidylarginine residues are converted to peptidylcitrulline by peptidylarginine deiminases (PADs) (Vossenaar et al., 2003) (Figure 1.7). In this reaction, the positively charged amino side-chain group of Arg is deiminated to the neutral carbonyl side chain of citrulline. PAD activity requires high concentrations of calcium, for instance calcium levels that occur extracellularly or in apoptotic cells (Baeten et al., 2001; Gyorgy et al., 2006). This causes changes in intra- and intermolecular interactions, which could lead to altered protein folding, enhanced degradation by proteases, and exposure of cryptic epitopes.

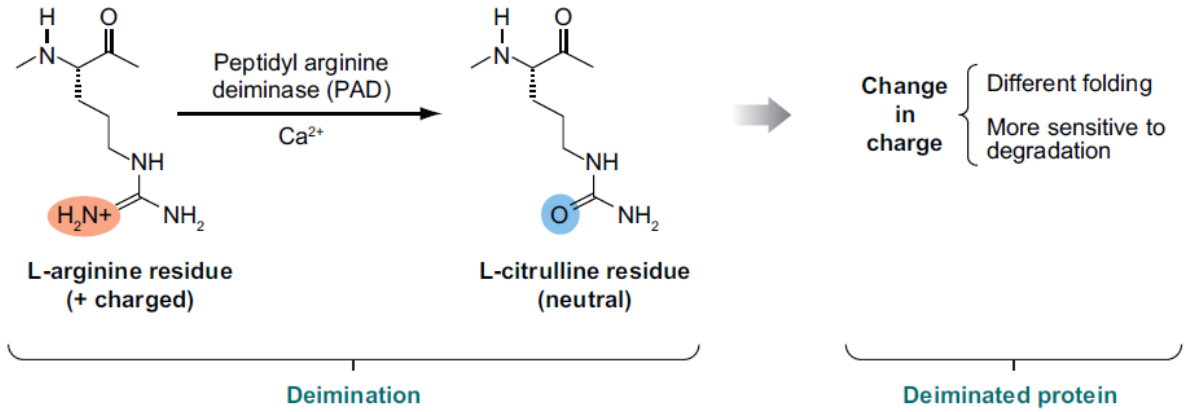

Figure 1.7. Deimination of peptidylarginine to peptidylcitrulline (Klareskog et al., 2008)

APF was described more than 30 years ago by the demonstration of a perinuclear staining pattern with RA sera using indirect immunofluorescence on buccal mucosal cells (Nienhuis et al., 1964) and AKA by a similar technique using rat oesophageal sections as substrate (Young et al., 1979). Both factors were found to be highly specific for RA and were shown to share specificity for the cytokeratin-filament aggregating protein fillaggrin (Sebbag et al., 2006). A great diversity of synthetic CCP based on citrulline-containing motifs of fillagrin have been shown to bind to APF/AKA positive sera emphasising the importance of citrullinated residues as key antigenic determinants recognised by these antibodies (Schellekens et al., 1998). The Sa antigen has been identified as the cytoskeletal protein vimentin, and once again antigenicity found to be dependent on citrullination as well as a high disease specificity (Hueber et al., 1999). Recent surveys have suggested that antibody reactivity occurred in a large number of other citrullinated proteins and peptides including deiminated forms of α and β fibrin (Masson-Bessiere et al., 2001), citrullinated peptides of collagens I and II (Koivula et al., 2005), citrullinated alpha-enolase (Kinloch et al., 2005), citrullinated fibrinogen

(Takizawa et al., 2006) and citrullinated eukaryotic translation initiation factor 4G1 (Okazaki et al. 2006). These data suggest that APF, AKA, anti-Sa and other ACPA are an overlapping heterogeneous group of antibodies directed against citrullinated antigens, for which the ELISA anti-CCP2 test can be considered as a proxy.

The level to which single antibody react against private epitope on different citrullinated peptides or proteins and the level to which there is a reaction to public epitopes shared by many citrullinated peptides/proteins are so far unknown, as well as the isotype distribution f the ACPA (Verpoort et al., 2006). The most outstanding data rising from studies of different ACPA are the high specificity for RA and the definition of a RA distinct subset. The majority of these studies were carried out using CPP-based assays. Characteristically, 50-70% of early RA patients possess ACPA (Avouac et al., 2006), and the phenotype is subsequently very stable, specifically, a small number of patients switch between an ACPA positive state to a negative one or the opposite, even after have being treated with disease modifying antirheumatic drugs (DMARDs) (Kastbom et al., 2004; Rönnelid et al., 2005). This qualitative phenotypic stability is also observed after treatment with TNF blocking agents, while ACPA concentrations remain stable in some studies (Caramaschi et al. 2005; De Rycke et al., 2005), however not in all of them (Alessandri et al., 2004; Chen et al., 2006). Relatively few individuals (approximately 2%) in a population of healthy controls are positive for ACPA. In contrast to RF, ACPA are to a certain extent specific for RA. Therefore, only moderately few patients with systemic inflammatory diseases, such as SLE, mixed connective tissue disease (MCTD), SS, or myositis, have ACPA. Most researchers have reported less than 10% of such patients to be ACPA positive. These diagnosed patients, when subgrouped by their ACPA phenotype, present detectable levels of ACPA, possessing as well RA-like features (including polyarthritic disease, erosions, RF), and can be classified as RA in addition to other diagnoses (Klareskog et al., 2008). This indistinctness has also been described for patients with psoriatic arthritis (PsA) (Bogliolo et al., 2005), juvenile idiopathic arthritis (JIA) (Low et al., 2004), SLE (Martinez et al., 2007), and MCTD (Takasaki et al., 2004). Altogether, these data suggest a possible new classification of arthritis, since some patients with polyarthritis and concomitant features of other systemic rheumatic conditions might share etiologic features with ACPA-positive classical RA. The actual classification criteria for RA could evolve a new ACPA-related classification, if taking in consideration further studies of common genetic and environmental determinants for ACPA-positive patients.

5.3. Other autoantibody systems

Several other antibodies have been characterized in RA like anti-RA33, anti-calpastatin and anti-BiP. Anti-RA33, a component of the spliceosome, was first demonstrated in 35% of patients with RA (Hassfeld et al., 1989). They are also present in 20% of patients with SLE and 40-60% with MCTD, usually concomitantly with antibodies to U1-snRNP however these antibodies do not occur in RA (Hassfeld et al., 1995). Interestingly anti-RA33 has been shown to occur in experimental model of arthritis (Hayer et al., 2005). Even though demonstrated to be present early in the disease, anti-RA33 has not been shown to have any predictive value for disease severity or progression (Goldbach-Mansky et al., 2000). Calpastatin is a natural inhibitor of the calpains which are over-expressed in diseased synovial tissue and secreted calpains can degrade components of articular cartilage (Yamamoto et al., 1992). Two studies described antibodies to calpastatin in approximately 50% of RA sera and although not specific for RA they occur at a higher frequency than in connective tissue diseases (Despres et al., 1995; Mimori et al., 1995). Autoantibodies to calpastatin may also potentiate joint destruction by interference with the calpain–calpastatin interaction (Menard and el-Amine, 1996). However they are not associated with disease severity and recent studies have suggested they could be less frequent than previously described (Lackner et al., 1998). BiP, a chaperone protein is member of a highly conserved family of proteins with critical roles in cellular homeostasis in both normal conditions as well as during cellular stress (Pockley, 2001). Anti-BiP have been identified completely independently by two studies in both human subjects and experimental models of RA (Blass et al., 2001; Corrigall et al., 2001) suggesting that BiP may indeed be an important autoantigen. Additionally, BiP is over-expressed in the rheumatoid joint and is present in both early and pre-disease sera (Bodman-Smith et al., 2004).

6. Rheumatoid Arthritis Treatment

The major input to progress in both assessment and best use of RA treatments has been the development of valid and responsive methods that measure, disease activity, functional status and joint damage. Strategies for treatment of RA have changed greatly over the past decade. Several factors have determined this alteration such the early and consistent reduction of inflammation (little joint damage), the target of specific molecular mechanisms implicated in RA pathogenesis and finally the different treatments efficacy for individual patients and at various time points. Findings of several studies have provided definite evidence that early and aggressive treatment with conventional DMARDs such as methotrexate (MTX), sulfasalazine, hydroxychloroquine, leflunomide, and glucocorticoids, can be highly helpful for control of

inflammatory activity and development of erosions in many patients (Möttönen et al., 1999; Svensson et al., 2005). The progress in the development of treatments that target distinct parts of the innate immune system was made from findings of basic studies of cloning and biological characterisation of TNF and from research into cytokine biology in arthritic joints (Brennan et al., 1989). The important role for TNF in clinical RA pathogenesis was first demonstrated in a clinical study of TNF blockade in RA patients (Elliott et al., 1993), and confirmed with randomised clinical trials (Elliott et al., 1994; Maini et al., 1998). Presently, TNF-blocking agents approved for clinical use are infliximab (chimeric anti-TNF), etanercept (soluble TNF receptor), and adalimumab (humanised anti-TNF). These drugs act by partly neutralising circulating and synovial TNF. From subsequent studies performed, we now know that TNF blockade is most effective when combined with MTX (Bathon et al., 2000; Lipsky et al., 2000; Klareskog et al., 2004), and this strategy not only reduces inflammation but also almost completely eradicates joint destruction, even in the presence of residual inflammatory activity (Smolen et al., 2005). TNF blockade success led to development and testing of a series of biological drugs that targeting several different molecules in inflammatory pathways (Figure 1.8). Anakinra is a recombinant version of the human IL-1R antagonist that competitively inhibits binding of IL-1 to its receptor. This drug had some effect on erosions in patients with RA (Bresnihan et al., 1998) but was never close to the effectiveness of TNF blockade in clinical practice (Burger et al., 2006). Abatacept is a recombinant fusion protein consisting of the extracellular domain of Cytotoxic T lymphocyte–associated antigen 4 (CTLA4) and a fragment of the Fc portion of IgG that inhibits co-stimulatory signals essential for T cell activation (Kremer et al., 2003). Rituximab is a monoclonal antibody that binds to CD20 on the surface of pre-B and matures B cells and depletes these cells from circulation (Edwards et al., 2004a). Tocilizumab is a monoclonal antibody directed against the IL-6R (Smolen et al., 2007). This drug is now approved for clinical use in Japan and Europe but not yet in other parts of the world. It seems to be efficient at reducing both inflammation and erosions (Smolen et al., 2008).

So far, treatment results with DMARDs and biological agents have revealed variable responses in individual RA patients. Biological explanations for these variations are not yet known, but tentative answers have been offered: large variability in cytokine expression has been noted between patients (Ulfgren et al., 2000), and findings of preliminary studies have suggested that people with high expression of TNF in their joints could be most responsive to TNF blockade (Wijbrandts et al., 2008) and individuals with high amounts of ACPA or RF

and many synovial B cells might be more responsive than others to B cell-directed treatments (Teng et al., 2007).

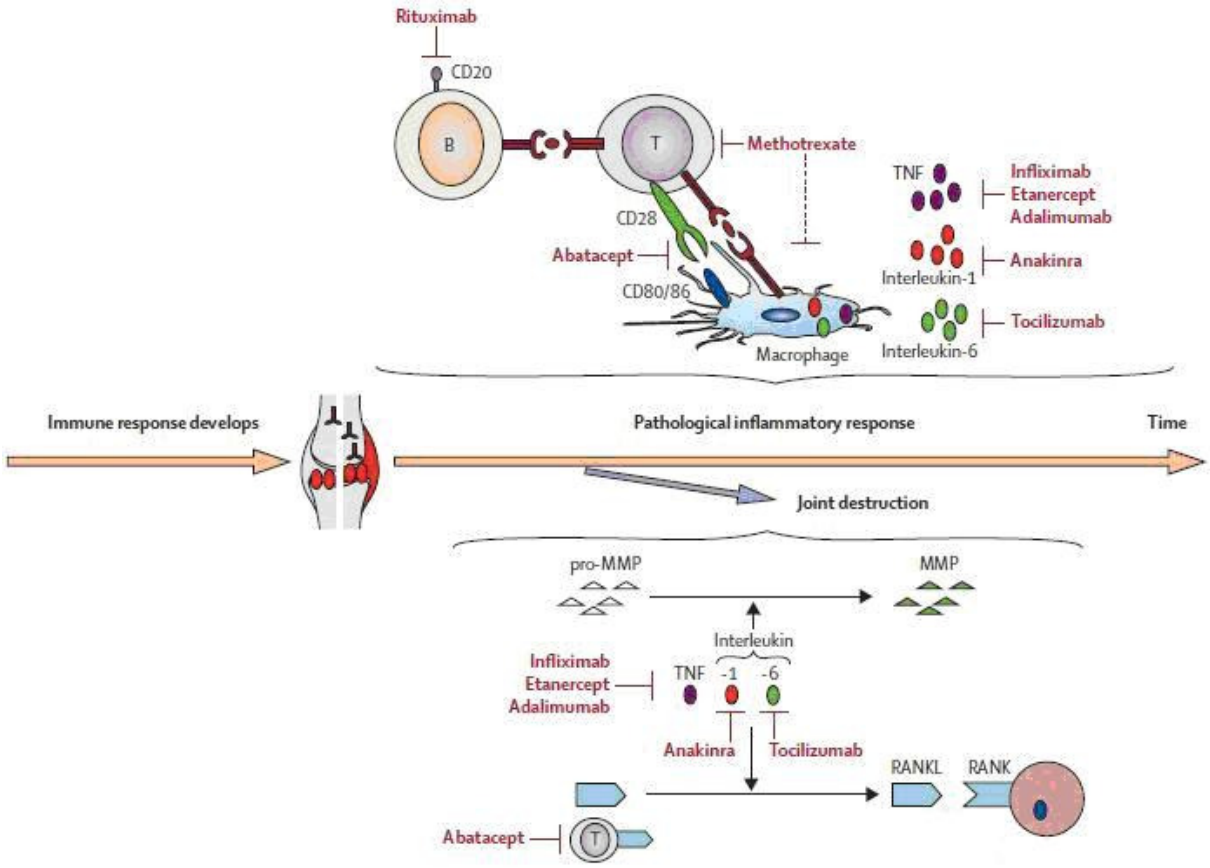

Figure 1.8. Modes of action of some currently used targeted treatments (adapted from Klaresgog et al., 2009).

7. Analytical epidemiology risk factors in Rheumatoid Arthritis

A risk factor is any factor (genetic, environmental or host) that increases the risk of developing a disease. There is a general agreement that RA is a multifactorial disease, resulting from the interaction of genetic, environmental and host factors, which contribute to its occurrence and expression (Seldin et al., 1999). There is epidemiologic evidence that genetic factors are related to an increased risk of RA. The nature and the impact of this genetic risk are becoming clearer during the last years (Bowes and Barton, 2008). On the other hand, several environmental and host factors have been suspected and studied as possibly related to an increased risk of RA, as well as to a worse or improved prognosis of the disease. However, the impact of most of these factors on the risk of developing RA and the expression of the disease remains still uncertain (Kobayashi et al., 2008a).

7.1. Genetics Etiology

Although the etiology of RA remains unknown, a genetic component of RA susceptibility has been established by data from familial aggregation and twin studies. Evidence of familial clustering was the first indication of a familial susceptibility to RA. The prevalence of RA ranges from 2% to 12% in first-degree relatives of patients (Deighton et al., 1989), whereas the population prevalence is only ~1%. Familial clustering is quantified using the λs coefficient, defined as the ratio of the prevalence in first-degree relatives to the population prevalence. The λs coefficient for RA has been estimated to range from 2 to 15 (Risch, 1990a). In twin studies, the concordance rates range from 12% to 30% in monozygotic twins and from 5% to 10% in same-sex dizygotic twins, confirming a role for genetic susceptibility factors (Aho et al., 1986; Silman et al., 1993b). From such studies, the heritability of RA, defined as the extent to which variation in liability to disease in a population can be explained by genetic variation, has been estimated at between 50% and 60% (MacGregor et al., 2000). These results suggest that genetic factors have a substantial influence on disease susceptibility and account for a major proportion of disease liability within populations but also leaving considerable space for shared environmental factors.

The first consistent RA genetic association, the human leukocyte antigen (*HLA*) loci, was introduced 33 years ago by Peter Stastny (1976). With the development of serological and molecular typing of *HLA* class II antigens/molecules it gradually became clear that RA was associated with several *HLA-DRB1* alleles (*0101, *0401, *0404, *0405, *0408, *1001, and *1402) *HLA* class II molecules constitute the most powerful recognized genetic factor for RA accounting for approximately one-third of the familial aggregation (Deighton et al., 1989; Wordsworth and Salmon, 1992).

To evaluate new RA susceptibility genetic factors, it is useful to understand some of the conceptual and analytic issues in genetic mapping. Three approaches have been used to identify new genetic variants that may contribute to any human phenotype, including autoimmune disorders. These approaches are linkage analysis in multiplex families, candidate gene association studies and genome wide association studies.

Because RA is a typical complex disease, genetic susceptibility to RA is likely to be polygenic, involving several genes of low penetrance with allelic as well as locus heterogeneity. Several microsatellites or single nucleotide polymorphism (SNP) based RA linkage analyses have been performed to identify the *HLA* and non-*HLA* loci involved in RA susceptibility (Cornelis et al., 1998; Shiozawa et al., 1998; Jawaheer et al., 2001; MacKay et al., 2002; Jawaheer et al., 2003; John et al., 2004; Eyre et al., 2004; Osorio et al., 2004;

Tamiya et al., 2005). However, with the exception of the chromosome 6p region containing the *HLA* complex, linkage has not been easy to replicate across studies. This is unsurprising; for rare genes, or genes having a weak effect, nonparametric linkage studies are likely to require several hundreds, possibly thousands, of affected sibling pairs (ASPs) to provide sufficient power to detect linkage (Risch, 1990b).

Recently, remarkable progress has been made in identifying further RA susceptibility variants and this has been achieved using both genome-wide association (GWA) and candidate gene approaches. Outside of the *HLA* region, the 1858C/T variant of the protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene is the most consistently and most strongly disease-associated variant identified in RA association studies performed in Caucasian populations (Begovich et al., 2004). *HLA-DRB1* and *PTPN22* genes account for 50% of the genetic susceptibility to disease in populations of European descent, although, interestingly, variation across the *PTPN22* gene does not appear to be associated with RA in Japanese or Korean populations (Mori et al., 2005). The Wellcome Trust Case Control Consortium (WTCCC) performed a GWA study that included 1860 RA cases and 2930 controls and confirmed association to these susceptibility variants, *HLA-DRB1* and *PTPN22* (WTCCC, 2007). In addition, nine other loci showed modest evidence for significance and association to one of these, a locus lying between the oligodendrocyte transcription factor 3 (*OLIG3*) and tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3*) genes on chromosome 6q23, has been unequivocally replicated in UK and US populations (Thomson et al., 2007; Plenge et al., 2007b). The tumor necrosis factor receptor-associated factor 1 (*TRAF1*)/Complement Component 5 (*C5*) locus has been identified by a GWA and candidate gene studies (Kurreeman et al., 2007; Plenge et al., 2007a). Furthermore, the results of a fine-mapping strategy investigating candidate genes mapping under a peak of linkage in US RA families has identified another RA susceptibility locus mapping to the signal transducer and activator of transcription 4 (*STAT4*) gene in US subjects (Remmers et al., 2007), which has been subsequently confirmed in European and Asian populations (Daha et al., 2009).

Recently, two more RA genetic risk factors were described in recent case-control and GWA studies and confirmed in a meta-analysis approach: *CTLA4* gene and 4q27 gene region (Plenge et al., 2005, Zhernakova et al., 2007; Raychaudhuri et al. 2008, Daha et al., 2009). Even if, these new confirmed genetic risk factors have small odds ratios (ORs) these new findings are very important to improve the knowledge of RA molecular pathways.

7.1.1. Rheumatoid Arthritis genetic approaches

7.1.1.1. Linkage studies in multiplex families

Linkage analysis aims to identify genomic regions containing disease predisposing genes by observing related individuals. The widely used approach is to study ASPs diagnosed for the disease. It is expected that affected relatives will show an excess sharing of haplotypes identical by descendent in the region of a disease-causing variant. Thus, chromosomal regions exhibiting increased allele sharing are likely to contain susceptibility genes (Dawn Teare and Barrett, 2005). In a linkage study the genome can be screened either partially or completely (whole genome scan), using multiallelic markers (microsatellites) or biallelic markers (SNPs). Linkage manifests as greater locus similarity in ASPs than predicted by Mendelian laws. For instance, if a polymorphic multiallelic marker is linked to a disease susceptibility gene, members of an ASP are more likely to have shared alleles than predicted by Mendelian laws. Increased allele sharing within ASPs is required for one or more markers. Chromosomal regions exhibiting increased allele sharing are likely to contain susceptibility genes. The main problem is limited power: except for the few loci showing major evidence of linkage (only *HLA* for RA), genetic factors are likely to be identified in only a small proportion of scans, for instance one in 10. In addition, with the statistical cutoffs used to increase the sensitivity of genome scans, false-positive results are common. For instance, with about 3000 microsatellite markers and an alpha risk set at 5%, 15 markers will yield false-positive results. With complex disorders such as RA, genome scans conducted in several genetically homogeneous populations can detect regions of interest. However, there is no certainty that all the major RA-susceptibility genes will be detected using this approach (Altmuller et al., 2001).

Genome scans meta-analysis

Several previously cited whole-genome linkage scans have been performed to identify the *HLA* and non-*HLA* loci involved in RA susceptibility. They identified several linkage loci for RA, but the findings of most of the studies have not been replicated with the exception of the chromosome 6p region containing the *HLA* complex. It is not surprising that linkage studies have shown inconsistent results, because they have been limited by small sample size, low statistical power and clinical or genetic heterogeneity (Altmuller et al., 2001). Meta-analysis combines the linkage results from several studies, providing greater statistical power. Meta-analysis may also identify regions where the genetic effect is too small to be detected in an individual study. Three RA genome scans meta-analysis have been performed using two

different statistical methods. Loesgen and colleagues (2001) developed a meta-analytic test that computes a weighted average estimate of score statistics. They proposed several weighting schemes in one of which the weights are based on a function of information content and sample size. Levinson and collegues (2003) reported the genome search meta-analysis method (GSMA), a non-parametric ranking method, developed to identify genomic regions that show consistent linkage evidence based on the linkage scores obtained in each scan.

Fisher and collegues (2003), integrated in this meta-analysis a US study (Jawaheer et al., 2001), a UK study (MacKay et al., 2002), a European study (Cornelis et al., 1998) and a Japanese study (Shiozawa et al., 1998). Using the GSMA method, they reported linkage signals (P<0.01) on 6p (*HLA* region), 6q, 12p and 16 (centromere); as well as novel regions (P<0.05) on 1q, 3q, 4q, 8p, 9q and 14q. Choi and collegues (2006) have used four RA whole-genome scans - a European study (Cornelis et al., 1998), two US studies (Jawaheer et al., 2001; Jawaheer et al., 2003) and a UK study (John et al., 2004) - to perform the meta-analysis. Using the GSMA method, linkage to the *HLA* region on chromosome 6 was confirmed. The Choi meta-analysis also revealed evidence of chromosomes 1p, 8p, 12, 16, 18q and subsequent areas on chromosome 6. Finally, Etzel and collegues (2006) included in the genome-search meta-analysis a European study (Cornelis et al., 1998), two US studies (Jawaheer et al., 2001; Jawaheer et al., 2003) and a UK study (Mackay et al., 2004) using the meta-analytical approach developed by Loesgen and collegues (2001). In this meta-analysis, the *HLA* region on chromosome 6 displayed overwhelming evidence of linkage to RA. This finding concurs with the previously cited meta-analysis of RA. This procedure also provided marginal evidence (P<0.05) of linkage on chromosome 1, 2, 5 and 18 and strong evidence (P<0.01) on chromosomes 8 and 16.

All these genome scans meta-analysis highlight several regions of genetic linkage for RA that can be further studied for gene localization and identification.

7.1.1.2. Candidate genes association studies

In genetic association studies the main objective is to detect if a genetic marker is implicated in disease susceptibility. The study design is based in the selection of a well define set of affected individuals (cases) and a healthy control group from the same population, to compare the distribution of certain genetic markers between the two groups (Woolf, 1955). This approach has greater power than linkage studies to detect small size effects contributing to disease predisposition. Nevertheless, a careful study design is essential in order to avoid false positive associations and population stratification. In case-control studies patients are

compared with matched (age and sex) controls, ideally with the same ethnicity, but differences in the genetic background of the two groups can be difficult to detect leading to the introduction of variables unrelated to the disease and might cause spurious associations due to stratification. Thus, to guarantee an adequate power of association studies, replication of findings in different unrelated populations and large population sizes are required. To circumvent stratification bias, a design based on a sample of nuclear families called trio families (each composed of one patient and both healthy parents) has been developed (Clarcke, 1961; Rubinstein et al., 1981). One of the advantages of the trio families design is that it provides accurate estimations of matched control subjects for each patient, an approach that is robust against population stratification. Furthermore, trio families contain additional information on haplotype phase compared to unrelated individuals, in the form of constraints imposed by the rules of Mendelian inheritance (Marchini et al., 2006).

Our laboratory coordinates the European Consortium of Rheumatoid Arthritis Families (ECRAF) which has available the DNA of 465 trio families: 319 French families and 146 from other continental West-European countries: 54 Italian, 40 Spanish, 24 Belgium, 14 Dutch, and 14 Portuguese. Several statistical tests are available to perform family-based association studies using trio families. Before association and linkage analysis, the Hardy–Weinberg equilibrium (HWE) must be tested in a control group (constituted by the non transmitted parental chromosomes from trio families). The Affected Family Based-Controls (AFBAC), which compares the allelic frequencies between chromosomes transmitted to the RA cases and non transmitted parental chromosomes (Thomson, 1995). The Transmission Disequilibrium Test (TDT) which compares, for a given allele, its transmission from heterozygous parents to RA patients, with the transmission expected from Mendel's law (*i.e.* 50 %) (Spielman et al., 1993). Finally, the Genotype Relative Risk (GRR) and the Haplotype Relative Risk (HRR), which compares the affected offspring's genotype or haplotype with the control genotype or haplotype derived from non transmitted parental chromosomes, respectively (Lathrop, 1983; Kruglyak et al., 1996).

Therefore, in association studies, candidate genes can be selected on the basis of their implication in the disease pathogenic mechanisms (functional candidate genes) and/or considering their location within genomic regions, which were previously in linkage to disease (positional candidate genes). Several genetic variants exist in a candidate gene and the selection of a candidate polymorphism to be analysed in an association study is difficult. First, the linkage disequilibrium (LD) block needs to be investigated to select a descriptive SNP representative of the block as described below (The International HapMap Consortium, 2003).

Secondly, the most interesting are putative functional genetic variants that might cause an alteration of gene function. Thus, acquire great relevance coding SNPs altering protein structure and function. In addition non-coding variants located in regulatory regions are of importance since they may alter promoter activity or RNA splicing and processing leading to differences in protein levels.

7.1.1.3. Genome-Wide Association Studies

In the last three years, GWA scans have dominated efforts in gene mapping for autoimmune diseases including RA. Like candidate gene studies, the analysis is based on association, but in the case of GWA scanning, no particular hypothesis is being addressed. Rather, hundreds of thousands of hypotheses are being addressed simultaneously, without regard to biologic plausibility. This is a purely discovery-driven approach to gene identification, free of the limitations imposed by *a priori* assumptions about which genes and pathways are likely to be involved in the disease under study (Gregersen and Olsson, 2009). The GWA scanning approach is essentially dependent on knowledge of the level and patterns of variation in the human genome. Even if the initial sequence of the human genome was an important first step, it is really the International HapMap Project that has provided the basis for a rational approach to GWA studies (The International HapMap Consortium, 2003). When considering SNPs, any two unrelated individuals in the population differ by approximately 0.1% across the 3.2 billion base pairs of the genome, or approximately 3 million SNPs. By studying 90 individuals in families from three major racial groups (Caucasian, Asian, and African), the HapMap Project has catalogued the mainstream of the common SNPs (e.g., SNPs with minor allele frequencies of 5% or greater) in these populations. This has provided a library of millions of SNP markers for use in GWA studies (Frazer et al., 2007). An important result of the HapMap Project has been the realization that to describe most of the common variations among individuals, it is not necessary to genotype all 3 million SNP differences among them, but only a subset of these, on the order of 300,000 to 500,000 SNPs. This is because SNP alleles are distributed nonrandomly among individuals, forming blocks of LD that may extend from thousands to many hundreds of thousands of base pairs. This results in a kind of bar code that can be used to define the common genetic variation across the genome of a given individual. This pattern of common variation across genomes has led to the concept of tagging SNPs (Chapman et al., 2003). This involves the use of a single SNP to tag a block of LD formed by many other SNPs, therefore allowing for the interrogation of a large section of the genome with a single marker. SNP tagging may be applied in a pairwise fashion, or may

also involve using several SNPs to predict the presence of a third. This ability to impute the likely presence of untyped SNPs is based on the information provided by the HapMap on the patterns of LD in the population under study.

Once a SNP association is observed and confirmed, much work remains to be done to establish which genetic variants in the region are actually responsible (e.g., causative) for the association. Furthermore, because many GWA studies employ hundreds of thousands SNPs across the genome, each addressing a separate hypothesis, the statistical significance levels must be adjusted for multiple testing. An overall *P* value of $< 5x10^{-7}$ is now widely accepted as compelling evidence of true association, although it is quite clear that lower degrees of statistical significance often reflect real associations. In any case, truly convincing association always requires multiple replications in independent data sets (Gregersen and Olsson, 2009). Finally, this overview of GWA studies would be incomplete without some mention of statistical power and sample size requirements. Most of the associations with RA involve the detection of ORs between 1 and 2, with many associations on the lower end of this range. The sample sizes required to generate statistical significance in the setting of GWA scan ($P<5x10^{-7}$) can be very large, depending on the allele frequency in the population and the ORs to be detected. For risk ratios on the order of 2 or more, sample sizes of 1000 are generally adequate. However, for risk ratios in the range of 1.2-1.3, even sample sizes of three or four thousand may have low statistical power depending on marker allele frequency (Iles et al., 2008). This magnitude of a population sample is now considered a minimum for a thorough analysis in the setting of a GWA scan, and truly comprehensive genetic studies will require considerably larger sample sizes to be studied in the future.

7.1.2. Rheumatoid Arthritis susceptibility genetic factors

7.1.2.1. Major Histocompatibility Complex

The MHC, located on the short arm of Chromosome 6, is one of the most extensively studied regions in the human genome because of the contribution of multiple variants at this locus in autoimmune, infectious, and inflammatory diseases and in transplantation. The murine MHC locus, *H2*, was identified almost 60 years ago by George Snell (1948). Shortly afterward, Jean Dausset (1958) recognized the human MHC, or *HLA* region, so named because Dausset originally observed MHC antigens on the surface of white blood cells. Subsequently, Baruj Benacerraf (1981) reported the importance of these antigens in the immune response. The classical MHC encompasses approximately 3.6 Mb on 6p21.3 and is divided into three

subregions: the telomeric class I, class III and the centromeric class II regions. The concept of the extended MHC (xMHC), spanning about 7.6 Mb of the genome, has been recently established by the finding that LD and MHC-related genes exist outside the classically defined locus. Of the 421 genes within the xMHC, 60% are expressed and approximately 22% have putative immunoregulatory function. The five subregions of the xMHC comprise the extended class I subregion, classical class I, classical class III, classical class II and the extended class II subregions (Horton et al., 2004). The MHC was first associated with disease when HLA-B antigens were found at increased frequency in patients with Hodgkin's lymphoma (Amiel, 1967). After that, variation within the MHC has been found to be associated with almost every autoimmune disease, as well as several infectious and inflammatory diseases. A complication in the identification of disease-causing variants at the MHC is the great variability exhibited by some of the genes within the MHC (such as the classical class I genes, *HLA-A*, *-B*, and -C and the classical class II genes, *HLA-DRB1*, *-DQA1*, and *-DQB1*), which require time-intensive typing strategies; indeed *HLA-B* is the most polymorphic gene known in the human genome (Fernando et al., 2008).

Association of the *HLA-DRB1* gene with Rheumatoid Arthritis

The involvement of HLA in RA etiology was introduced 30 years ago by Peter Stastny (1976) who discovered that HLA-DW4 as defined by mixed lymphocyte culture (MLC) typing was associated with RA. His discovery was triggered by Astorga and Williams (1969) who observed 5 years earlier that MLC reactions between cells from RA patients were often negative or low. With the development of serological and molecular typing of HLA class II antigens/molecules it gradually became clear that RA was associated with several *HLA-DRB1* alleles (*0101, *0401, *0404, *0405, *0408, *1001, and *1402). The products of these alleles appeared to carry a similar five-AA sequence (^{70}Q/R K/R R A A^{74}) in the third hypervariable region of the DRB1 molecule (Zoschke and Segall, 1986; Nepom et al., 1987). Based on the molecular genetic epidemiological data Gregersen and colleagues (1987) proposed the so called shared epitope (SE) hypothesis. This hypothesis assumes that *HLA-DRB1* alleles encoding a highly conserved amino acid sequence - RAA pattern at positions 72-74 of the third hypervariable region of different *HLA-DRB1* chains - are associated with RA susceptibility. HLA-DR molecules are heterodimers that present antigenic peptides to T lymphocytes. The peptide-binding groove is composed of two α-helical "walls" and a "floor" of β-pleated sheet. The *HLA* SE is in the α-helix wall of the peptide-binding groove. Structural studies provide evidence that the SE can influence peptide binding as well as

contact between HLA-DR and the T cell receptor. The crystallographic structures of shared-epitope-expressing HLA-DR1 (*HLA-DRB1**0101) and HLA-DR4 (*HLA-DRB1**0401) complexed with an influenza hemagglutinin peptide or with a peptide from human collagen II demonstrate that position 71 (Arg71 for *DRB1**0101, Lys71 for *DRB1**401) interacts with the peptide amino acid in the P4 anchoring pocket. The side chains of the shared-epitope residues 72-74 either point away from the peptide-binding groove or are too far away from the peptides to make contact (Dessen et al., 1997; Hennecke and Wiley; 2002; Rosloniec et al., 2006) (Figure 1.9).

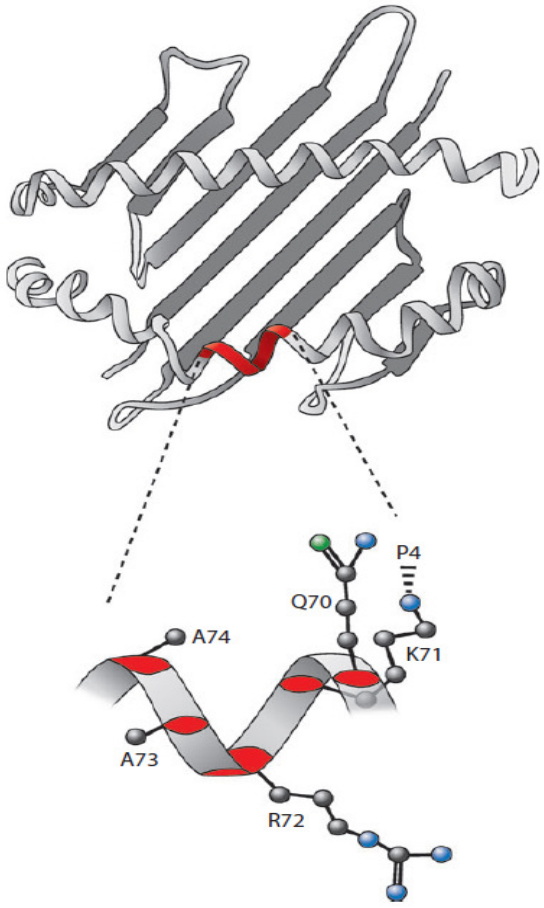

Figure 1.9. Schematic representation of the SE encoded by *HLA-DRB1**0401 (Imboden, 2008).

Studies of the structure of a covalently stabilized complex of T cell receptor, HLA-DR1, and the influenza peptide reveal that the T cell receptor contacts Gli70 within the SE. Residues 70 and 71 of the SE contact, respectively, the T cell receptor and the antigenic peptide. These

results, therefore, suggest that the products of SE *HLA-DRB1* alleles may predispose to RA by presenting peptides to T cells (Hennecke et al., 2000).

The frequency of RA-associated SE alleles has been found to vary considerably depending on ethnic group. For example, the alleles *0401 and *0404 are predominantly associated with RA in Caucasian populations, *0405 and *901 alleles in Asian populations and *0101 in Israeli Jews (Bowes and Barton, 2008). A further layer of complexity is that combinations of SE alleles can carry a greater risk than homozygosity for those alleles. For example, the heterozygous combination of *DRB1**0401/0404 is strongly associated with early onset and a more severe form of disease than homozygosity for either allele. Additionally, presence of one of the *HLA-DR* alleles conferring susceptibility to RA is noted in 40% of individuals in the general population and in 70% of patients with RA, indicating that these alleles are neither necessary nor sufficient to cause the development of RA in a given individual (Newton et al., 2004).

Several studies have indicated that the influence of *HLA-DRB1* alleles on RA is due to effects on disease severity and progression rather than susceptibility. This was initially reported by investigators in the UK for *HLA-DR4* and appeared to be related to the extent of bone erosions (Young et al., 1984; Jaraquemada et al., 1986). However, the association with disease severity varies among different ethnic populations. Thus, a well-defined association exists between severe disease outcome and presence of the SE, particularly in populations from Northern Europe and North America (Young et al., 1984; Jaraquemada et al., 1986; Weyand et al., 1992; Moreno et al., 1996; Wagner et al., 1997; Toussirot et al., 1999; Meyer et al., 1999). This observation also has been reported by del Rincón and Escalante (1999) in Mexican Americans carrying the SE sequence. Likewise, in the region of northwest Spain, erosive disease was associated with carriage of the SE, particularly *HLA-DRB1**0101 and *DRB1**0404, whereas RF positivity was associated mainly with *DRB1**0401 (Hajeer et al., 2000).

Since 1998, several already cited genome linkage scans have been conducted, in populations from France, UK, USA, and Japan and the results consistently support a role for the *HLA* locus in the genetic susceptibility to RA. Furthermore, some recently GWA studies confirm the *HLA* locus in the RA genetic susceptibility (WTCCC, 2007; Plenge et al., 2007a).

Several modifications of *HLA-DRB1* classification were suggested and studied by other investigators to develop more sophisticated explanations for the distribution of disease-associated alleles in RA (Mattey et al., 2001; de Vries et al., 2002; du Montcel et al., 2005). These reshaped classifications attempted to classify all *HLA-DRB1* alleles with regard to the

magnitude of risk for RA based on the amino acid sequence of HLA-DRB1. One of these new classifications of *HLA-DRB1* alleles that reconsiders the SE hypothesis was introduced by du Montcel and colleagues (2005) In terms of susceptibility to RA, this new classification, described in Figure 1.10, suggests that the risk of developing RA depends on whether the RAA sequence occupies positions 72-74 but the risk is modulated by the amino acids at position 71 (K confers the higher risk, R an intermediate risk, A and E a lower risk) and at position 70 (Q or R confers a higher risk than D) complexifying the classical SE epitope classification based on the presence of RAA in positions 72-74. Briefly, the *HLA-DRB1* alleles were first divided into two groups according to the presence or absence of the RAA sequence at positions 72-74 and were denoted S and X alleles, respectively. The S alleles were subsequently divided into four groups according to the amino acid at position 71: an alanine (A), a glutamic acid (E), a lysine (K), or an arginine (R). Different groups were thus defined in the new classification: S1 for ARAA and ERAA, S2 for KRAA, S3 for RRAA, and X for all non-RAA patterns. Since an aspartic acid (D) at position 70 was reported to be protective against RA susceptibility in comparison with a glutamine (Q) or an arginine (R) at the same position, two additional groups were defined: S3D for DRRAA and S3P for QRRAA or RRRAA. In terms of structural severity of RA, this new classification allowed the differentiation of predisposing or protective alleles (two effects) - respectively characterized by the DRRAA or by the DERAA amino acid pattern at positions 70-74 - which was not possible using the classical SE epitope classification based on the only presence of RAA in positions 72-74.

HLA-DRB1 allele	Amino acid position								Classification of du Montcel and colleagues
	69	70	71	72	73	74	75	76	
HLA-DRB1*0101	E	**Q**	**R**	**R**	**A**	**A**	V	D	**S3P**
HLA-DRB1*0102	-	**Q**	**R**	**R**	**A**	**A**	-	-	**S3P**
HLA-DRB1*0103	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*03	-	-	K	-	G	R	-	-	**X**
HLA-DRB1*0401	-	Q	**K**	**R**	**A**	**A**	-	-	**S2**
HLA-DRB1*0402	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*0403	-	-	-	-	-	E	-	-	**X**
HLA-DRB1*0404	-	**Q**	**R**	**R**	**A**	**A**	-	-	**S3P**
HLA-DRB1*0405	-	**Q**	**R**	**R**	**A**	**A**	-	-	**S3P**
HLA-DRB1*0407	-	-	-	-	-	**E**	-	-	**X**
HLA-DRB1*0408	-	**Q**	**R**	**R**	**A**	**A**	-	-	**S3P**
HLA-DRB1*0411	-	-	-	-	-	E	-	-	**X**
HLA-DRB1*07	-	D	-	-	G	Q	-	-	**X**
HLA-DRB1*08	-	D	-	-	-	L	-	-	**X**
HLA-DRB1*0901	-	R	-	-	-	E	-	-	**X**
HLA-DRB1*1001	-	**Q**	**R**	**R**	**A**	**A**	-	-	**S3P**
HLA-DRB1*1101	-	**D**	**R**	**R**	**A**	**A**	-	-	**S3D**
HLA-DRB1*1102	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*1103	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*1104	-	**D**	**R**	**R**	**A**	**A**	-	-	**S3D**
HLA-DRB1*12	-	**D**	**R**	**R**	**A**	**A**	-	-	**S3D**
HLA-DRB1*1301	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*1302	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*1303	-	D	**K**	**R**	**A**	**A**	-	-	**S2**
HLA-DRB1*1323	-	D	**E**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*1401	-	R	-	-	-	E	-	-	**X**
HLA-DRB1*1402	-	**Q**	**R**	**R**	**A**	**A**	-	-	**S3P**
HLA-DRB1*1404	-	R	-	-	-	E	-	-	**X**
HLA-DRB1*15	-	Q	**A**	**R**	**A**	**A**	-	-	**S1**
HLA-DRB1*16	-	**D**	**R**	**R**	**A**	**A**	-	-	**S3D**

Figure 1.10. HLA–DRB1 amino acid sequence for alleles observed among RA patients and their classification according to du Montcel and colleagues (2005). The conventional classification of the amino acids was used, here divided into three biochemical subgroups, as follows: group 1 = G for glycine, A for alanine, V for valine, L for leucine [aliphatic amino acids (nonpolar hydrophobic)]; group 2 = K for lysine, R for arginine [basic amino acids (polar and positively charged)]; group 3 = E for glutamic acid, Q for glutamine (the amide corresponding to E), D for aspartic acid, and N for asparagine (the amide corresponding to D) (acidic amino acids and corresponding amides are very hydrophilic; acidic amino acids are polar and negatively charged at physiologic pH, amides are polar and uncharged, and not ionizable) (adapted from Gourraud et al., 2007).

Subsequent studies evaluated the strength of these classifications, but controversy remains and the best model has not yet been confirmed (Morgan et al., 2008). One good example of an exception to these classifications is the *HLA-DRB1* *0901 allele in Asian populations. This allele is common in Asian populations, with reported frequencies of 15% in Japanese (Wakitani et al., 1998; Kochi et al., 2004), 8% in Korean (Lee et al., 2004) and 7% in Malaysian (Kong et al., 2002), but is less frequent in European populations. Although the *0901 allele does not contain the classical SE and is not classified into the group of disease-risk alleles in the new classifications, evidence of an association with RA-susceptibility in Asian populations has been accumulating.

More recently, as the clinical importance of ACPA has been recognized in RA, light has again been shed on the genetic role of *HLA-DRB1*. Several studies examining the relationship between ACPA and *HLA-DRB1* have shown that the presence of ACPA is associated with possession of *HLA-DRB1* SE alleles in both European and Asian populations (Irigoyen et al., 2005; Verpoort et al., 2005; Klareskog et al., 2006; Furuya et al., 2007). Intriguingly, the *DRB1*0301 allele does not contain the SE and is associated with ACPA negative RA in European populations (Irigoyen et al., 2005; Verpoort et al., 2005). A similar association with negative status of ACPA has been described for the *DRB1*0901 allele in Japanese RA patients (Furuya et al., 2007), though the sample set examined was relatively small and the results need further confirmation. Moreover, citrullination of peptides increases the affinity to HLA-DR molecule and activates CD4+ T cells in human HLA-DR4 transgenic mice (Hill et al., 2003a).

Despite considerable progress in understanding the association of *HLA-DRB1* with RA and the structure and function of HLA-DR molecules, the mechanisms by which inheritance of particular *HLA-DRB1* alleles predisposes to the development of RA remain unknown. Proposed models include the selection of pathogenic T cells during thymic selection, the presentation of “arthritogenic peptides” to class II–restricted peripheral-effector T cells and the failure to generate appropriate Treg cells (Firestein et al., 2003).

Amino acids "DERAA" and Non-inherited maternal antigen

As described before, at the same position as the SE, the amino acids ''DERAA'' (*i.e.* the amino acids aspartic acid, glutamic acid, arginine, alanine, alanine) can be present in other HLA-DRB1 molecules (*i.e. HLA-DRB1**0103, *0402, *1102, *1103, *1301, *1302 and *1323). People carrying *HLA-DRB1* alleles that express this ''DERAA'' sequence show a lower susceptibility to develop RA and have less severe disease than people with ''neutral'' (SE- and ''DERAA''-negative) *HLA-DRB1* alleles. The OR of people carrying *HLA-DRB1* alleles that express the ''DERAA'' sequence compared with that of people with ''neutral'' (SE- and ''DERAA''- negative) *HLA-DRB1* alleles to develop RA is 0.5-0.7, indicating that ''DERAA''-positive subjects have a lower susceptibility to develop RA (Mattey et al., 2001; van der Helm-van Mil et al., 2005a; Shadick et al., 2007). The protective effect associated with ''DERAA'' is also observed after stratification for the presence or absence of *HLA* SE alleles. This findings indicate that the protective effect associated with ''DERAA'' expression can not be explained by an over-representation of SE alleles in patients, resulting automatically in a lower frequency of other *HLA* alleles in patients with RA. Thus, the ''DERAA''-containing *HLA-DRB1* alleles are independently associated with a reduced risk of developing RA (van der Helm-van Mil et al., 2005a). It is uncertain whether the entire ''DERAA'' motif is essential for the protection or whether only certain amino acids of this motif confer the same effect. In contrast to several studies showing the protective effects by ''DERAA''-containing *HLA-DRB1* alleles to the development and severity of RA (Mattey et al., 1999; Orozco et al., 2008a), other reports hypothesized that the amino acids ''RAA'' at position 72-74 in the third hypervariable region influence the susceptibility to RA development, whereas the amino acids at positions 70 and 71 modulate this effect (du Montcel et al., 2005; Barnetche et al., 2008).These studies have shown that *HLA* alleles expressing the ^{70}ERAA^{74} sequence or the aspartic acid (D) at position 70 both have a lower frequency in patients with RA than in healthy controls. Therefore, despite these differences in nomenclature and stratification, it is becoming increasingly clear that some *HLA* alleles confer susceptibility, whereas others are associated with protection. The mechanism of protection is unknown, but it has been proposed that it is mediated by the recognition by T cells of peptides containing the ''DERAA'' sequence presented by HLA-DQ molecules (Snijders et al., 2001).

The biological effect of non-inherited maternal antigen (NIMA) was described initially by Owen and colleagues (1954). They reported that Rhesus D (RhD) negative children were

tolerant to the RhD antigen when they had RhD-positive mother, probably owing to exposure to the RhD antigens during pregnancy.

Recently, Feitsma and colleagues (2007) revealed that HLA-DRB1 molecules that contain the amino acid sequence ‘‘DERAA’’ when presented as NIMA also have a protective effect on the development of RA. Using a cohort of Dutch patients with RA together with their parents, they observed that the mothers of patients with RA had a significantly lower frequency of ‘‘DERAA’’-containing *HLA-DRB1* alleles than the Dutch control population. In opposite, the frequencies of ‘‘DERAA’’-containing *HLA-DRB1* alleles in fathers of the patients with RA and members of the healthy control group were comparable. In the same study, these findings were replicated in the English multicase families from Manchester. When the patients from the UK and the Netherlands were pooled, the OR of the RA ‘‘DERAA’’-negative patients having a ‘‘DERAA’’-positive mother compared with a ‘‘DERAA’’-positive father was 0.25 (P=0.003). These results together show that there is a protective effect of ‘‘DERAA’’-containing *HLA-DRB1* alleles as NIMA on development of RA in the child. Thus, together these data indicate that both ‘‘DERAA’’- containing *HLA-DRB1* alleles inherited from one of the parents and the presence of ‘‘DERAA’’-containing *HLA-DRB1* alleles as a NIMA protect against the development of RA. Additionally, the effect of ‘‘DERAA’’-containing *HLA-DRB1* alleles as NIMA is as strong as the effect observed when the ‘‘DERAA’’ alleles are inherited directly from one of the parents. Although not significant (over 7000 families would be required to determine whether the inherited and non-inherited protection differ significantly or not), these data indicate that both effects are of the same magnitude (Feitsma et al., 2008).

The presence of ‘‘DERAA’’-containing HLA-DRB1 molecules can protect a person against the development of RA. The ‘‘DERAA’’-containing HLA-DRB1 molecules can either be present because an individual has inherited them directly or because an individual has a ‘‘DERAA’’-positive mother and acquired some of the ‘‘DERAA’’-containing HLA-DRB1 molecules during fetal or neonatal life, or both. The protective effect that is acquired in either way is of similar strength, which suggests that already a small number of cells can initiate this protective effect. Additional research is required to elucidate the mechanism of protection of both the inherited and the NIMA effect of the ‘‘DERAA’’-containing HLA-DRB1 molecules (Feitsma et al., 2008).

7.1.2.2. Protein Tyrosine Phosphatase Non-receptor 22 gene

The minor allele of a non-synonymous SNP (rs2476601 C/T - 1858C/T) in *PTPN22* gene, located on chromosome 1p13, has been found to be associated with multiple autoimmune diseases including RA. This variant confers the second largest genetic risk to the development of RA, with an effect size of 1.8 (Vang et al., 2008). The associated non-synonymous SNP results in an amino acid substitution of arginine (Arginine 620) for tryptophan (Tryptophan 620) in one of the several proline-rich motifs within the non-catalytic C-terminal end of the lymphoid tyrosine phosphatase (LYP). This motif enables the physical binding of LYP with the Src homology 3 (SH3) domain of the negative regulatory kinase, C-terminal Src kinase (Csk). The resulting LYP-Csk complex is known to synergistically inhibit the TCR signalling pathway by down-regulating the tyrosine kinase, lymphocyte-specific protein tyrosine kinase (LCK), which is involved in early stages of T cell activation (Hill et al., 2002).

The first study of association of the minor T allele with an autoimmune disease came from a candidate-gene study of two independent case-control cohorts of patients with T1D (Bottini et al., 2004), and this association with T1D has since been replicated in a number of studies (Smyth et al., 2004; Qu et al., 2005; Zheng and She, 2005). At about the same time, Ann Begovich and colleagues (2004) initiated a 'functional' genome-wide SNP scan of several thousand likely candidate genes, and the prioritization was informed by linkage data, including linkage at chromosome 1p13, the chromosomal location of *PTPN22*. This study reported an association of the same *PTPN22* variant with RA. This was followed by several other studies in which the association with RA has been unequivocally confirmed in populations of European descent, including UK (Hinks et al., 2005), France (Dieudé et al., 2005, Michou et al., 2007), Finnish (Seldin et al., 2005), US and Swedish (Plenge et al., 2005), Dutch (Zhernakova et al., 2005a), Spanish (Orozco et al., 2005) populations. Additionally, this association has been also confirmed by both major GWA studies (WTCCC, 2007; Plenge et al., 2007a). Interestingly, a study in a Japanese population could not test for association as the causal variant was found to have a very low minor allele frequency (Ikari et al., 2006). Following studies have extended these observations and have shown that the same variant is also associated with susceptibility to SLE (Kyogoku et al., 2004), Graves' disease (GD) (Velaga et al., 2004), JIA (Hinks et al., 2006a), and generalized vitiligo (Canton et al., 2005). Nevertheless, no associations with CD (van Oene et al., 2005), multiple sclerosis (Begovich et al., 2005), Psoriasis (Ps) (Nistor et al., 2005) have been detected, suggesting that there may be differences in the aetiology of these subsets of autoimmune diseases.

This polymorphism resides in a rather large haplotype block encompassing the entire *PTPN22* gene as well as several flanking loci. Indeed, in the recent WGA scan reported by the WTCCC (2007), the *PTPN22* association was actually picked up by a marker that is outside of the *PTPN22* gene itself. Thus, as with all association studies, the question is whether the polymorphism used to identify the association is actually the causative variant.

Resequencing of the *PTPN22* locus by Carlton and colleagues (2005) showed that the 1858T allele was the only variant that distinguished the risk haplotype from a second non associated haplotype. Subsequent studies have supported the notion that 1858C/T is the only SNP in *PTPN22* associated with RA (Wesoly et al., 2007; Hinks et al., 2007). The importance of the 1858T allele is further supported by the fact that there is no association with *PTPN22* in the Asian population, and indeed Asian populations rarely carry the 1858T variant (Kochi et al., 2009). Recently, a small study of Asiatic Indians with RF positive RA showed an association of the 1858C/T polymorphism with RA (Mastana et al., 2007); in this population the allele frequency of the risk allele was lower than in Caucasians. Attempts to identify additional *PTPN22* variants that may associate with RA in Asian population have not been successful (Lee et al., 2009). Thus, although the genetic data are not totally comprehensive across the entire risk haplotype, it is highly likely that the 1858T allele is directly responsible for the associations with RA. Similarly, T1D patients have also been investigated with regard to other SNPs in *PTPN22*. Whereas one report suggested that a promoter SNP in *PTPN22* (-G1123C, rs2488457) confers increased risk of T1D (Kawasaki et al., 2006), a later study could not confirm this connection (Cinek et al., 2007), again supporting a role for 1858C/T as the major T1D-associated SNP in *PTPN22* (Onengut-Gumuscu et al., 2006).

Knockout animals for PEP (the mouse ortholog of PTPN22) exhibit enhanced T-cell activation in combination with an increased production of antibodies (Hasegawa et al., 2004). This is consistent with the ability of PTPN22 to dephosphorylate Lck at the activating phosphotyrosine 394. Until now, rather surprisingly, it is increasingly apparent that the consequence of the 1858T risk allele is a lower degree of T cell activation (an increased threshold for TCR signalling) (Vang et al., 2005; Rieck et al., 2007). It is not clear if this particular activity is affected by the 1858T allele in *PTPN22*. Delogu and collegues (2009) have proposed a model for interactions between Lck, PTPN22 and Csk that may explain the elevation of thresholds for TCR signalling, with the overall implication that reduced rather than elevated T cell triggering may be part of the phenotypic predisposition to autoimmunity. A similar tendency to increased thresholds for receptor triggering has also been reported in B

cells (Rieck et al., 2007). However, PTPN22 is widely expressed in many hematopoietic cell types, and the function of PTPN22 in these cells is largely unknown.

Finally, the study of *PTPN22* is informative because it emphasizes the importance of taking population substructure into account when performing case-control analysis. The 1858T allele shows dramatic frequency differences among the major racial groups (the allele is virtually absent in Asians) and shows a striking gradient of increasing frequency going south to north in European populations (Begovich et al., 2004). This emphasizes that matching cases and controls by ancestry, even within the major racial groups, is essential for carrying out reliable genetic association studies. Therefore, candidate gene association studies that do not attempt to take account of ancestry are likely to be flawed, especially when modest effects are being examined. Ancestry determination by self-report is not a reliable method of doing this, and in any case, this information is frequently not available. Recent advances in the analysis of European population substructure (Price et al., 2008; Kosoy et al., 2009) allows the selection of panels of SNP markers that can correct for this source of error analytically (Seldin and Price, 2008).

7.1.2.3. Tumor Necrosis Factor Receptor-Associated Factor 1/Complement Component 5 locus

The region encompassing *TRAF1* and *C5* genes has been recently reported to be a genetic risk factor involved in RA. Using a GWA approach, Plenge and colleagues (2007a) identified this locus on 9q33-34 as a novel genetic risk for ACPA positive RA in European descent populations [North American Rheumatoid Arthritis Consortium (NARAC) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA)]. Simultaneously, Kurreeman and colleagues (2007) reported the same *TRAF1-C5* locus association by a candidate gene approach in Caucasian RA case-control populations (Dutch, The Netherlands, Swede and US populations). They observed that this genetic risk factor may be predominant in the autoantibody-positive subset (RF and ACPA) of RA patients. The robustness of this association was demonstrated by its prevalent risk in Dutch, Swedish and North America populations. Furthermore, a recent replication study in a large British cohort in combination with imputed data from the WTCCC genome-wide scan has confirmed the association of the *TRAF1-C5* region with RA, with stronger evidence of association for the ACPA positive RA patients (Barton et al., 2008). Additionally, a recent study performed by Chang and colleagues (2008) was found variants in the plant homeodomain-finger protein 19 (*PHF19*)-*TRAF1-C5* region on chromosome 9q33.2 that show strong and consistent association across three

independent RA case-control studies, paralleling and extending the results of the GWA approach and the candidate gene study, described before. This detailed genetic analysis of this region, incorporating HapMap information, localizes the RA-susceptibility effects to a 70 kb region that includes a portion of *PHF19*, all of *TRAF1*, and the majority of the *TRAF1-C5* intergenic region, but excludes the *C5* coding region. Although the evidence from the SNPs genotyped in their sample sets most strongly points towards *TRAF1* variants as being the most highly consistent with a disease model, the high LD that extends from the 5' end of *PHF19* through *TRAF1* and into the *TRAF1-C5* intergenic region precludes conclusively determining causative genes or functional motifs through genetic means in these samples. Mapping studies in additional sample sets with a different LD architecture and/or functional studies will be required to resolve the molecular relevance of these findings. Two meta-analysis performed in this locus also confirmed the susceptibility risk of *TRAF1-C5* with RA (Raychaudhuri et al., 2008; Patsopoulos and Ioannidis, 2009).

This locus has been also associated with JIA (Albers et al., 2008; Behrens et al., 2008) and SLE (Nishimoto et al., 2009; Kurreeman et al., 2009) suggesting that this region is likely to be part of a shared mechanism underlying several autoimmune diseases.

Both *TRAF1* and *C5* are compelling candidate genes for RA. *C5* codes for complement component 5 that could contribute to the development of RA through tissue destruction as well as the mobilization of inflammatory and synovial cells (Ravetch and Clynes, 1998). C5-deficient mice as well as mice treated with antibodies directed against C5a are protected from collagen induced arthritis (Wang et al., 1995; Wang et al., 2000). Complement components and regulatory molecules have long been known to be present in synovial tissues (Ward and Zvaifler, 1974; Neumann et al., 2002). Indeed, inhibition of C5a receptor signalling is an appealing therapeutic target, although recent human trials have been disappointing (Vergunst et al., 2007). Nevertheless, the central role of complement in the inflammatory process makes *C5* a plausible candidate gene in this region.

TRAF1 is a member of the TNF receptor associated factor family, a group of adaptor proteins that link TNF receptor family members (for example, TNF-α) to downstream signalling (Arch et al., 1998). The molecules are involved in signalling pathways that play a role in cell proliferation and differentiation, apoptosis, bone remodelling and activation or inhibition of cytokines. The role of TRAF1 in apoptosis has been demonstrated in mice, where overexpression of TRAF1 resulted in a reduced antigen-induced apoptosis of the CD8+ T-lymphocytes (Speiser et al., 1997). TRAF1 also has a antiproliferative effect: TRAF1 knock-out mice respond to TNF signalling through the TNF receptor 2 with an enhanced T-cell

proliferation and activation of the NF-κB signalling pathway (Tsitsikov et al., 2001). TRAF1 is also regulated by, and has a role in, CD40 signalling (Bishop et al., 2004), a provocative fact given that *CD40* is likely to be associated with risk for RA (Raychaudhuri et al., 2008). Such studies suggest that associations in the *TRAF1/C5* region with RA may be related to alterations in the regulatory activity of TRAF1. Finally, PHF19 is involved in differentiation and cell cycle regulations and is also in LD with the SNPs RA associated in the *TRAF1-C5* region. Based on its function (Wang et al., 2004), *PHF19* does not seem to be an obvious candidate an RA susceptibility gene, although additional studies will clearly be necessary to determine which gene/variant in the *TRAF1-C5* region is ultimately responsible for the increased susceptibility to RA.

7.1.2.4. Chromosome 6q23 region

The WTCCC (2007) identified, through a GWA study, 9 new putative RA susceptibility loci, in addition to *HLA* and *PTPN22*. One SNP located in the chromosomal region 6q23, rs6920220, was unequivocally replicated in a validation study and reported to be stronger in patients with ACPA or RF positivity than in the ACPA-negative or RF-negative subgroup (Thomson et al., 2007). Interestingly, association with the same SNP and a second, independently associated polymorphism in the region (rs10499194) was detected in a GWA study in a RA ACPA-positive US population, confirming the locus as important in RA causation (Plenge et al., 2007b). Increased risk for RA is conferred by the minor allele of rs6920220 and protection by the minor allele of rs10499194. The SNP rs6920220 identified in the WTCCC study (2007) is located only 3.8 kb away from marker rs10499194. However, fine mapping of the region followed by regression analysis showed that these two signals are independent, and a haplotype analysis using these two SNPs showed that a two-allele model of risk provided the strongest risk predictor. Construction of a haplotype tree indicated the haplotype tagged by rs1099194 as protective, whereas the haplotype tagged by rs6920220 is the risk haplotype. Two recent studies in the Spanish population reported an absence of association between these two polymorphisms (rs6920220 and rs13207033, which is a perfect proxy of rs10499194) and RA. However after autoantibodies stratification they observed, in the first study, an association between the rs13207033 SNP and RA only in the patients with ACPA (Dieguez-Gonzalez et al., 2009) and an association of the rs6920220 with RA in ACPA or RF-positive patients, in the second study (Perdigones et al., 2009).

These RA-associated SNPs in the 6q23 region are located at more than 150 kb distance from the nearest genes, *TNFAIP3* and *OLIG3*. A fine-mapping performed in this region found 3

SNPs associated with RA in the UK population (Orozco et al., 2009). The SNPs showing the strongest association were rs6920220 and rs13207033. Additionally, they found a new potential RA marker, rs5029937, located in the intron 2 of *TNFAIP3*. They reported that the combination of the carriage of both risk alleles of rs6920220 and rs5029937 together with the absence of the protective allele of rs13207033 was strongly associated with RA when compared to carriage of none. This equates to an effect size of 1.50 compared to controls and is higher than that obtained for any SNP individually. Moreover, this locus has been associated with the rate of joint destruction in RA in the Dutch population with early RA (Scherer et al., 2009). In this study, two polymorphisms rs675520 and rs9376293 located in this region were associated with severity of radio-graphic joint damage in ACPA+ patients. Two meta-analysis performed in this locus also confirmed the susceptibility risk of 6q23 region with RA (Raychaudhuri et al., 2008; Patsopoulos and Ioannidis, 2009). This region has been also identified as a new susceptibility locus in SLE (Graham et al., 2008; Musone et al., 2008), T1D (Fung et al., 2009), Ps (Nair et al., 2009) and Coeliac disease (Trynka et al., 2009).

Until now, all studies published point to a role of *OLIG3* in the development of neuronal cells, without any specific involvement in immune function (Muller et al., 2005; Ding et al., 2005). In contrast, *TNFAIP3*, also known as *A20*, is an attractive candidate gene for autoimmunity. A20 is a TNF-inducible zincfinger protein that acts in the cytoplasm to regulate and restrict the duration of both TNF and TLR induced NF-κB signals (Wertz et al., 2004; Boone et al., 2004). Overexpression of the protein leads to a block of NF-κB activation including that of the TNF and TLR signalling pathways, while A20-deficient mice show severe inflammation affecting multiple organs including the joints (Lee et al., 2000). An already described recent analysis of the associations of *A20* in SLE show that at least three independent genetic effects exist with the *A20* locus, and one of these variant results in an amino acid changes in the A20 protein (phe127cys) (Musone et al., 2008). Preliminary studies suggest that this amino acid change has functional consequences in terms of the ability to inhibit TNF-induced NF-κB activation. These findings suggest that *TNFAIP3*/*A20* plays a critical role in autoimmunity, including RA, and may act in pathways suggested by other genetic findings such as TRAF1.

7.1.2.5. Signal transducer and activator of transcription 4 gene

The discovery of the *STAT4* gene association with RA was the result of genetic mapping efforts focused on a linkage peak on chromosome 2q. While linkage peaks in RA have historically been challenging to replicate using traditional microsatellite markers, a replication

of linkage using a more robust SNP genotyping methodology revealed significant linkage on chromosome 2q (Amos et al., 2006). Follow-up dense association mapping in the region led to definitive evidence for association with *STAT4* in both RA and SLE (Remmers et al., 2007). This fine mapping of this region in North American and Swedish populations revealed that four SNPs within the third intron of *STAT4* were associated with risk of RA, rs7574865 being the most significant. This association was also demonstrated in Spanish, Dutch, Swedish (Orozco et al., 2008b), UK (Barton et al., 2008), Colombian (Palomino-Morales et al., 2008), Crete (Zervou et al:, 2008) and Dutch (Daha et al., 2009) Caucasian populations. Interestingly, the GWA studies in RA have not pointed strongly to *STAT4* as a risk gene, and this undoubtedly relates to the very modest ORs for this association (WTCCC, 2007; Plenge et al., 2007a). In contrast, the ORs for *STAT4* associations with SLE are considerably stronger, and thus *STAT4* emerges as a prominent association signal in GWA studies in SLE (Harley et al., 2008; Hom et al., 2008). Unlike *PTPN22*, the *STAT4* associations with both RA and SLE are also observed in Asian populations (Lee et al., 2007a; Kobayashi et al., 2008b), thus confirming STAT4 as an important common risk gene for these two diseases in both Caucasian and Asian populations. Moreover, STAT4 is also associated with SS (Korman et al., 2008). STAT4 is a member of the STAT family of transcription factors, of which there are six main members, each with distinct roles in cytokine receptor signalling (Levy and Darnell, 2002). STAT4 is a key molecule for IL-12 signalling in T cells and NK cells, leading to the production of IFN-γ and differentiation of CD4 T cells into a Th1 phenotype (Jacobson et al., 1995). Upon IL-12R binding by IL-12, STAT4 is phosphorylated and forms homodimers. These homodimers are translocated in the nucleus where they initiate transcription of STAT4 target genes, including IFN-γ (Watford et al., 2004). Thus, *STAT4* $^{-/-}$ mice, do not respond to IL-12, lack Th1 responses and have a predominantly Th2 immune response phenotype (Kaplan et al., 1996). Interestingly, these mice are also resistant to experimental arthritis (Hildner et al., 2007). Relatively little is known about how the expression of STAT4 itself is regulated at the transcriptional level. STAT4 is expressed in resting CD4+ T cells and NK cells and in Jurkat cells. STAT4 transcription has been shown to be regulated in part by Ikaros, a zinc-finger transcription factor known to be involved in hematopoietic cell differentiation (Yap et al., 2005). In contrast, STAT4 is not constitutively expressed by monocytes or immature DCs, but can be induced upon activation and maturation (Fukao et al., 2001). In the case of DCs, NF-κB/Rel proteins have been shown to upregulate STAT4 transcription during the differentiation into mature human DCs in response to LPS (Remoli et al., 2007).

7.1.2.6. Chromosome 4q27 region

The chromosome 4q27 region was originally associated with the inflammatory disorders Coeliac disease and T1D in two GWA studies, suggesting a general role of this region with autoimmune disorders (Todd et al., 2007; van Heel et al., 2007). This hypothesis was supported by a subsequent study confirming the role of the region in T1D and detecting an association with RA (Zhernakova et al., 2007). In this case–control study, in the Dutch population, five SNPs (rs4505848, rs11732095, rs6822844, rs4492018 and rs1398553) of the block were investigated, showing a significant association of the rs6822844-T allele and the rs6822844-GT genotype and of a specific haplotypes with RA. Additionally, Raychaudhuri and colleagues (2008) performed a meta-analysis of two published GWA studies (WTCCC, 2007; Plenge et al., 2007a) providing further support for the role of this region in RA. Furthermore, RA 4q27 association has been replicated in the UK (Barton et al., 2009) and Dutch populations (Daha et al., 2009). The 4q27 region was also associated with Ps and PsA (Liu et al., 2008), SLE (Sawalha et al., 2008), inflammatory bowel diseases (IBD) [CD and ulcerative colitis (UC)] (Festen et al., 2009) and JIA (Albers et al., 2009). Thus, the 4q27 region provides a reprise of the general theme that common genes provide risk for multiple autoimmune disorders.

The associated region contains 4 genes: *KIAA1109*, testis nuclear RNA-binding protein (*Tenr*), *IL-2* and *IL-21*. Tenr is exclusively expressed in the testis (Schumacher et al., 1995); *KIAA1109* is more widely expressed, but the function of this gene is unknown. In contrast, both *IL-2* and *IL-21* have obvious potential relevance to the pathogenesis of RA. *IL-21* is produced by activated CD4+ and NK T cells, co-stimulates the proliferation and differentiation of T and B cells and NK cells (Leonard and Spolski, 2005). IL-21 has recently been implicated in the development of Th17 cells (Yang et al., 2008), and appears to be uniquely required for the initiation of this differentiation pathway in naive CD4 cells. Peripheral blood and synovial T cells of RA patients show higher proliferation as well as an enhanced secretion of the proinflammatory cytokines TNF-α and IFN-γ after stimulation with IL-21 than T cells from healthy individuals and patients with OA (Li et al., 2006). Arthritis mouse and rat models treated with IL-21 receptor Fc fusion proteins show an improvement of the disease-related symptoms and histologic parameters (Young et al., 2007). These studies suggest a direct role of IL-21 in the development of RA and also a potential therapeutic target. IL-2 exerts its effects on many cell types, the most prominent of which is the T lymphocyte. Accordingly, a major function of IL-2 is to promote proliferation and expansion of both antigen-specific clones of CD4+ and CD8+ T cells as well as to induce production of other

cytokines. In CD4+ T cells, IL-2 plays a non redundant role in the development of Treg cells. Accumulating evidence supports CD4+ $CD25^{high}$ Treg cells playing an essential role in controlling and preventing autoimmunity (Chistiakov et al., 2008). Therefore, IL-2 pathways are clearly a prime candidate for causative risk alleles in human autoimmunity, including RA. In addition, the WTCCC study (2007) provided evidence of association of the IL-2 receptor with RA as well as T1D. Thus, both *IL-2* and *IL-21* are leading candidate genes for RA susceptibility in the 4q27 region.

7.1.2.7. Cytotoxic T lymphocyte-associated antigen 4 gene

CTLA4 molecule is a homolog for CD28, and both molecules and their common ligands (B7-1 and B7-2) constitute the B7/CD28-CTLA4 co-stimulatory pathway for T-cell activation. Whereas the CD28/ligand interaction plays a critical role in increasing and maintaining the T-cell response initiated through T-cell antigen receptor engagement, the CTLA4/ligand interaction has an inhibitory effect on T-cell activation and might contribute to peripheral tolerance (Thompson and Allison, 1997). Therefore, *CTLA4* is a functional candidate gene to susceptibility to RA. Several polymorphisms have been described in the *CTLA4* gene, some of them are: -1722T/C (Johnson et al., 2001) and -319C/T (Deichmann et al., 1996), both within the promoter region; +49A/G in the exon 1 (Donner et al., 1997); and a microsatellite $(AT)_n$ 3'UTR polymorphism (Polymeropoulos et al., 1991) and a CT60A/G polymorphism (rs3087243), which has been 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 CT60-G allele was shown to be associated with lower mRNA levels of soluble CTLA4 isoform that could increase T-cell activation and might have an important role in determining susceptibility to autoimmune diseases (Ueda et al., 2003). Various *CTLA4* RA association studies have shown encouraging, yet inconsistent, results for this locus (Barton et al., 2004; Orozco et al., 2004; Lei et al., 2005; Zhernakova et al., 2005b; Plenge et al., 2005; Tsukahara et al., 2008). Plenge and colleagues (2005) provided evidence that the differences between studies could be due to insufficient power in some of the studies. Recently, several studies have confirmed the association of the CTLA4 CT60A/G polymorphism with RA. Raychaudhuri and colleagues (2008) performed a meta-analysis of two published GWA studies (WTCCC, 2007; Plenge et al., 2007a) providing further support for the role of *CTLA4* gene in RA. Furthermore, RA association of this polymorphism has been replicated in UK (Barton et al., 2009) and Dutch populations (Daha et al., 2009). Furthermore, a meta-analysis of five published studies of disease association with this

polymorphism was performed confirming an overall association of this locus with RA in Caucasians (Daha et al., 2009). Interestingly, in this meta-analysis, *CTLA4* in the Caucasian population was found to predispose to ACPA-positive disease only, and not to ACPA-negative disease. Finally, the fusion protein, Abatacept (Orencia), a biologic drug with proven efficacy in RA, is a CTLA4 analogue, highlighting the fact that relatively modest genetic effects may identify targets for treatment which are highly effective in large numbers of patients (Kremer et al., 2008).

7.1.2.8. Other Rheumatoid Arthritis susceptibility genes

In the past two years, the first wave of GWA studies has improved the understanding of the RA genetic basis. These studies have provided the identification of new possible RA susceptibility loci, such as those mapping to the *CD40*, chemokine (C-C motif) ligand 21 (*CCL21*), kinesin family member 5A (*KIF5A*), protein kinase C, theta (*PRKCQ*), membrane metallo-endopeptidase-like 1 (*MMEL1*), cyclin-dependent kinase 6 (*CDK6*) (Raychaudhuri et al., 2008), *IL2RA*, *IL2RB* (Barton et al., 2008), and Kruppel-like factor 12 (*KLF12*) (Julià et al., 2008). Some of these genes were replicated in the UK population (Orozco et al., 2009). However, validation studies using large sample sizes and ethnically different populations as well as meta-analysis will be essential to robustly define these new potential susceptibility loci. Several other genes like peptidyl arginine deiminase, type IV (*PADI4*), solute carrier family 22, member 4 (*SLC22A4*), Fc receptor-like 3 (*FCRL3*) (Tokuhiro et al., 2003; Mori et al., 2005; Kochi et al., 2005; Plenge et al., 2005; Caponi et al., 2005; Hu et al., 2006; Kang et al., 2006; Raychaudhuri et al., 2008; Burr et al., 2009), protein kinase C, eta (*PRKCH*) (Takata et al., 2007), have been reported as associated with RA susceptibility in Caucasian or/and Asian populations, with variable degrees of statistical evidence. In many instances, this is likely to reflect a real, but modest, effect on risk. Such small risk increases can only be detected in very large cohorts explaining the lack of unequivocal association in smaller cohorts, including the major published GWA studies. Some genes as *PADI4*, *SLC22A4*, *FCRL3*, described before, have been associated with RA in studies of Asian patients, but the associations were weak or negative in populations of European ancestry. Other risk factors like caspase 7 (*CASP7*) and interferon regulatory factor 5 (*IRF5*) genes have been linked with RA genetic susceptibility in the Spanish population (García-Lozano et al., 2007) and in a subset of ACPA-negative RA in Suedish and Dutch populations (Sigurdsson et al., 2007), respectively. However, these associations need replication to corroborate definitive RA association.

7.2. Rheumatoid Arthritis environmental and host factors

The development of autoimmune multifactorial diseases like RA depends on the interaction between genetic background and a number of environmental factors. The onset of clinical disease occurs at a threshold reached by the cumulative action of genetic and environmental factors beyond which the abortion of an auto-aggressive immune response becomes impossible (Edwards and Cooper, 2006). Estimates of heritability suggest that genetic factors are responsible for at least 50% to 60% of the risk of developing RA (MacGregor et al., 2000). This means that gene-environment interactions and environmental factors must explain the rest. In recent years advances in molecular biology including GWA studies have permitted the identification of genes important in RA (WTCCC, 2007; Plenge et al., 2007a). In contrast with the detailed study of the role of genes in the aetiology of RA, lower attention has been directed towards the elucidation of environmental and host factors. Studies carried out looking for environmental and host factors important in RA have identified numerous candidates but as described below only tobacco smoking was well established. These candidates' factors include airway products exposures, a number of infectious agents, sex hormones, pregnancy and diet.

7.2.1. Tobacco smoking

When RA is defined according to ACR criteria, several studies have found a link between smoking and risk of RA (Hazes et al., 1990; Heliovaara et al., 1993; Voigt et al., 1994; Stolt et al., 2003). In some studies, the association of cigarette smoking with the development of RA seemed to be higher for men than for women (Reckner-Olsson et al., 2001; Krishnan et al., 2003). However, prospective cohort studies have demonstrated that tobacco exposure also increases the risk of RA in women (Karlson et al., 1999; Criswell et al., 2002; Costenbader et al., 2006). This relationship seems to be independent of the hormonal status, as suggested by the findings obtained from Costenbader and colleagues (2006), in which the risk of developing RA during the premenopausal years for all women was similar to the risk observed among postmenopausal women ever smokers. In the same stdudy, they observed that both increasing duration and intensity of cigarette smoking amplified the risk of RA which was significantly elevated with 10 pack-years or more of smoking and increased linearly with increasing pack-years. Importantly, the risk of incident RA remained substantially elevated until 10-20 years after smoking cessation.

When the definition of RA is based on the presence or absence of RF, and notably ACPA, a relationship between tobacco exposure and certain RA subtypes has been observed. Prior to

implementation of ACPA for RA diagnosis, several studies found an association between smoking and RF-positive RA in both genders (Mattey et al., 2002; Stolt et al., 2003). Additionally, recent studies demonstrated an unexpected high increase in risk associated with exposure to smoking in the presence of SE alleles of the *HLA-DRB1* gene, with regard to susceptibility to ACPA-positive and/or RF-positive RA (Padyukov et al., 2004; Linn-Rasker et al., 2006; Klareskog et al., 2006; Van der Helm-van Mil et al., 2007; Verpoort et al., 2007; Pedersen et al., 2007). A strong gene-environment interaction between tobacco exposure and the SE in the ACPA-positive subset of patients has been repeatedly demonstrated in several studies in Europe (Padyukov et al., 2004; Linn-Rasker et al., 2006; Van der Helm-van Mil et al., 2007; Pedersen et al., 2007), whereas neither smoking nor the SE confers an increased risk of ACPA-negative RA. However, when replication of the demonstrated gene-environment interaction was assessed in 3 North American cohorts by Lee and colleagues (2007), evidence of a gene-environment interaction between smoking and SE alleles for ACPA formation could be observed in only one of those cohorts.

While the mechanisms responsible for the influence of smoking in RA are not clear, some studies have shown an association between RA and the toxic compounds found in cigarette smoke, such as nicotine, 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) and reactive oxygen species (Tamaki et al., 2004; Mirshafiey and Mohsenzadegan, 2008). Recently, Kobayashi and colleagues (2008c) reported that the mRNA and protein levels of aryl hydrocarbone receptor (AhR) were higher in RA synovial tissue than in OA tissue, and that TCDD upregulated the expression of IL-1B, IL-6 and IL-8 through binding to AhR, with this effect transmitted *via* the NF-κB and extracellular signal-regulated kinase signaling cascades. Additionally, AhR expression in synovial cells was upregulated by TNF-α. These data suggest that TNF-α activates AhR expression in RA synovial tissue, and that cigarette smoking and exposure to TCDD enhance RA inflammatory processes. Therefore, TCDD exposure, such as smoking, appears to exacerbate RA pathogenesis.

7.2.2. Other environmental and host factors

Several infectious agents have been reported to be risk factors for RA, including human parvovirus B19, Epstein–Barr virus, retroviruses, hepatitis B virus, Mycobacterium tuberculosis and Mycoplasma (Kobayashi et al., 2008a). These associations between RA and infectious agents have been supported by increased antibody titers or DNA to the infectious organism in RA patients compared with other individuals. However, some epidemiological studies oppose these possible links, and there has been no consistent evidence that a single

infectious agent or other environmental factor is responsible for the effect of the environment on RA. Therefore, the pathological role of these and other infectious agent's needs to be further explored (Kobayashi et al., 2008a).

Sverdrup and colleagues (2005) investigated the association between exposure to mineral oil and the risk of developing RA, and performed a separate analysis on the major subphenotypes for the disease; namely, RF-positive RA, RF-negative RA, ACPA-positive RA and ACPA-negative RA. They found that among men, exposure to any mineral oil was associated with a 30% increased RR of developing RA. When cases were subdivided into the major subphenotypes, RF-positive RA, an increased risk was only observed for RF-positive and ACPA-positive RA. The findings are of particular interest since the same mineral oils can also induce polyarthritis in rats (Kleinau et al., 1994). Silica dust has also been found as environmental risk factor for RA (Klockars et al., 1987). Finally, in a historic report, researchers described a severe form of RA (Caplan's syndrome) in charcoal workers (Caplan, 1959).

The increased risk of RA in women has led to evaluation of the role of sex hormones in disease susceptibility. It is well known that the levels of male sex hormones, particularly testosterone, are lower in men who have RA. By contrast, the levels of female sex hormones do not differ significantly between RA patients and control individuals (Heikkilä et al., 1998). Exogenous hormones have also been reported to influence RA disease risk. Several studies have shown that women who use oral contraceptive pills have a reduced risk of developing RA (Spector and Hochberg, 1989; Brennan et al., 1997a). On the other hand, Walitt and colleagues (2008) reported that there were no statistically significant differences in the risk of developing RA or the severity of RA between postmenopausal hormone therapy groups and placebo groups. The molecular mechanisms underlying hormone involvement in RA pathogenesis require further elucidation.

It is widely accepted that RA frequently remits during pregnancy. Although the mechanism for this is unclear, Nelson and colleagues (1993) observed that the amelioration of RA during pregnancy is associated with a disparity in *HLA* class II antigens between mother and fetus. These investigators suggested that the maternal immune response to paternal HLA antigens may play a role in pregnancy-induced RA remission. By contrast, nulliparous women have an increased risk of developing RA, although there is no increased risk in women who are single (Silman, 1994). These reports support the hypothesis that pregnancy is related to the development of RA. Some studies have also suggested that the risk of disease development is increased during the postpartum period, particularly after the first pregnancy (Nelson and

Ostensen, 1997). Subsequent investigations demonstrated that much of this increased risk is associated with breastfeeding and that woman who breastfeed after their first pregnancy are at the greatest risk of developing RA (Brennan and Silman, 1994). These researchers also suggested that the association among breastfeeding, pregnancy and RA may be related to either increased prolactin levels or an abnormal, pro-inflammatory response to prolactin (Brennan et al., 1997b).

Few reports have addressed the influence of diet on RA development and progression. The addition of omega-3 fatty acids to the diets of RA patients has been associated with improvement in RA (Ariza-Ariza et al., 1998). Furthermore, diets high in eicosapentaenoic acid have a favourable effect on the clinical outcome of RA (Volker et al., 2000). Although the association between diet and RA onset is unclear, it is accepted that such fatty acids compete with arachidonic acids, the latter of which are involved in inflammation. Some reports have demonstrated the influence of vitamins on RA. For example, greater intake of vitamin D, primarily from fish and fish products, has been associated with a lower risk of RA (Cutolo et al., 2007). In addition, Okamoto and colleagues (2007) recently reported that vitamin K, which is primarily derived from vegetables and legumes could inhibit the proliferation of fibroblast-like synoviocytes and the development of CIA. Finally, some data also indicate that moderate alcohol consumption can reduce risk for RA (Aho and Heliovaara, 1993; Kallberg et al., 2009), and it diminishes risk and severity of experimental arthritis in rodents (Jonsson et al., 2007).

8. Large-scale gene expression profiling studies in Rheumatoid Arthritis

During the last 6 years, gene expression profiling studies using microarrays technology have been developed for large-scale clinical research and routine to identify genes involved in disease states. At present, microarrays comprising the whole human genome have become commercially available and their potential to identify abnormal gene activity in disease is now well recognized. Array-based expression analysis is based on the hybridisation of an ordered set of probes attached to a surface with a target consisting of cell/tissue isolated mRNAs. In general, these are mRNAs isolated under different biological situations, eg, health and disease, or before and after treatment. The hybridisation pattern reflects the relative abundance of each mRNA and leads to the identification of genes up- or downregulated in the test condition compared with the reference. By grouping sets of differentially expressed genes

according to function, information can be obtained about key pathways related to disease or treatment.

Peripheral blood mononuclear cells (PBMCs) gene expression profiling allows both pathogenetic and pathophysiological processes identification as demonstrated in several types of diseases: cancer (Alizadeh et al., 2000), asthma (Brutsche et al., 2001), SLE (Mandel and Achiron, 2006), cardiovascular diseases (Henriksen and Kotelevtsev, 2002) and psychiatric disorders (Colangelo et al., 2002). PBMCs - and in particular lymphocytes - are particularly convenient for medical research and diagnostic applications, because they can easily and repeatedly be collected in sufficient quantities in the course of the disease. Altered function of lymphocytes in diseases is a result of abnormal expression of genes for numerous cytokines, receptor components, signal transduction pathways, and modulators of transcription and translation. In addition, polymorphism of the genes affects their functional properties. Despite intensive studies, the pathophysiological and pathogenetic mechanisms behind several diseases including RA are still poorly understood. Interest of gene expression of PBMCs concerns with identifying both pathogenetic and pathophysiological processes. Pathogenetic processes are primarily associated with the cause of a disease. Then, microarrays could lead to the identification of abnormal genes and gene activities that may not be only limited to PBMCs, but could occur in cells of pathological tissue as well. In contrast, pathophysiological changes in lymphocytic gene expression are considered an essentially normal reaction of the immune system to a pathological stimulus. Therefore, pathophysiological gene profiles may be shared in a variety of diseases, whereas pathogenetic gene expression is expected to be disease specific (Gladkevich et al., 2005). The differences in expression profiles provide opportunities to stratify RA patients based on molecular criteria that may require different treatment strategies. There has been special interest in these technologies to elucidate the genetics of heterogeneous autoimmune diseases, such as RA. The broad goals of expression profiling in RA are to (i) improve our understanding of the pathogenic mechanisms underlying RA, (ii) identify new drugs targets, (iii) assess activity of the disease, (iv) predict future outcomes, such as responsiveness therapy, overall disease severity, and organ specific risk and (v) develop new diagnostic tests (Baechler et al., 2006).

Expression profiling studies in RA can be classified roughly into two categories: (1) those focused on finding (new) candidate genes for disease aetiology and understanding its pathogenesis, and (2) those focused on identifying expression patterns typical for a state of RA [mild *vs* severe disease or drug-responsive *vs* non-responsive patients (pharmacogenomics studies)]. In the first category, expression analysis is often the first step in elucidating gene

function, and in the second it is aimed at reducing phenotypic heterogeneity or at identifying expression profiles that can serve as diagnostic tools predicting, disease outcome or response to disease-modifying anti-rheumatic drugs (TNF blocking agents) (Toonen et al., 2008). In RA patients, several gene expression profiling studies have been done in PBMCs and synovial tissues using microarrays technology.

8.1. Gene expression profiling studies in peripheral blood mononuclear cells

Several gene expression profiling studies using PBMCs were performed in RA patients and/or healthy controls. Four studies were focused on finding new candidate genes for RA and understanding its pathogenesis. One study was performed to identify a gene expression pattern typical of a specific state of the disease. One study was performed to categorize gene expression profiles concerning genetic (*HLA* SE), autoantibodies (RF and ACPA) and disease activity score 28 (DAS 28) status. All these studies were summarized in Table 1.3.

Table 1.3. Overview of RA gene expression profiling studies using peripheral blood mononuclear cells.

Study	No. of the individuals	No. of transcripts studied	Up-regulated genes (biological processes) A *vs* B	Down-regulated genes (biological processes) A *vs* B
Bovin et al. (2004)	**A.** 8 RA RF-positive **B.** 6 RF-negative	12 626 cDNAs	No significant differences in expression patterns between RF-positive and RF-negative patients	
	A. 14 RA patients **B.** 7 healthy controls		23 genes (immunity and defense, G-protein signalling, extracellular matrix maintenance, haemoglobin)	2 genes (immunity and defense)
Olsen et al. (2004)	**A.** 11 early RA patients **B.** 8 established RA patients	4 329 cDNAs	9 genes (immune/growth factor, neuromuscular, transcription	40 genes (immune/inflammatory, cancer/neoplasia, transcription, translation, cell cycle, growth factors, Metabolism, Cartilage)
Batliwalla et al. (2005).	**A.** 29 RA patients **B.** 21 healthy controls.	12 626 cDNAs	52 genes (40% 'monocyte-enriched' genes)	29 genes (10% 'T-cell-enriched' genes, signal transduction in lymphoid cells)
Van der Pouw Kraan et al. (2007)	**A.** 35 RA patients **B.** 15 healthy controls	42 000 cDNAs	259 genes (immunity and defense - type I interferon signature, lipid and fatty acid transport, transport, ligand-mediated	318 genes (cytotoxic functions, unknown functions)

signalling)

Table 1.3. Overview of RA gene expression profiling studies using peripheral blood mononuclear cells (continued).

Study	No. of the individuals	No. of transcripts studied	Up-regulated genes (biological processes) A *vs* B	Down-regulated genes (biological processes) A *vs* B
Edwards et al. (2007)	**A.** 9 RA patients **B.** 13 healthy controls	6 937 cDNAs	330 genes differentially expressed (calcium binding, chaperones, cytokines, transcription, translation, signal transduction, extracellular matrix, plasma membrane, intracellular membrane, mitochondrial)	
Junta et al. (2008)	**A.** 15 RA patients HLA SE/SE or SE/X **B.** 8 RA patients HLA X/X	4500 cDNAs	13 genes exclusively associated with the presence of HLA-SE alleles (signal transduction, phosphorylation and apoptosis)	
	A. 13 RA patients DAS-28>5.0 **B.** 10 RA patients DAS-28<5.0		91 genes associated with disease activity (signal transduction, apoptosis, response to stress and DNA damage)	
	A. 15 RA patients ACPA positive **B.** 8 RA patients ACPA-negative		101 genes associated with the presence of ACPA (signal transduction, cell proliferation and apoptosis)	

8.2. Gene expression profiling studies in synovial tissue

van der Pouw Kraan and colleagues (2003a) were the first group that generates a molecular description of synovial tissue from RA patients. They used 18,000 cDNAs to profile gene expression, with a focus on immune-related genes, in affected joint tissues from 21 RA patients and in tissues from 9 OA patients as a control. The gene expression signatures of synovial tissues from RA patients showed considerable variability, resulting in the identification of at least two molecularly distinct forms of RA tissues. One class of tissues revealed abundant expression of clusters of genes indicative of an involvement of the adaptive immune response. Detailed analysis of the expression profile provided evidence for a prominent role of an activated signal transducer and activator of transcription 1 (*STAT1*) pathway in these tissues. The expression profiles of another group of RA tissues revealed an increased tissue remodelling activity and a low inflammatory gene expression signature. The gene expression pattern in the latter tissues was reminiscent of that observed in the majority of OA tissues.

Another study of the same group (van der Pouw Kraan et al., 2003b) was used 15 different RA tissues to subclassify RA patients and disclose disease pathways in rheumatoid synovium. Hierarchical clustering of gene expression data identified two main groups of tissues (RA-I and RA-II). A total of 121 genes were significantly higher expressed in the RA-I tissues, whereas 39 genes were overexpressed in the RA-II tissues. Among the 121 genes overexpressed in RA-I tissues, a relative majority of nine genes are located on chromosome 6p21.3. An interpretation of biological processes that take place revealed that the gene expression profile in RA-I tissues is indicative for an adaptive immune response. The RA-II group showed expression of genes suggestive for fibroblast dedifferentiation. Within the RA-I group, two subgroups could be distinguished; the RA-Ia group showed predominantly immune-related gene activity, while the RA-Ib group showed an additional higher activity of genes indicative for the classical pathway of complement activation. All tissues except the RA-Ia subgroup showed elevated expression of genes involved in tissue remodelling.

Devauchelle and colleagues (2004) aimed to evaluate the possibility of different molecular signature of RA comparatively to OA in synovial tissues. A set of 48 genes was selected, based, more specifically, on their overexpression or underexpression in 5 RA samples compared to 10 OA samples. Detailed analysis of the expression profile of the selected genes provided evidence for dysregulated biological pathways such as transcription and the cell cycle, signal transduction, metabolism and proteases and inhibitors, pointed out to chromosomal location and revealed novel genes potentially involved in RA.

Kasperkovitz and colleagues (2005) intended to determine if the heterogeneity of gene expression patterns and cellular distribution between RA synovial tissues was also reflected at the level of the FLS cultured from RA synovial tissues. Gene expression profiles in FLS cultured from synovial tissues obtained from 19 RA patients were analyzed. Hierarchical clustering identified 2 main groups of FLS characterized by distinctive gene expression profiles. FLS from high-inflammation synovial tissues revealed increased expression of *TGF-β*-inducible gene profile that is characteristic of myofibroblasts, a cell type considered to be involved in wound healing, whereas increased production of growth factor (insulin-like growth factor 2/insulin-like growth factor binding protein 5) appeared to constitute a characteristic feature of FLS derived from low-inflammation synovial tissues. The molecular feature that defines the myofibroblast-like phenotype was reflected as an increased proportion of myofibroblast-like cells in the heterogeneous FLS population. Myofibroblast-like cells were also found upon immunohistochemical analysis of synovial tissue.

Lindberg and colleagues (2006a) aimed to investigate variations in gene expression in synovial tissues within and between patients with RA. This was done by applying microarray technology on multiple synovial biopsies obtained from the same knee joints. In this way the relative levels of intra-patient and inter-patient variation could be assessed. The biopsies were obtained from 13 different patients: 7 by orthopedic surgery and 6 by rheumatic arthroscopy. They found that the average number of differentially expressed genes between biopsies from the same patient was about three times larger in orthopedic than in arthroscopic biopsies. Using a parallel analysis of the tissues by immunohistochemistry, they also identified orthopedic biopsies that were unsuitable for gene expression analysis of synovial inflammation due to sampling of non-inflamed parts of the tissue. Removing these biopsies reduced the average number of differentially expressed genes between the orthopedic biopsies from 455 to 171, in comparison with 143 for the arthroscopic biopsies. Hierarchical clustering analysis showed that the remaining orthopedic and arthroscopic biopsies had gene expression signatures that were unique for each patient, apparently reflecting patient variation rather than tissue heterogeneity.

Galligan and colleagues (2007) have compared gene expression profiling of FLS cells in RA, OA and control trauma joint tissues (non-RA, non-OA). Thirty-four genes specific to RA and OA FLS cells were identified. Five genes were highly and exclusively expressed in RA and six genes were expressed only in OA. Expression heterogeneity for patients with the same disease was also found. To address disease heterogeneity in RA FLS cells, they examined the effects of clinical disease parameters (Health Assessment Questionnaire (HAQ) score, C-

reactive protein (CRP), erythrocyte sedimentation rate (ESR) and RF)) and drug therapies (MTX/prednisone) on RA FLS cell gene expression. Eight specific and unique correlations were identified: *HLA-DQA2* with HAQ score; *CLEC12A* with RF; *MAB21L2*, *SIAT7E*, *HAPLN1* and *BAIAP2L1* with CRP level; *RGMB* and *OSAP* with ESR.

Recently, Huber and colleagues (2008) aimed to elucidate the contribution of expression variances in synovial membrane of RA patients, OA patients, and controls to RA pathogenesis. Five hundred sixty eight genes were found with significantly different variances between RA and controls whereas 333 genes were found significantly expressed between RA and OA. Ten pathways/complexes significantly affected by higher gene expression variances were identified in RA compared with controls, including cytokine–cytokine receptor interactions, the TGF-β pathway, and anti-apoptosis. Compared with OA, three pathways with significantly higher variances were identified in RA (for example, B-cell receptor signalling and vascular endothelial growth factor signalling). Functionally, the majority of the identified pathways are involved in the regulation of inflammation, proliferation, cell survival, and angiogenesis.

8.3. Pharmacogenomics expression profiling studies

A pharmacogenomic study by Lequerré and colleagues (2006) set out to identify genes predictive of responsiveness to infliximab, a TNF blocking agent, in PBMCs of RA patients. Thirty-three patients with highly active disease refractory to MTX treatment were included; 16 patients were classified as responders to infliximab, 17 as non-responders. Unsupervised hierarchical clustering of 41 mRNAs differentially expressed in PBMCs prior to treatment perfectly discriminated responders from non-responders. These transcripts included *CYP3A4*, *LAMR1* and *KNG1*, genes that were also differentially expressed in several other studies and related to disease severity. Twenty of the 41 transcripts were assessed by quantitative polymerase chain reaction in a second set of 10 responders and 10 non-responders to validate their predictive value. This set of transcripts provided 90% sensitivity and 70% specificity for the classification of responders and non-responders.

Lindberg and colleagues (2006b) investigated gene expression profiles in synovial tissue of 10 RA patients before and after infliximab treatment. Three patients were found to be good responders, five patients to be moderate responders and two patients to be non responders. A significant difference between the good responding and non responding patients were detected. They found 279 differentially expressed genes involved in processes such as chemotaxis, immune function, signal transduction and inflammatory responses. Among the

identified genes, *MMP-3* was significantly upregulated in good responders compared with nonresponders, providing further support for the potential of *MMP-3* as a marker for good responses to therapy.

In a similar study, large-scale gene expression profiling was performed in synovial biopsies, obtained before initiation of treatment with infliximab, from 18 RA patients with active disease. Nine biological processes were found more actively expressed in 12 responding patients: immunity and defence, T-cell mediated immunity, cell surface receptor mediated signal transduction, *MHC* class II-mediated immunity, cell adhesion, cytokine and chemokine mediated signalling pathway, cell adhesion-mediated signalling, signal transduction, and macrophage-mediated immunity. The transcript expression patterns of synovial tissues shown that almost all patients with high transcript levels of inflammation-related genes responded to infliximab (van der Pouw Kraan et al. 2008).

Finally, Koczan and colleagues (2008) aimed to discriminate molecular differences of responsiveness to etanercept, another TNF blocking agent, in PBMCs of RA patients. RNA was extracted in 19 RA patients before the first application of the TNFα blocker etanercept as well as after 72 hours. Early downregulation of expression levels secondary to TNFα neutralization was associated with good clinical responses, as shown by a decline in overall disease activity 3 months after the start of treatment. Informative gene sets include genes (for example, *NFKBIA*, *CCL4*, *IL8*, *IL1B*, *TNFAIP3*, *PDE4B*, *PPP1R15A* and *ADM*) involved in different pathways and cellular processes such as TNFα signalling *via* NF-κB, NF-κB-independent signalling *via* cAMP, and the regulation of cellular and oxidative stress response. Pairs and triplets within these genes were found to have a high prognostic value, reflected by prediction accuracies of over 89% for seven selected gene pairs and of 95% for 10 specific gene triplets.

There were some limitations in these gene expression profiling studies due to the large heterogeneity concerning different sample sets, designs (case-healthy control, case-other pathologies, subsets of RA), platforms, probe sets and statistical analysis. It seems very important to reduce heterogeneity between studies and increase power in future. Nevertheless, the advent of expression profiling in RA research has served several important purposes: (1) long assumed concepts of RA got additional support on the molecular level and confirmed the heterogeneous nature of RA giving insight into the distinct pathogenic mechanisms contributing to the disease (van der Pouw Kraan et al., 2003a; van der Pouw Kraan et al., 2007) and (2) clinical biomarkers to aid disease diagnosis, prognosis and treatment outcome can be extracted from the genes differentially expressed between patients with RA and

controls. Recent papers suggest feasibility of predicting treatment response by pharmacogenomics, potentially leading to more individualised treatment strategies (Lequerré et al., 2006; Lindberg et al., 2006b; van der Pouw Kraan et al., 2008; Koczan et al., 2008) Especially Lequerré and colleagues (2006) and Koczan and colleagues (2008) - by measuring transcript levels at baseline in blood suitable for implementation into clinical practice - showed that a small subset of discriminative transcripts can provide a tool to predict infliximab and etanercept efficacy in RA, respectively. The genes identified in the treatment response studies were also found differentially expressed in studies related to disease severity, strengthening the view that the same genes and genetic mechanisms may underlie disease severity and response to anti-TNF treatment (Padyukov et al., 2003).

9. Interactions between Genes, Antibodies and Tobacco smoking in Rheumatoid Arthritis

The first gene-environment interaction study has done concerning tobacco smoking and *HLA-DRB1* SE alleles for RF subset (Padyukov et al., 2004). This study showed that the presence *HLA-DRB1* SE alleles was a significant risk factor only for the RF+ but not for the RF- subset of RA, and also that the risk conferred by smoking was entirely restricted to the RF+ subset of RA. The RR for RF+ RA patients homozygous for *HLA-DRB1* SE and who were also smokers was ~16. This means a dramatic gene-environment interaction between *MHC* class II and smoking - something that strongly points towards a biological mechanism involved in the initiation of RA *via* a *MHC* class II dependent T cell activation. This remarkable gene-environment interaction occurring in one subset of RA but not in the other required a biological explanation, which was difficult to find in relation to the RF status. More recently, Klareskog and colleagues (2006) have demonstrated that the occurrence of ACPA was linked to the presence of *HLA-DRB1* SE alleles in a gene-dose dependent manner. Furthermore, the earlier described correlation between the presence of *HLA-DRB1* SE alleles and the presence of RF was shown to be a consequence of a primary link between *HLA-DRB1* SE and the presence of ACPA antibodies. The presence of RF in the absence of ACPA was thus not associated with presence of *HLA-DRB1* SE alleles, whereas an increased frequency of *HLA-DRB1* SE was seen in patients positive for ACPA but negative for RF. They also show that smoking was a risk factor for ACPA positive but not for ACPA negative disease. The gene-environment interaction between smoking and presence of *HLA-DRB1* SE alleles was striking

when analysed for the ACPA positive subset only, with a RR of >21 for the development of RA among smokers carrying two copies of the SE genes.

Such an effect of tobacco exposure on ACPA production only in the context of *HLA* SE has been confirmed in other European case-control studies (Padyukov et al., 2004; Linn-Rasker et al., 2006; Van der Helm-van Mil et al., 2007; Pedersen et al., 2007) whereas neither smoking nor the SE confers an increased risk of ACPA-negative RA. Moreover, in a recent study, Lundström and colleagues (2009) have demonstrated that all SE *DRB1* alleles strongly interact with smoking in the development of ACPA-positive RA. They also observed that the SE alleles do not seem to confer an increased risk of ACPA-negative RA, either on their own or in combination with smoking.

When replication of the demonstrated gene-environment interaction was assessed in 3 North American cohorts by Lee and colleagues (2007), evidence of a gene-environment interaction between smoking and SE alleles for ACPA formation could be only observed in one of those cohorts. This discrepancy could possibly be explained by different procedures for recruiting controls and patients, diverse methodologies for evaluation of smoking, and the existence of different sorts of environmental exposure. Therefore, in the North American population other environmental factors, such as coffee consumption or air pollution, might be confounding factors that obscure the association of ACPA production and smoking in this geographic location (Chang et al., 2006).

Like *HLA-DRB1* SE alleles, *PTPN22* polymorphism is also associated to ACPA production. Indeed, RA patients carrying the *PTPN22*-1858C/T and -T/T genotypes have significantly higher RF and ACPA levels than those carrying the *PTPN22*-1858C/C genotype (Kokkonen et al., 2007; Kallberg et al., 2007).

Two EIRA studies demonstrated that the presence of both genetic factors is associated with a higher risk of developing ACPA antibodies. The OR of developing ACPA-positive RA for subjects having at least one copy of the *PTPN22*-T allele and at least one *HLA-DRB1* SE allele was 9.9 compared with patients without risk alleles (Klareskog et al., 2006; Kallberg et al., 2007). However, Kallberg and colleagues (2007) did not found an interaction between *PTPN22* and cigarette smoking. The RRs for development of RA in positive or negative for ACPA with two different genetic variations (*HLA-DRB1* SE alleles and *PTPN22* 1858C/T allele) and one tobacco smoking in these two EIRA studies were represented in Figure 1.11.

Finally, only one case-control study of Caucasian women found an interaction between *PTPN22* and cigarette smoking in RA (ACPA not studied) (Costenbader et al., 2008).

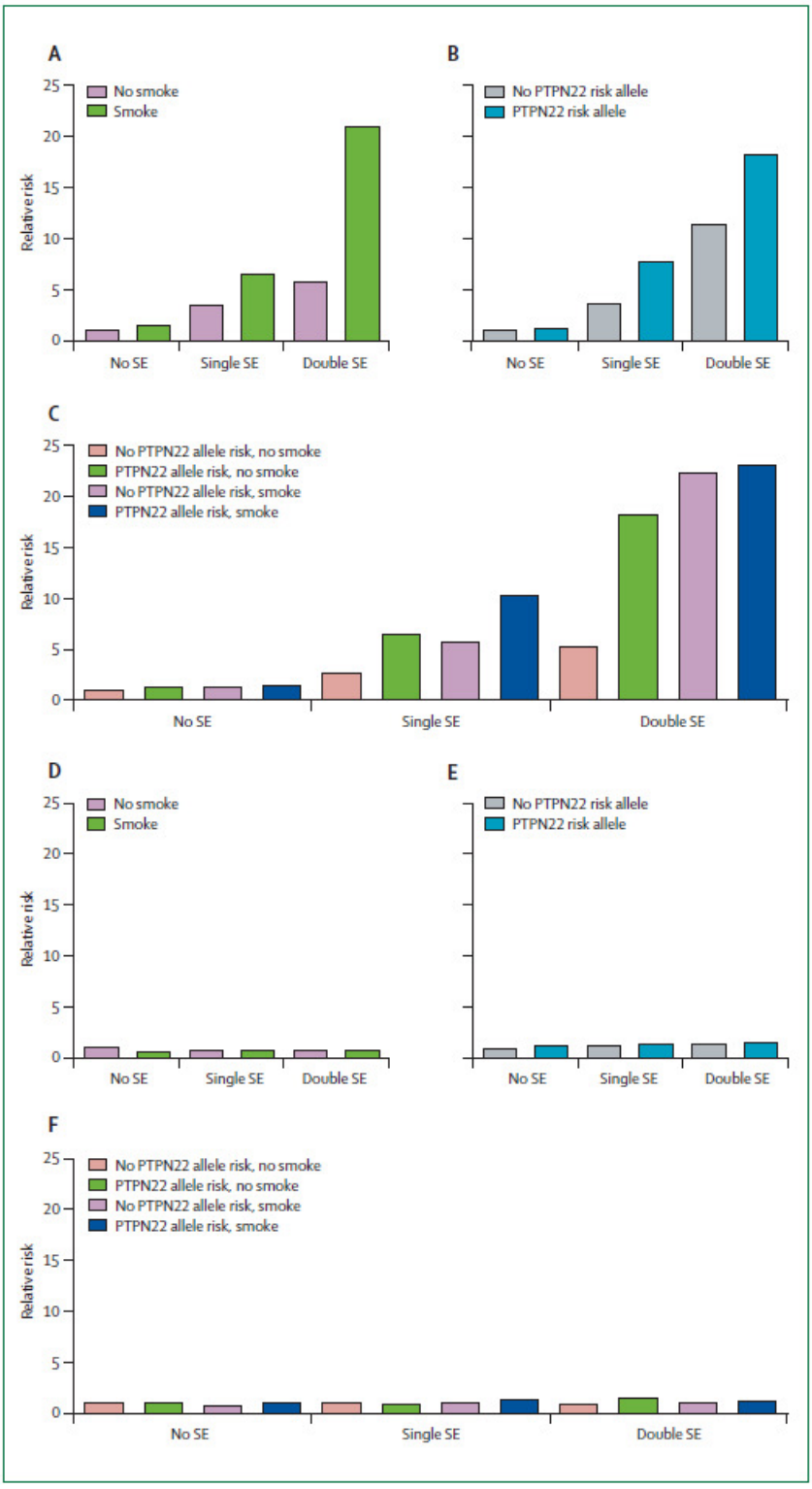

Figure 1.11. Gene-environment and gene-gene interactions determining risk for RA. Genetic variations are absence or presence of one or two copies of HLA DRB1 alleles containing the SE, and absence or presence of the 1858C/T allele of *PTPN22*. The environmental variation is smoking status, either no smoke (for individuals who never smoked) or smoke (for those who ever smoked cigarettes). A, B and C represents the risk for RA positive ACPA. D, E and F represents the risk for RA negative ACPA. (A) and (D) represent gene–environment interactions, (B) and (E) gene–gene interactions, and (C) and (F) gene–gene–environment interactions (Klaresgog et al., 2009).

10. Rheumatoid Arthritis subclassification into distinct disease subsets

Growing evidence shows that the RA consists of at least two subsets, with different causes and severity. This subdivision has been built classically on presence or absence of RF (Bukhari et al., 2002), but increasingly the separation is made on the basis of presence or absence of ACPA (De Rycke et al., 2004). The ACPA method is more specific for RA than is RF and is, thus, more informative as a diagnostic test for early disease. For prognosis in cases of already established RA, ACPA and RF define largely overlapping populations of patients. Notably, joint destruction, comorbidities such as cardiovascular disease, and other extra-articular manifestations are all most prominent in the subset of patients positive for RF and ACPA (Schellekens et al., 1998; De Rycke et al., 2004).

Assembling the data from the last years, it emerges that the *HLA-DRB1* SE and *PTPN22* risk alleles are associated only with a subgroup of RA, defined by the presence of ACPA and/or RF (Padyukov et al., 2004; Klareskog et al., 2006; Kallberg et al., 2007; Lee et al., 2007b). These studies suggest that the involvement of adaptive, B-cell, and T-cell-mediated immunity in pathogenesis is applicable only for the ACPA positive or RF-positive disease subset. These data suggest as well that all further causal studies consisting of genetics should consider these subsets of RA as separate entities. Genetics research of complex diseases took a strong advantage of new technologies, permitting GWA studies of risk alleles (WTCCC, 2007; Plenge et al., 2007a). These studies confirmed that the MHC region possesses the most important genetic risk factors for RA ACPA-positive disease, being *PTPN22* the second most important gene. Numerous further risk alleles for the disease have been identified in gene regions containing *TRAF1-C5* locus, *STAT4* and *OLIG3-TNFAIP3* genes (Plenge et al., 2007a; Remmers et al., 2007; Plenge et al., 2007b). These new findings, and data from complementary candidate gene studies (Kurreeman et al., 2007; Sigurdsson et al., 2007; Thomson et al., 2007) indicate that the genetic risk for RA is a result of a series of genetic variations, confirming different patterns for subsets of RA positive and negative for ACPA or RF (van der Helm-van Mil et al., 2007).

However, small ORs for the majority of these individual risk factors slight these findings to use as prediction of disease risk. Alternatively, the main value these data is the prospective to identify distinct molecular pathways in which several genes work together during development of different forms of RA. As pointed out previously, the best established environmental risk factor for RA is cigarette smoking. Several studies regarding the effect of smoking indicated it as a risk factor for the RF-positive or ACPA-positive subset of RA and having no or few effect on the autoantibody-negative subset (Padyukov et al., 2004; Linn-

Rasker et al., 2006; Klareskog et al., 2006; Pedersen et al., 2007). A main environment interaction was distinguished between *HLA-DR* risk alleles and smoking in RA positive patients for RF or ACPA, in three European investigations (Linn-Rasker et al., 2006; Klareskog et al., 2006; Pedersen et al., 2007 and to a less important level in one North American study (Lee et al., 2007b). These findings indicate three main facts.: (1) that ACPA-positive RA patients are essentially different from those who are ACPA-negative with respect to genetic and environmental risk factors; (2) that an environmental exposure (in this case smoking) could modify significantly the importance of a genetic association in a complex disease; and (3) that these prominent data from genetic epidemiological studies require biological explanations for the combined effects of genetic and environmental risk factors and to understand the different behaviour in RA subsets divided by anti-citrulline immunity (Klareskog et al., 2009).

Klareskog and colleagues (2008) have hypothesized an etiological model for the development of ACPA-positive RA (Figure 1.12). In the first stage (A), when the lung encounters smoke macrophages are activated and some cells go into apoptosis an/or necrosis. This process could lead to increased synthesis and activity of enzymes called PAD, which cause citrullination in certain proteins in the lungs. Some of these post-translationally modified proteins bind specifically to HLA-DR molecules on antigen-presenting cells that contain the SE peptide-binding motif. This process determines the strength of the immune response to citrullinated peptides. Smoking might further contribute to T-cell and B-cell activation by triggering APCs in the lung, thus enhancing cell–cell interactions which finally result in high titres of ACPA. In a second stage (B), a joint-specific inflammatory event is initiated by an unknown and unspecific stimulus, for example, infection or trauma. Inflammatory cells are recruited to the joint and are activated by the unknown “trigger”. In this inflammatory environment, PAD becomes activated and deiminates proteins present in the joint. Finally (C), circulating ACPA enter the joint, bind to the citrullinated proteins and form immune complexes. In the third stage, the immune complexes of ACPA and citrullinated proteins will stimulate APCs, by binding to complement and Fc receptors. Activated APCs present more citrullinated antigens, activate more T and B cells, increase the ACPA production but also RF production. The increased production of proinflammatory cytokines, including TNF, IL-1, and IL-6, recruits more immune cells into the joint perpetuating the inflammatory process. Activation of PAD generates more citrullinated proteins, establishing a vicious cycle that ultimately leads to the development of chronic RA.

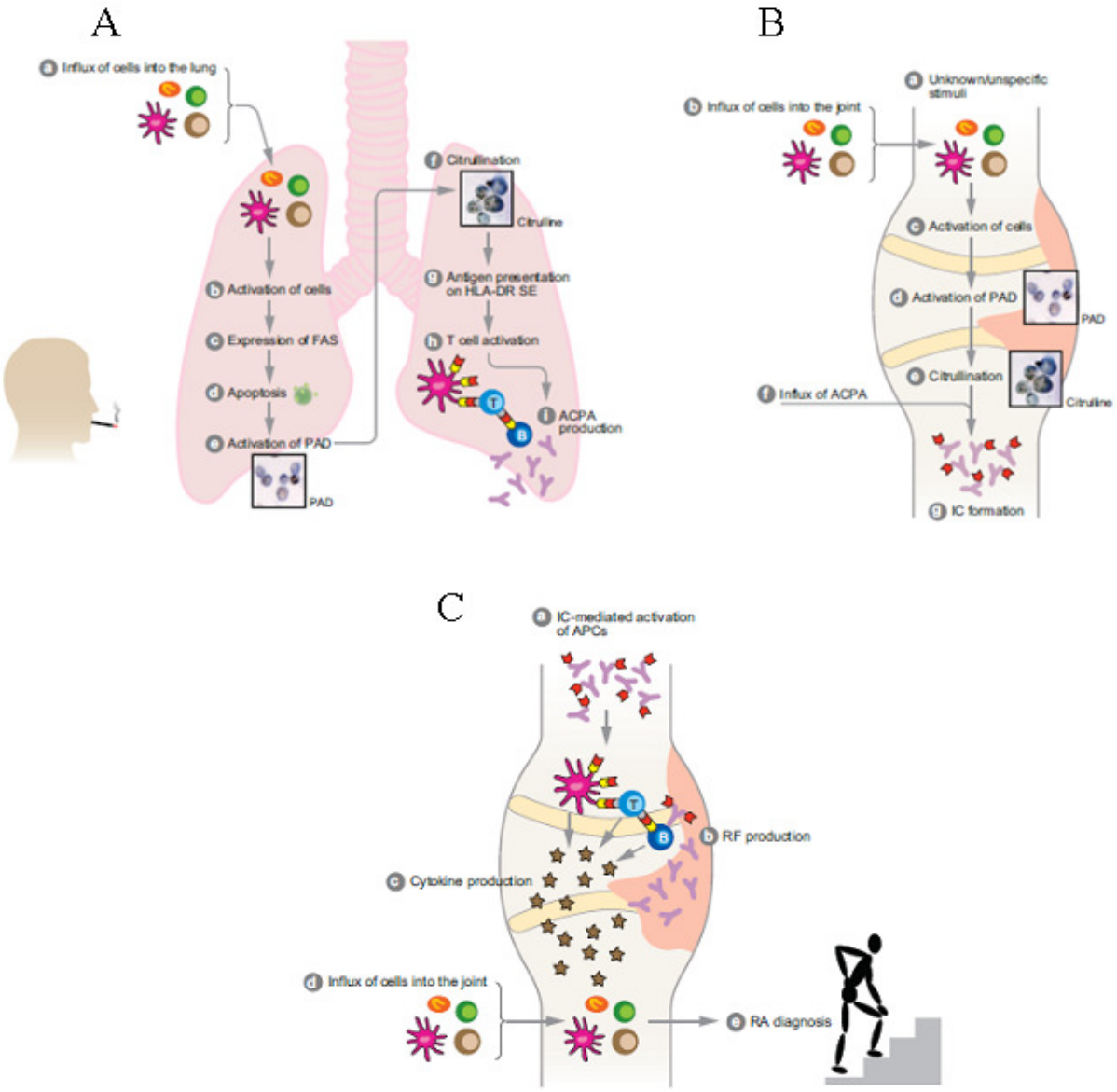

Figure 1.12. Etiological model for the development of ACPA-positive RA. **A** - Immune response; **B** - Pathologic inflammatory response; **C** - Chronic RA; (adapted from Klareskog et al., 2008).

Chapter 2

Objectives

Rheumatoid Arthritis, the most common systemic autoimmune disease affecting about 1% of the adult population worldwide, is characterized by immune cell-mediated destruction of the joint architecture. The development of autoimmune multifactorial diseases like RA depends on the interaction between genetic background and a number of environmental factors. The onset of clinical disease occurs at a threshold reached by the cumulative action of genetic and environmental factors beyond which the abortion of an auto-aggressive immune response becomes impossible. Although the etiology of RA remains unsolved, a genetic component of RA susceptibility has been established by data from familial aggregation and twin studies. Estimates of heritability suggest that genetic factors are responsible for at least 50% of the risk of developing RA. Several genome scans have suggested multiple RA loci and association studies have suggested new RA genes. Until 2006, only the *HLA-DRB1* locus and *PTPN22*-1858T allele have consistently demonstrated both RA linkage and association. Concerning transcriptome analysis, there are only few RA large-scale gene expression profiling studies which have been done in PBMCs of RA patients. Expression profiling studies in RA can be classified roughly into two categories: (1) those focused on finding (new) candidate genes for disease aetiology and understanding its pathogenesis, and (2) those focused on identifying expression patterns typical for a state of RA (eg, mild versus severe disease or drug-responsive versus non-responsive patients). In the first category, expression analysis is often the first step in elucidating gene function, and in the second it is aimed at reducing phenotypic heterogeneity or at identifying expression profiles that can serve as diagnostic tools predicting, eg, disease outcome or response to disease-modifying anti-rheumatic drugs.

In contrast with the detailed study of the role of genes in the aetiology of RA, lower attention has been directed towards the elucidation of environmental factors. Studies carried out looking for environmental factors important in RA have identified numerous candidates but only tobacco smoking was now well established.

The overall aim of this thesis was the identification and characterization of candidate genes in RA.

GenHotel laboratory coordinates the ECRAF which has available a huge numbers of RA trio families (one patient and both healthy parents) from France and West Europe. This biological source allows performing family-based association and linkage studies. This approach permits the circumvention of the stratification bias originated by case-control studies. Thus, trio families design provides accurate estimations of matched control subjects for each patient, an approach that is robust against population stratification. In chapters 3 and 4, several family-

based association and linkage studies are performed in order to identify and/or confirm new RA susceptibility genes in French and European population. Furthermore, expression studies on some candidate genes are carried out to complete genetic analysis.

Large-scale gene expression profiling allows the elucidation of RA genetics because it permits the understanding improvement of the pathogenic mechanisms underlying RA, the identification of new drugs targets, the prediction of future outcomes, such as responsiveness therapy, overall disease severity and organ specific risk, and the development new diagnostic tests. In chapter 5 we performed a large-scale gene expression profiling of PBMCs from RA patients and healthy controls to gain insights into RA molecular mechanisms.

In addition, to elucidate and confirm the association of tobacco smoking in RA etiology we investigated the interaction between genes (*HLA-DRB1* and *PTPN22*) and tobacco smoking, separately as well as combined, and serological markers (RF and ACPA) in a French population with RA (Chapter 6).

Finally, we performed several studies in collaboration with other laboratories. We aimed to investigate new susceptibility genes in RA and other auto-immune diseases like Pemphigus, in different ethnic populations, using family-based and/or case-control association studies. All these studies were presented in Chapter 8 (Annexes).

Chapter 3

Genetic analysis of candidate gene regions in Rheumatoid Arthritis

3.1. Testing for linkage and association with Rheumatoid Arthritis a *PTPN22* promoter polymorphism reported to be associated and linked with Type 1 Diabetes in the Caucasian population

Based on: Dieudé P, Teixeira VH, Pierlot C, Cornelis F, Petit-Teixeira E, ECRAF. (2008) Ann Rheum Dis. 67(6):900-901.

3.1.1. Abstract

A new protein tyrosine phosphatase non-receptor 22 (*PTPN22*) single nucleotide polymorphism (SNP), -1123G*/C*, was reported to be independently linked to and associated with diabetes type 1 (T1D). The aim of this study was to test the hypothesis of an over-transmission of *PTPN22* –1123G allele compared to that previously observed for the *PTPN22* +1858T allele.

200 French Caucasian Rheumatoid Arthritis (RA) trio families previously genotyped for the *1858T/C* SNP were genotyped for the –1123G/C SNP and analysed using the Transmission Disequilibrium Test (TDT), the Affected Family-Based Controls (AFBAC) and the Genotype Relative Risk (GRR).

As previously reported significant linkage to Rheumatoid Factor (RF) positive RA was observed with an excess of transmission of the +1858T allele. No RA linkage was observed as there was no over-transmission of the –1123G allele from heterozygous parents. The GRR analysis showed an association between RA and the homozygous genotype -1123G*/G*. Both +1858T and –1123G alleles were in close Linkage Desiquilibrium (LD).

Our findings do not support the hypothesis of an involvement of the *PTPN22* –1123G allele independent of the +1858T allele in RA genetic susceptibility in the French Caucasian population

3.1.2. Introduction

The *PTPN22* SNP +1858 C/T (rs2476601) was found to be associated with various autoimmune diseases including RA (Bottini et al., 2004; Begovich et al., 2004; Kyogoku et al., 2004; Velaga et al., 2004; Hinks et al., 2005). The *PTPN22* gene, encodes the LYP which is involved in the suppression of T cell activation. The functional polymorphism rs2476601 resulting in the R620W amino-acid substitution, affects a proline-rich motif of LYP involved in protein-protein interactions. The significance of the R620W substitution remains to be clarified, as reciprocally gain and loss of function of LYP have both been suggested (Vang et al., 2005; Bottini et al., 2006). In addition, an ethnic diversity of the +1858T/*C* allelic variation is observed: the frequency of the susceptibility +1858T allele range from 2% of South Europeans to 15% of North Europeans, and is not observed in Asian population (Gregersen et al., 2006), suggesting the involvement of other disease–associated *PTPN22* functional polymorphisms either independently or in combination with +1858T. The hypothesis of an involvement of other susceptibility functional polymorphisms of *PTPN22* may be of major relevance to explain the molecular mechanisms of the disease susceptibility. If *PTPN22* was reported to be associated with multiple autoimmune disorders, surprisingly most of the association studies have focused their investigation to the +1858T susceptibility allele. Carlton and colleagues (2005) identified two SNPs (rs3811021 and rs3789604) associated with RA independently of rs2476601. However, both polymorphisms rs3811021 and rs3789604 are in quite absolute LD and a two recent association studies performed in the Korean and the Caucasian population failed to replicate the association of rs3789604 with RA (Gregersen et al., 2006; Hinks et al., 2007). More recently, a resequencing study of *PTPN22* performed in the T1D Japanese population lead to the identification of a new frequent variant located in the promoter (-1123 G/C, rs2488457) for which the –1123G allele was found to be associated with T1D (Kawasaki et al., 2006). The same allele was also tested in the T1D UK Caucasian population, using multiplex families and found in LD with the +1858T allele. The TDT showed an excess of transmission of the –1123G allele (P=0.024) compared to that observed for the +1858T allele (P=0.062), suggesting that the –1123G allele was a more likely causative variant (Kawasaki et al., 2006). Recently, a haplotype-based analysis of the *PTPN22* locus was performed in Caucasian T1D nuclear families suggesting that the –1123G allele could not be an independent risk variant (Onengut-Gumuscu et al., 2006). Thus, in this study, tacking advantage of 200 RA French Caucasian trio families previously genotyped for

+1858T/C SNP (Dieudé et al., 2005), we tested the hypothesis of an over-transmission of -1123G allele compared to that previously observed for the +1858T allele.

3.1.3. Patients and Methods

Patients

200 RA nuclear families were recruited through a national media campaign in France, followed by the selection of individuals who fulfilled the ACR 1987 revised criteria for RA (Arnett et al., 1998), according to the rheumatologist in charge of the patient. All clinical data were reviewed by former university fellow rheumatologists of our team. Families with an additional affected sibling and RA patients younger than 18 years of age were excluded. All individuals provided informed consent and the ethics committee of the Bicêtre Hospital, France, approved the study. Characteristics of the RA sample were reported in Table 3.1.1.

Table 3.1.1. Characteristics of RA index cases from the investigated sample.

Characteristic	**RA patients (*n* = 200)**
Females (%)	88.5
Mean age (± standard deviation, SD) at disease onset (years)	31.5 (± 8)
Mean (± SD) disease duration (years)	17 (± 7.5)
RA patients with bone erosions (%)	84.5
RA patients seropositive for rheumatoid factor (%)	78.5
RA patients carrying at least one *HLA-DRB1*shared epitope allele (%)*	79

* - *DRB1**0101, *DRB1**0102, *DRB1**0401, *DRB1**0404, *DRB1**0405, *DRB1**0408, *DRB1**1001

Molecular Genotyping Method.

Genomic DNA was purified from fresh peripheral blood leukocytes by standard methods. Genotyping of the *PTPN22* rs2488457 polymorphism was carried out with a Taqman 5' allelic discrimination assay on an ABI 7500 real time PCR machine (assay: C__16027865_10). In order to test the quality of genotyping, we performed a control quality test by randomly genotyping again 10% of the samples.

Statistical Analysis

Using the previously reported allelic frequencies of 32,3 % in T1D Caucasian patients and 24,6% in controls, (Kawasaki et al., 2006), power to detect an association was calculated using the method described by Garnier and colleagues (2007).

The Hardy-Weinberg equilibrium was checked in the control group (constituted by the non transmitted parental chromosomes) prior to analysis.

The linkage analysis relied on the TDT, which compares, for a given allele, its transmission from heterozygous parents to RA patients, with the transmission expected from Mendel's law (*i.e.* 50 %) (Spielman et al., 1993). The association analysis relied first on the AFBAC (Thompson, 1995), which compares the allelic frequencies between chromosomes transmitted to the RA cases and non transmitted parental chromosomes (Thomson, 1995) and secondly on the Genotype Relative Risk (GRR) and the Haplotype Relative Risk (HRR), which compares the affected offspring's genotype or haplotype with the control genotype or haplotype derived from non transmitted parental chromosomes, respectively (Lathrop, 1983; Kruglyak et al., 1996). The OR and 95% C.I. were estimated by using the method of Woolf (1955), as modified by Haldane (1956). Statistical significance was considered for $P<0.05$.

3.1.4. Results

Power calculation

Using the previously reported allelic frequencies of 32.3 % in T1D Caucasian patients and 24.6% in controls, (Kawasaki et al., 2006) we had a 75% power to detect a significant association ($P<0.05$).

Hardy-Weinberg equilibrium check

Both *PTPN22* SNPs rs2476601 and rs2488457 were in hardy-Weinberg equilibrium in the control sample investigated.

Test for linkage and association in RA trio families

PTPN22 rs2476601

As we have previously reported, the *PTPN22* +1858T allele was significantly increased in RA compared to controls (18.5% *vs* 13%, *P*=0.05), this difference was more important in the RF positive subgroup (19.1% *vs* 12.4%, *P*=0.03). Significant linkage to RF+ RA was observed with an excess of transmission of the +1858T allele (55 transmitted *vs* 35 untransmitted, *P*=0.035) (Table 3.1.2). The GRR analysis was concordant as we observed a statistically significant increase of the frequency of genotypes homozygous for +1858T allele in RA cases compared to the controls (*P*=0.042). Furthermore, GRR analysis showed a statistically significant increase of the frequency of genotypes homozygous for +1858T allele and of genotypes carrying at least one +1858T allele in RF+ RA cases compared to the controls (*P*=0.046 and *P*=0.018, respectively) (Table 3.1.3).

PTPN22 rs2488457

We observed an increase of frequency of the suspected –1123G allele in RA index cases compared to controls (30.5% *vs* 27.3%, *P*=0.31). However, the difference did not reach statistical significance even in the RF+ RA subgroup (32.2% *vs* 27.1%, *P*=0.16). No RA linkage was observed: there was no over-transmission of the –1123G allele from heterozygous parents (84 transmitted *vs* 71 untransmitted, *P*=0.29) (Table 3.1.2). The GRR analysis showed increase of the frequency of genotypes homozygous for the G allele in RA cases compared to the controls (12% *vs* 8.5%, *P*=0.047) and in RF+ RA cases compared to the controls (13.4% *vs* 8.9%, *P*=0.023) (Table 3.1.3).

Linkage analysis of *PTPN22* rs2476601 - rs2488457 haplotypes

No RA linkage was observed as none of the 4 haplotypes were over-transmitted, nevertheless, we observed in the global analysis a trend for an excess of transmission of the haplotype 4 (h4) carrying both –1123G and +1858T allele (52 transmitted *vs* 36 untransmitted, *P*=0.09). *PTPN22* linkage to RF+ RA was significant for h4 (45 transmitted *vs* 27 untransmitted, *P*=0.03).

Table 3.1.2. AFBAC and TDT analysis between both *PTPN22* rs2476601 and rs2488457

ele		AFBAC				TDT	
		AFBAC frequency	Transmitted frequency	*P*-value	Odds Ratio (95%CI)	Transmitted	Untransmitted
	All RA index cases (n=200)	0.13	0.185	0.05	1.45 (0.99-2.13)	66	46
	RF+ RA index Cases (n=157)	0.191	0.124	0.03	1.62 (1.05-2.5)	55	35
	All RA index cases (n=198)	27.3	30.5	0.31		84	71
	RF+ RA index Cases (n=155)	27.1	32.2	0.16		70	54

SNPs and RA.

Table 3.1.3. *PTPN22* rs2476601 and rs2488457 genotypes distribution.

***PTPN22* SNP**	**Genotypes**	**All RA cases (n= 200) n (%)**	**Controls (n=200) n (%)**	***P*-value**	**RF+ RA cases (n=157) n (%)**	**Controls (n=157) n (%)**	***P*-value**
+1858C/*T* (rs2476601)	**CC**	134 (67)	150 (75)	0.076*	104 (66.2)	120	0.046*
	CT	58 (29)	46 (23)	0.042‡	46 (29.3)	34	0.018‡
	TT	8 (4)	4 (2)		7 (4.5)	3	
-1123C/*G* (rs2488457)	**CC**	102 (51)	108 (54)	0.68*	77 (49)	86 (54.8)	0.44*
	CG	74 (37)	75 (37.5)	0.047‡	59 (37.6)	57 (36.3)	0.023‡
	GG	24 (12)	17 (8.5)		21 (13.4)	14 (8.9)	

*GRR: heterozygous genotype carrying the suspected allele compared to the remaining homozygous genotypes

‡GRR: homozygous genotype for the suspected allele compared to the remaining genotype

3.1.5. Discussion

In this study, we aimed at testing the *PTPN22* -1123G allele for linkage and association with RA, as an independent genetic susceptibility factor. The -1123G allele frequency was concordant with that previously reported in the Caucasian population (Kawasaki et al., 2006; Onengut-Gumuscu et al., 2006). We observed an increase of frequency of the suspected allele in RA. No linkage evidence for the -1123G allele was observed. However, the GRR showed an association between the genotype homozygous for -1123G allele and RA, in the global set and in the RF+ RA subgroup. Those results are consistent with the significant association observed between the *PTPN22* haplotype carrying both -1123G and +1858T alleles and RA. In order to detect a putative contribution of the -1123G allele independent of the +1858T allele and to avoid any distortion in the transmission of the -1123G allele as both -1223G and +1858T alleles were in strong LD (*D'*=0.975) we investigated RA trio families for which both parents and the affected offspring were homozygous for the +1858C allele. Unexpectedly, we observed a decrease of transmission of the -1123G allele (18 transmitted *vs* 24 untransmitted, *P*=0.86). The GRR analysis was consistent with the TDT results as no increased frequency of genotypes carrying or homozygous for the -1123G allele was found (data not shown). Our results were obtained with a particularly robust methodology which avoid for imperfect population match between patients and controls. Hence, those results allowed us to exclude the *PTPN22*-1123G allele as RA genetic susceptibility factor in this population. Recently, two *PTPN22* haplotype analysis performed in both RA UK and Dutch Caucasian population testing multiples *PTPN22* SNPs (but not the rs2488457) failed to detect an association independent of the +1858T allele (Gregersen et al., 2006; Wesoly et al., 2007). Meanwhile, a *PTPN22* disease associated variant, independent of the well characterised +1858T allele could not be excluded. In this way, linkage study performed in the T1D population identified a new *PTPN22* mutation, which was preferentially transmitted to affected offspring (Onengut-Gumuscu et al., 2006). This mutation located in exon 18 leads to missplicing and deletion of exon 18 and could be involved in other autoimmune disease such as RA. However, to test this hypothesis, tacking into account the minor allele frequency of 0.006, a huge sample size would therefore be required.

In conclusion, our findings do not support the hypothesis of an involvement of the *PTPN22*-1123G allele in RA genetic susceptibility in the French Caucasian population. Nevertheless, it remains to be clarified whether other *PTPN22* variants, independent of the +1858T susceptibility allele, are associated with RA.

3.2. Association study of the *RANK* locus in white European Rheumatoid Arthritis families

Based on: Teixeira VH, Dieudé P, Michou L, Migliorini P, Balsa A, Westhovens R, Barrera P, Alves H, Vaz C, Fernandes M, Pascual-Salcedo D, Bombardieri S, Dequeker J, Radstake TR, Van Riel P, van de Putte L, Lopes-Vaz A, Bardin T, Cornelis F, Petit-Teixeira E. (2009) Ann Rheum Dis. 68(3):448-449.

3.2.1. Abstract

The last genome-wide meta-analysis on Rheumatoid Arthritis (RA) provided evidence of linkage with RA on chromosome 1, 2, 5 and 18q. The receptor activator of nuclear factor-kappa B (*RANK*) gene is located in the chromosome 18q and has been shown to be critically involved in the osteoclast differentiation and in bone resorption. The aim of our study was to use RA familial material to test three *RANK* tagSNPs for association with RA in European Caucasian families.

RANK rs8086340, rs1805034 and rs9646629 tagSNPs were genotyped in 99 French Caucasian trio (set 1), in 91 French Caucasian trio (set 2) and in 258 European Caucasian trio (set 3) using Taqman® allelic discrimination assay. The genetic analyses for association were performed using the Family-Based Association Test (FBAT).

We observed a significant RA association for the *RANK* haplotype G-C of the rs8086340 (C/G)-rs1805034 (C/T) SNPs in the set 1 (P=0.03) and the same trend in replication set 2 (P=0.1). The two sets combined showed a more significant RA association (P=0.008). In the set 3, the G-C haplotype was under-transmitted to RA (P=0.4).

Our findings did not support the hypothesis of an association of *RANK* with RA in European Caucasian population.

3.2.2. Introduction

The etiology of RA remains unknown, but appears to have a complex genetic component. Whole-genome scans identified several linkage loci for RA (Choi et al., 2006), but have shown inconsistent results, because they have been limited by small sample size, low statistical power and clinical or genetic heterogeneity (Altmuller et al., 2001). One way to circumvent and minimize these problems is to use a meta-analysis, in which data across independent genome scans are combined. In the last RA meta-analysis, Etzel and colleagues (2006) applied a novel meta-analytical method developed by Loesgen and colleagues (2001) to Caucasian populations and reported overwhelming evidence of linkage in the HLA region, strong evidence of 8p and 16p ($P<0.01$), and marginal evidence of 1q, 2q, 5q, and 18q ($P<0.05$). The 18q locus has also shown evidence for linkage with T1D, SLE and GD in humans, and the orthologous region involved in animal models of SLE, multiple sclerosis, and experimental allergic encephalomyelitis (Jawaheer et al., 2003). A possible candidate gene on chromosome 18q is the *RANK* gene which encodes receptor activator of NF-κB, which has been shown to be critically involved in the osteoclasts differentiation playing a pivotal role in bone resorption (Li et al., 2000). Several studies have demonstrated that osteoclasts are essential effector cells for focal erosion of bone and cartilage in inflammatory arthritis such as RA, for which an accelerated osteoclastogenesis takes place at disease sites outside of the marrow cavity, resulting in abnormal bone resorption, irreversible joint damage, and bone pain (Goldring, 2003).

A single *RANK* haplotype not specified was associated with RA at a significance level of 0.02 in a candidate gene study using about 400 NARAC cases and matched controls (Criswell and Gregersen, 2005). Furthermore, activating mutations in the *RANK* gene, that disrupts the signal peptide region of the protein resulting in the lack of normal cleavage of the signal peptide and an increase in RANK-mediated signaling, results in different bone disorders as early-onset Paget's, expansile skeletal hyperphosphatasia and familial expansile osteolysis (Vega et al., 2007).

The aim of our study was to use RA familial material to test three *RANK* tagSNPs for RA association in European Caucasian families.

3.2.3. Patients and Methods

Study design and study population

Patients with RA and family members were recruited through a national media campaign, followed by the selection of individuals fulfilling the ACR 1987 criteria for RA (Arnett et al., 1988), according to the rheumatologist in charge of the patient. All clinical data were reviewed by rheumatologists of our team. Families with an additional affected sibling and patients with RA aged<18 years were excluded. All individuals provided informed consent, and the ethics committee of the Bicêtre Hospital, France, approved the study. Set 1, 2 and 3 consisted of 99 French Caucasian trio families (one RA patient and both parents) with the four grandparents of French Caucasian origin, of 91 French Caucasian trio families, and of 258 European Caucasian trio families (France, Italy, Portugal, Spain, Belgium, and The Netherlands), respectively. Characteristics of the all RA trio families are reported in Table 3.2.1.

Table 3.2.1. Characteristics of RA index cases from the investigated samples

Characteristic	**Set 1 (*n* = 99)**	**Set 2 (*n* = 97)**	**Set 3 (*n* = 258)**
Females, *n* (%)	86 (86.9)	87 (89.7)	222 (86.0)
Mean age at disease onset, years ± standard deviation (SD)	32.1 ± 9.9	31.1 ± 8.7	30.3 ± 9.5
Mean disease duration, years± SD	14.1 ± 7.3	11.8 ± 8.7	8.2 ±7
RA patients with bone erosions, *n* (%)	89 (89.9)	78 (80.4)	181 (70.1)
RA patients seropositive for rheumatoid factor, *n* (%)	80 (80.8)	74 (76.3)	182 (70.5)
RA patients seropositive for anti-cyclic citrullinated peptides antibodies, *n* (%)	79 (77.9)	79 (77)	Not available
RA patients carrying at least one *HLA-DRB1* shared epitope allele, *n* (%)*	79 (77.9)	82 (79.7)	Not available

* - *DRB1**0101, *DRB1**0102, *DRB1**0401, *DRB1**0404, *DRB1**0405, *DRB1**0408, *DRB1**1001.

Molecular Genotyping Method

Genomic DNA of the 454 European Caucasian RA Trio families was isolated and purified from fresh peripheral blood leucocytes according to standard protocols. Genotyping of the *RANK* rs8086340, rs1805034, rs9646629 tagSNPs was carried out with a Taqman 5' allelic discrimination assay on an ABI 7500 real time PCR machine (assays: C__477989_10, C__8685532_20, C__1444406_10, respectively). CEPH (Centre d'Etude du Polymorphisme Humain) DNA samples (1347–02 and 884–15) were co-genotyped with all our samples for quality control. Moreover, ten percent of the samples chosen at random were genotyped twice.

Statistical Analysis

The Hardy-Weinberg equilibrium was checked in the control group (constituted by the non transmitted parental chromosomes from trio) prior the analysis.

We carried out single-locus tests of association with each individual SNP using two different methods. We conducted an allelic test of association using the Family-Based Association Test (FBAT) (Horvath et al., 2001). FBAT is based on the original TDT method testing for linkage and association on family data. We then carried out multi-locus tests of association testing haplotypes of adjacent SNPs using HBAT. HBAT is an elaboration of FBAT that allows for family-based association tests of haplotypes, even when the phasing of the haplotypes is ambiguous. We used a sliding window approach to test haplotypes of the three *RANK* tagSNPs. Significance values were obtained for tests of each specific haplotype. The significance of the p value was assessed at 5%, leading to replication tests in set 2 and 3.

RR associated to haplotype genotypes was performed using OR and 95% C.I. by the method of Woolf (1955), as modified by Haldane (1956).

3.2.4. Results

Hardy-Weinberg equilibrium check.

The *RANK* rs8086340, rs1805034, rs9646629 tagSNPs in the three sets investigated were in Hardy-Weinberg equilibrium.

Test for association in set 1.

A total of 297 French Caucasian individuals from 99 trio families were analysed. The results of the single locus tests of association between *RANK* rs8086340, rs1805034, rs9646629

SNPs and RA using FBAT did not show any significant association (data not shown). However, in the multi-locus tests of association, we observed a significant association (*P*=0.03) between the haplotype G-C of the rs8086340 (C/G)-rs1805034 (C/T) SNPs and RA (Table 3.2.2). RR associated to the haplotype genotypes containing G-C haplotype did not reveal a significant risk associated to these particular genotypes (G-C/G-C and G-C/X) (data not shown). Association test was performed in subgroups stratified for cyclic citrullinated peptide positivity, presence of erosion and presence of the *HLA-DRB1* SE and no significant association was detected (data not shown).

Test for association in set 2.

The significant association observed for the *RANK* rs8086340 (C/G)-rs1805034 (C/T) SNPs G-C haplotype in the set 1 led to a replication test in the set 2 with the hypothesis of a G-C haplotype association with RA. A total of 291 French Caucasian individuals from 97 trio families were analyzed. In this set, a trend for association of the G-C haplotype with RA was observed (*P*=0.1) (Table 3.2.2).

Test for association in the combined sets 1 + 2.

The combination of the two sets, authorized by the absence of any significant clinical difference between them (data not shown), showed a significant association (*P*=0.008) of the G-C haplotype with RA (Table 3.2.2).

Test for association in set 3.

The significant association of the G-C haplotype in the combined sets 1 + 2 led to a larger replication test (258 families, set 3) with the hypothesis of a G-C haplotype association with RA. We observed an under-transmission of the G-C haplotype to RA (*P*=0.4) (Table 3.2.2).

Test for association in the combined sets 1 + 2 + 3.

We observed a non significant over transmission of the G-C haplotype to RA (*P*=0.3) (Table 3.2.2) in the global sample of 449 RA families.

Table 3.2.2. Results from Haplotypes rs8086340 (C/G) - rs1805034 (C/T) tests using FBAT.

Haplotypes	Sets	RA Informative Trio Families (*n*)	S	E(S)	Var(S)	*P*-values
G-C	1	47	46.3	38.1	13.9	0.03
C-T	1	52	32.3	36.6	6.3	0.3
G-T	1	68	58.7	61.4	21.1	0.6
C-C	1	55	36.7	37.9	17.0	0.8
G-C	2	55	50.3	43.9	17.5	0.1
G-C	1 + 2	102	96.7	82.0	31.4	0.008
G-C	3	153	110.8	117.1	41.8	0.4
G-C	1 + 2 + 3	255	207.5	199.1	73.0	0.3

S - Statistic relied on the haplotype observed in RA patients; E(S) – Expected value of S; Var(S) – Variance, *n* = number.

3.2.5. Discussion

This study examined the association of three tag polymorphisms in *RANK* gene with susceptibility to RA. We studied the *RANK* gene, a good RA candidate gene for its function implicated in the osteoclasts differentiation and in bone erosions in RA, and its chromosomal location (18q locus, suggested to be linked to RA). This 80 kb gene region contains 30 LD blocks with 28 tagSNPs (Minor Allele Frequency up to 10%)

We observed a significant RA association for the *RANK* haplotype G-C of the rs8086340 (C/G)-rs1805034 (C/T) SNPs in the set 1 (*P*=0.03) and the same trend in replication set 2 (*P*=0.1). The two sets combined provided a higher significant RA association (*P*=0.008). Replication in set 3 revealed a contradictory result with a non significant (*P*=0.4) under-

transmission of this haplotype to RA. Consequently, G-C haplotype association in the totality of the 449 RA families was not detected. Heterogeneity between sets 1/2 and set 3 could be considered, as disease duration, erosion and RF percentage showed non significant deviation in values reported in Table 3.2.1. However, allelic and haplotype frequencies are not significantly different between the three sets (data not shown). Lack of power can not be excluded in our sample. However, using the haplotype frequencies observed in sets 1 + 2, association analysis in the 259 trio families in set 3 would provide a 100% power to reach statistical significance (P<0.05). Nevertheless, the two last GWA studies in Caucasian populations found new RA associated regions and failed to replicate the 18q locus (WTCCC, 2007; Plenge et al., 2007a).

RANKL is also a factor involved in the regulation of osteoclasts. Binding of RANKL to its receptor RANK provides the crucial signal to drive osteoclast development from haematopoietic progenitor cells as well as to activate mature osteoclasts (Wada et al., 2006). Furthermore, blockade of osteoclastogenesis using monoclonal antibodies directed against RANKL (denosumab) has been proposed to prevent bone erosion in RA (Cohen et al., 2006). Therefore, three *RANKL* tagSNPs were tested for RA association in set 1 but no significant result was found (data not shown).

To our knowledge, this study is the first to report association study of *RANK* gene with RA in families using three tagSNPs distributed over the gene. Following the involvement of RANK in the pathophysiology of bone resorption, further studies are required to investigate the influence of this *RANK* haplotype on the functional and structural progression of RA. In addition, other *RANK* and *RANKL* tagSNPs and independent samples should be used to exclude this gene in genetics of RA in Caucasian population.

In conclusion, our findings failed to identify an association of the *RANK* gene with RA in European Caucasian population. However, further investigations should be done at the 18q locus as there are many candidate genes in this region as transcription factor 4 (*TCF4*), mucosa-associated lymphoid tissue lymphoma translocation protein 1 (*MALT1*), PMA-induced protein 1 (*PMAIP1*) and B-cell CLL/lymphoma 2 (*BCL2*).

3.3. Replication of the Tumor Necrosis Factor Receptor Associated Factor 1/Complement Component 5 Region as a Susceptibility Locus for Rheumatoid Arthritis in a European Family-Based Study

Based on: Kurreeman FA, Rocha D, Houwing-Duistermaat J, Vrijmoet S, Teixeira VH, Migliorini P, Balsa A, Westhovens R, Barrera P, Alves H, Vaz C, Fernandes M, Pascual-Salcedo D, Michou L, Bombardieri S, Radstake T, van Riel P, van de Putte L, Lopes-Vaz A, Prum B, Bardin T, Gut I, Cornelis F, Huizinga TW, Petit-Teixeira E, Toes RE, ECRAF. (2008) Arthritis Rheum. 58(9):2670-2674.

3.3.1. Abstract

Kurreeman and colleagues (2007) showed, using a candidate gene approach in a case-control association study, that a 65-kb block encompassing the tumor necrosis factor receptor-associated factor 1 (*TRAF1*)/Complement Component 5 (*C5*) genes is strongly associated with Rheumatoid Arthritis (RA). Compared with case-control association studies, family-based studies have the added advantage of controlling potential differences in population structure and are not likely to be hampered by variation in population allele frequencies, as is seen for many genetic polymorphisms, including the *TRAF1*/*C5* locus. The aim of this study was to confirm this association in populations of European origin by using a family-based approach.

A total of 1,356 western European white individuals from 452 RA trio families were genotyped for the rs10818488 polymorphism, using the TaqMan allelic discrimination assay.

The family-based analysis was performed using the basic TDT combined with parental phenotype information (parenTDT). Genotypic Odd Ratios (ORs) were calculated using a conditional logistic regression model stratifying on matched pairs.

We observed evidence for association, demonstrating departure from Mendel's law, with an overtransmission of the rs10818488-A allele (A=55%; *P*=0.036). By taking into consideration parental phenotypes, we also observed an increased A allele frequency in affected versus unaffected parents (A=64%; combined *P*=0.015). Individuals carrying the A allele had a 1.2-fold increased risk of developing RA (allelic OR 1.24, 95% CI 1.04–1.50).

Using a family-based study that is robust against population stratification, we provide evidence for the association of the *TRAF1*/*C5* rs10818488-A allele and RA in populations of European descent, further substantiating our previous findings. Future functional studies should yield insight into the biologic relevance of this locus to the pathways involved in RA.

3.3.2. Introduction

Environmental as well as genetic factors are thought to play an important role in both the onset and the progression of the disease (Firestein, 2003). Because the genetic contribution to RA has been estimated to be 50–60%, the identification of genes contributing to the disease is important for the understanding of underlying biologic mechanisms (Van der Helm-van Mil et al., 2005b). In addition to *HLA*, the first identified genetic risk factor (Stastny et al., 1976), and 3 other replicated regions including the *PTPN22* gene (Begovich et al., 2004), the 6q23 locus near *TNFAIP3* (Thomson et al., 2007; Plenge et al., 2007b), and the *STAT4* gene (Lee et al., 2007a; Remmers et al., 2007), Kurreeman and colleagues. (2007) have recently reported a new genetic locus associated with RA. This region encompasses the *TRAF1* and *C5* genes, both of which are immune regulators and potential perpetuators of inflammation. They identified 1 SNP, rs10818488, located within a 65-kb haplotype block, explaining most of the association signal in this region in several populations, including Dutch, Swedish, and US sample sets consisting of 2,719 patients with RA and 1,999 control subjects (OR 1.28, 95% CI 1.17-1.39, P=1.40x10^{-8}). Interestingly, the same genetic association was described in a recent whole-genome association study (Plenge et al., 2007a). Kurreeman and colleagues (2007) data revealed that although the case–control allele frequency increase in different sample sets ranged from 4% to 9%, the population frequency ranged from 38% to 46% in populations of European ancestry. Given that association studies compare the frequencies in patients versus healthy individuals, unknown biases in control frequencies may lead to spurious associations. Thus, family-based association studies remain important to definitively establish association, especially when small effect sizes as well as variability in allele frequency in different populations are observed. Therefore, with the aim to further substantiate the association at the *TRAF1*/*C5* locus, we took advantage of one of the largest reported European family resources dedicated to RA family–based studies.

3.3.3. Patients and Methods

Study population

DNA was available from 452 white trio families from western Europe, through the ECRAF. Each family consisted of 1 patient with RA and both of his or her parents. Ethnicity was determined by the origin of the grandparents. At the time of inclusion in the study, all patients with RA fulfilled the ACR 1987 revised criteria (Arnett et al., 1988). All individuals provided written informed consent, and the study was approved by the ethics committees in each

country. For each patient, the characteristics collected were sex, age at the onset of RA, disease duration (years), presence of bone erosions on radiographs, presence of rheumatoid nodules, and seropositivity for RF. RF status was not available for 9 of the patients with RA. Because the ACPA status was available for only a small proportion of the patients (n=197), we did not perform further analyses for ACPAs. The 452 families included 313 families from France, 53 from Italy, 37 from Spain, 22 from Belgium, 13 from The Netherlands, and 14 from Portugal. The characteristics of the French and European sample sets are summarized in Table 3.3.1.

Table 3.3.1. Characteristics of RA index cases from the investigated samples.

Characteristic	All (n =452)	French (n=313)	European (n=139)
Female sex, no. (%)	393 (86.9)	275 (87.8)	118 (84.9)
Age at onset of RA, mean-SD years	30.8-9.4	30.7-9.4	30.9-9.4
Disease duration, mean-SD years	10.3-7.9	12.0-8.1	6.6-5.9
Bone erosions, no. (%)	340 (75.2)	246 (78.6)	94 (67.6)
Rheumatoid factor positivity, no. (%)	321 (71.0)	221 (70.6)	100 (71.9)
Nodules, no. (%)	72 (15.9)	55 (17.6)	17 (12.2)

Genotyping

All DNA samples were genotyped using the TaqMan allelic discrimination assay according to the manufacturer's instructions (Applied Biosystems). At least 2 positive controls and 1 negative control were performed in each plate, and no inconsistencies were detected. Furthermore, the concordance rate between 10% random samples genotyped in duplicate was ~99%. The genotyping success rate in the 452 trio families was 100% (parents and probands included).

Statistical analysis

The family-based analysis was performed using the basic TDT combined with parenTDT, as implemented in Haploview 4.0 (Barrett et al., 2005). The parental discordance test is based on counting the number of alleles in affected versus unaffected parents, treating each nuclear family parental pair as a matched pair. These counts combined with the transmitted and untransmitted counts of the basic TDT give a combined test statistic (Purcell et al., 2005).

The current data set included 42 families with 1 affected parent. The control genotypes were derived from the untransmitted parental chromosomes, using Unphased version 3.0.10 (Dudbridge, 2003). By combining the case–control data from the probands with parental data, genotypic ORs were calculated using a conditional logistic regression model stratifying on matched pairs (each proband–pseudocontrol as a matched pair; each affected and unaffected parent as a matched pair). Robust standard errors were computed, taking into account the dependency between pairs from the same family. These analyses were performed using Stata version 9.0 software (www.stata.com).

3.3.4. Results

Hardy-Weinberg equilibrium check.

No deviation from Hardy-Weinberg equilibrium was detected in parental genotypes (n=904; *P*=0.548).

Test for association in RA trio families

We observed deviation from Mendel's first law, with a 55% overtransmission of the A allele to the patients in the 452 families (*P*=0.036) (Table 3.3.2) along with an increased prevalence of the A allele in affected parents (64% increase versus unaffected parents). By applying a parenTDT test that takes into consideration both the transmission from parents to patients and the occurrence of alleles in discordant parents (discordant for both disease status and genotype), we obtained a combined statistic (*P*=0.015). There were no differences between families of French origin and those from other continental western European countries (55% and 56% overtransmission, respectively) as well as no differences between paternal and maternal transmissions (56% and 57% overtransmission, respectively).

Table 3.3.2. Family-based association of the *TRAF1-C5* region with RA.

		rs10818488 A allele					
Sample Set	**Trio Families**	**Transmitted**	**Untransmitted**	**T[a] (%)**	***P*‡**	**Discordant parents #**	***P****
All	452	231	188	55	0.036	64	0.015
RF+	335	175	136	56	0.027	67	0.0099
RF-	108	47	49	49	0.92	40	0.84

a - Percent transmission of the rs10818488 A allele from heterozygous parents; ‡ - By standard TDT, as implemented in Haploview 4.0; **#** - Percent of the rs10818488 A allele from the affected parents in discordant parent pairs; *** -** Combined TDT with parental phenotype information statistic, as described by Purcell and colleagues (2005).

One of the advantages of family trio data is that such data provide perfectly matched control subjects for each patient investigated. Each patient chromosome transmitted by a given parent is perfectly matched for the population of origin with the untransmitted chromosome of each heterozygous parent. We observed an rs10818488-A allele frequency of 36% in control subjects, increasing to 41% in the patients with RA (data not shown). Using conditional logistic regression for the combined data set of case-control and parental discordant pairs, we observed an allelic OR of 1.24 (95% CI 1.04-1.50) (Table 3.3.3). Because RA is a heterogeneous disease, and distinct subsets of patients are characterized by the presence of autoantibodies such as RF and ACPAs, we also performed a stratified analysis for the presence or absence of RF (only limited data were available for ACPAs (see Patients and Methods). In concordance with our previous findings, we observed an overtransmission (A transmitted = 56%) as well as a higher prevalence of the A allele in affected parents (A = 67%; *P*=0.0099, by parenTDT) in the 335 RF-positive families (Table 3.3.2). Among RF-positive individuals, harboring 1 copy of the risk allele yielded an OR of 1.47 (95% CI 1.03-2.10), and homozygous individuals had an almost 2-fold increased risk of developing RA (OR 1.93, 95% CI 1.01-3.69) (Table 3.3.3). Interestingly, this effect was not detected in the RF-negative subgroup, as reflected by transmission of 49% and an A allele frequency in affected parents of 40% in the 108 RF-negative families (*P*=0.84) (Table 3.3.2), resulting in an allelic OR of 1.00 (95% CI 0.69-1.45) (Table 3.3.3).

Table 3.3.3. Case-control association of *TRAF1*/*C5* rs10818488 with RA.

Sample Set	AA‡	AG‡	Allelic#
All	1.48 (0.87–2.51)	1.37 (1.01–1.86)	1.24 (1.04–1.50)
RF+	1.93 (1.01–3.69)	1.47 (1.03–2.10)	1.31 (1.06–1.63)
RF-	0.56 (0.18–1.69)	1.16 (0.63–2.12)	1.00 (0.69–1.45)

‡ **-** Genotype-specific ORs (95% CIs), using GG as the referent; **# -** Allelic ORs (95% CIs), using the G allele as the referent.

3.3.5. Discussion

In the current family-based study, we observed evidence of association between RA and the *TRAF1*/*C5* rs10818488-A variant in a western European sample set, replicating our initial findings. One of the advantages of the family trio design is that it provides accurate estimations of matched control subjects for each patient, an approach that is robust against population stratification. In this set of perfectly matched cases and controls, we observed a 5% increase in the A allele frequency in the overall sample set and a 6% increase in the group of RF-positive patients of western European descent. Because these differences are well within the previously observed effect in the Dutch (9%), Swedish (4%), and American (7%) populations, these data together indicate that the contribution of the TRAF1/C5 locus to RA is not likely caused by underlying stratification in populations of European descent, and that the effect size is modest (OR ~1.3). Although we observed association in the overall sample set as well as in the RF-positive subset of patients with RA, we did not observe any overtransmissions in the RF-negative subset of patients with RA (49% versus the expected 50%), indicating that the effect of this genetic risk factor in the overall sample set is likely to be attributable to the RF-positive subset of patients with RA. Given the recent finding of this locus by a genomewide SNP association study in ACPA-positive patients with RA, the current evidence further substantiates the role of the *TRAF1*/*C5* region in the autoantibody-positive subset of patients with RA. However, whether this association extends to the autoantibody-negative subset of patients remains to be determined, because our RF-negative sample set possesses only 26% power to detect small effect sizes (OR ~1.3) at a significance

level less than 0.05. Therefore, our data do not allow a conclusion regarding the (lack of) contribution of the *TRAF1*/*C5* locus to autoantibody-negative RA. The currently identified polymorphism lies in an intergenic region between *TRAF1* and *C5*. Because TRAF1 is involved in TNF-mediated signaling and C5 generates C5a, the most potent chemoattractant involved in inflammation, both genes possess characteristics relevant to the pathogenesis of RA. LD patterns have so far revealed that the haplotype block surrounding this polymorphism encompasses the *TRAF1* gene as well as the 3' region of *C5*. Interestingly, Albers and colleagues (2008) have recently observed an association of this SNP with the polyarticular form of juvenile idiopathic arthritis. It is therefore plausible that the association of the *TRAF1*/*C5* variant may not be restricted to RA as such but may be relevant for other diseases. Furthermore, because current HapMap phase II data suggest that the frequency of the rs10818488-A allele is high in Chinese, Japanese, and Yoruban populations (44-69%), it would be interesting to investigate whether this genetic risk factor is also relevant across various ethnic populations. In conclusion, this study provides evidence of the association of the *TRAF1*/*C5* locus as one of the widely confirmed genetic risk factors for RA in white individuals of European descent. Endeavors to characterize the functional relevance of this polymorphism and/or others highly linked to it will yield insights into the biologic effects of this locus and will generate further crucial information on the pathways underlying disease.

3.4. Testing for the association of the *KIAA1109/Tenr/IL2/IL21* gene region with Rheumatoid Arthritis in a European family-based study

Based on: Teixeira VH, Pierlot C, Migliorini P, Balsa A, Westhovens R, Barrera P, Alves H, Vaz C, Fernandes M, Pascual-Salcedo D, Bombardieri S, Dequeker J, Radstake TR, Van Riel P, van de Putte L, Lopes-Vaz A, Bardin T, Prum B, Cornelis F, Petit-Teixeira E; ECRAF. (2009) Arthritis Res Ther. 11(2):R45.

3.4.1. Abstract

A candidate gene approach, in a large case–control association study in the Dutch population, has shown that a 480 kb block on chromosome 4q27 encompassing *KIAA1109*/*Tenr*/*IL2*/*IL21* genes is associated with Rheumatoid Arthritis (RA). Compared with case-control association studies, family-based studies have the added advantage of controlling potential differences in population structure. Therefore, our aim was to test this association in populations of European origin by using a family-based approach.

A total of 1,302 West European white individuals from 434 trio families were genotyped for the rs4505848, rs11732095, rs6822844, rs4492018 and rs1398553 polymorphisms using the TaqMan Allelic discrimination assay. The genetic association analyses for each single nucleotid polymorphism (SNP) and haplotype were performed using the the Transmission Disequilibrium Test (TDT)) and the Genotype Relative Risk (GRR).

We observed evidence for association of the heterozygous rs4505848-AG genotype with RA (P=0.04); however, no significance was found after Bonferroni correction. In concordance with previous findings in the Dutch population, we observed a trend of undertransmission for the rs6822844-T allele and rs6822844-GT genotype to RA patients. We further investigated the five SNP haplotypes of the *KIAA1109*/*Tenr*/*IL2*/*IL21* gene region. We observed, as described in the Dutch population, a nonsignificant undertransmission of the AATGG haplotype to RA patients.

Using a family-based study, we have provided a trend for the association of the *KIAA1109*/*Tenr*/*IL2*/*IL21* gene region with RA in populations of European descent. Nevertheless, we failed to replicate a significant association of this region in our RA family sample. Further investigation of this region, including detection and testing of all variants, is required to confirm RA association.

3.4.2. Introduction

RA is a common autoimmune inflammatory disease of unknown cause, in which both genetic and environmental risk factors have been implicated (Firestein, 2003). Since the genetic contribution to RA has been estimated to be between 50% and 60%, identification of genes contributing to disease is important for the understanding of underlying biological mechanisms (Van der Helm-van Mil et al., 2005b). In addition to *HLA* (Stastny et al., 1976) – the first identified genetic risk factor – and four other replicated regions – including the *PTPN22* gene (Begovich et al., 2004), the *TRAF1*/*C5* locus (Kurreeman et al., 2007; Plenge et al., 2007a; Kurreeman et al., 2008), the 6q23 locus near the *TNFAIP3* gene (Thomson et al., 2007; Plenge et al., 2007b), and the *STAT4* gene (Lee et al., 2007a; Remmers et al., 2007) – a new genetic region associated with RA was described in the Dutch population (Zhernakova et al., 2007). This region encompassing *KIAA1109*/*Tenr*/*IL2*/*IL21* is contained in a large block (480 kb) of LD located on chromosome 4q27 and includes the *IL2* and *IL21* genes, which are both plausible functional candidate loci for RA. Five SNPs (rs4505848, rs11732095, rs6822844, rs4492018 and rs1398553) of the block were investigated in a sample set consisting of 1,047 RA patients and 929 controls, showing a significant association of the rs6822844-T allele and the rs6822844-GT genotype (P=0.0002, OR = 0.72, 95% CI = 0.61 to 0.86; and P=0.0009, OR = 0.71, 95% CI = 0.58 to 0.87, respectively) and of a specific haplotype (AATGG) (P=0.00025, OR = 0.70, 95% CI = 0.57 to 0.85) with RA (Zhernakova et al., 2007). Furthermore, in the NARAC and EIRA populations, rs6822844 and rs1398553 SNPs were significantly associated with RA (P=0.01, OR = 0.84, 95% CI = 0.74 to 0.96; and P=0.003, OR = 1.17, 95% CI = 1.05 to 1.30, respectively) (Plenge et al., 2007a). In addition, the last meta-analysis performed with the NARAC, EIRA and WTCCC studies, with 3,393 RA patients and 12,462 controls, observed evidence of association of 4q27 with RA in an independent replication, suggesting that 4q27 is a true-positive association (Raychaudhuri et al., 2008). Given that association studies compare the frequencies in patients versus healthy individuals, unknown biases in control frequencies may lead to spurious associations. Family-based association studies therefore remain important to definitively establish association, especially when small effect sizes as well as variability in allele frequency in different populations are observed. With the aim to further substantiate the association at the *KIAA1109*/*Tenr*/*IL2*/*IL21* gene region, we therefore took advantage of one of the largest reported European family resources dedicated to RA family-based studies.

3.4.3. Patients and methods

Study design and study population

DNA was available from 434 white trio families from Western Europe through the ECRAF collection followed by the selection of individuals fulfilling the ACR 1987 criteria for RA (Arnett et al., 1988), according to the rheumatologist in charge of the patient. Each family consisted of one RA patient and both parents. Characteristics of the 434 RA European Caucasian families are reported in Table 3.4.1. The 434 families included 308 families from France, 51 families from Italy, 32 families from Spain, 20 families from Belgium, nine families from the Netherlands and 14 families from Portugal. Families with RA patients aged <18 years were excluded. All individuals provided written informed consent and the study was approved by the Ethics Committee of l Kremlin-Bicêtre Hospital (Paris, France).

Table 3.4.1. Characteristics of RA index cases.

Characteristic	**RA families (*n* = 434)**
Females (*n* (%))	375 (86%)
Mean age at disease onset (years) (± standard deviation)	31.1 (9.7)
Mean disease duration (years) (± standard deviation)	9.8(8.5)
With bone erosions (*n* (%))	336 (77%)
Seropositive for rheumatoid factor (*n* (%))	323 (76%) (out of 425 RA patients)
Seropositive for anti-cycle citrullinated peptides antibodies (*n* (%))	214 (76%) (out of 282 RA patients)
Carrying at least one *HLA-DRB1* shared epitope allele (*n* (%))[a]	152 (77%) (out of 197 RA patients)
Carrying at least one PTPN22-1858T allele (*n* (%))	115 (27%)
Carrying the *TRAF1/C5* rs10818488-A variant (*n* (%))	277 (64%)

a - *DRB1**0101, *DRB1**0102, *DRB1**0401, *DRB1**0404, *DRB1**0405, *DRB1**0408, *DRB1**1001.

Genotyping

All samples were genotyped using the TaqMan allelic discrimination assay according to the manufacturer's instructions (Applied Biosystems). Centre d'Etude du Polymorphisme Humain DNA samples were co-genotyped with all our samples, with no inconsistencies detected. The genotyping success rate in the 434 trio families was 100% (parents and probands included).

Statistical analysis

Using the previously reported frequencies for the rs6822844-T allele in Dutch RA patients (14.1%) and in controls (18.5%) (Zhernakova et al., 2007), the power to detect an association was calculated using the method described by Garnier and colleagues (2007).

The HWE was checked in the control group (constituting the nontransmitted parental chromosomes from the trio) prior to analysis.

The association analysis relied on the TDT (Spielman et el., 1993) and on the GRR (Lathrop, 1983). ORs were calculated and 95% CIs were approximated using Woolf's method (1955), with Haldane's correction (1956). Haplotypes were generated using GeneHunter software (Kruglyak et al., 1996). We then carried out multi-locus tests of association testing haplotypes of SNPs using the TDT and HRR. *P* values were adjusted with Bonferroni correction (Bonferroni, 1936) for each independent test performed.

3.4.4. Results

Power calculation

Using the previously reported frequencies for the rs6822844-T allele in Dutch RA patients (14.1%) and in controls (18.5%) (Zhernakova et al., 2007), association analysis of 434 trio families provides an 80% power to reach statistical significance ($P<0.05$).

Hardy–Weinberg equilibrium check

The five SNPs in the sample investigated were in HWE in the control group.

Test for association in RA trio families

A total of 1,302 European individuals from 434 trio families (one RA case and both parents) were analyzed. Three hundred and eight families were of French origin and 126 were from other continental Western European countries. With the rs6822844 (G/T) SNP we observed

deviation from Mendel's first law, with a nonsignificant undertransmission of 45.3% of the T allele to the patients in the 434 families (*P*=0.24) (Table 3.4.2). No significant association with RA was detected for the four other SNPs (Table 3.4.2). Since RA is a heterogeneous disease and distinct subsets of patients are characterized by the presence of autoantibodies such a RF and anti-citrullinated protein antibodies, we also performed a stratified analysis for the presence or absence of RF or anti-citrullinated protein antibodies; no associations were detected (data not shown). Furthermore, the same tests were performed in subgroups stratified for the presence of the *HLA-DRB1* SE, *PTPN22*-1858T and *TRAF1/C5* rs10818488-A variant, and no associations were detected (data not shown)

Table 3.4.2. Family-based association of the chromosome 4q27 gene region with RA: TDT.

4q27 region SNPs	Allele	Transmitted allele	Untransmitted allele	Transmission[a] (%)	*P*-value
rs450584	A	185	189	49.5	0.84
rs1173209	A	96	78	55.2	0.17
rs6822844	T	97	114	45.3	0.24
rs4492018	G	183	174	51.3	0.63
rs1398553	G	182	182	50	1

a - Percentage of transmission from heterozygous parents.

One of the advantages of family trio data is that they provide perfectly matched controls for each patient investigated. Each patient chromosome, transmitted by a given parent, is perfectly matched for the population of origin with the untransmitted chromosome of each heterozygous parent. The GRR analysis of the rs4505848 (A/G) SNP showed an association of the heterozygous AG genotype with RA (*P*=0.04, OR = 1.32, 95% CI = 1.01 to 1.72) (Table 3.4.3). The significance threshold was not reached, however, after Bonferroni correction. In the four other SNPs, no association with RA was detected (Table 3.4.3). In concordance with previous findings in the Dutch population, we observed an undertransmission of the rs6822844-T allele and the rs6822844-GT genotype, but without significance (Tables 3.4.2 and 3.4.3).

Table 3.4.3. Family-based association of the chromosome 4q27 gene region with RA: GRR.

SNP	Genotype	RA cases (*n*)	Controls (*n*)	OR (95% CI)	*P*-value[a]
rs4505848	GG	38	51	0.72 (0.46 to 1.12)	0.18 (GG vs. AA + AG)
	AG	213	183	1.32 (1.01 to 1.72)	0.04 (AG vs. GG + AA)[a]
	AA	182	199	0.85 (0.65 to 1.11)	0.28 (AA vs. AG + GG)
rs11732095	GG	7	9	0.77 (0.28 to 2.09)	0.8 (GG vs. AA + AG)
	AG	87	101	0.83 (0.6 to 1.15)	0.28 (AG vs. GG + AA)
	AA	340	324	1.23 (0.9 to 1.68)	0.23 (AA vs. AG + GG)
rs6822844	TT	8	11	0.72 (0.29 to 1.81)	0.64 (TT vs. GG + TG)
	TG	99	110	0.87 (0.64 to 1.19)	0.43 (TG vs. TT + GG)
	GG	327	313	1.18 (0.87 to 1.6)	0.32 (GG vs. TT + TG)
rs4492018	GG	235	232	1.03 (0.79 to 1.35)	0.89 (GG vs. AA + AG)
	AG	174	171	1.03 (0.78 to 1.35)	0.89 (AG vs. GG + AA)
	AA	25	31	0.79 (0.46 to 1.36)	0.49 (AA vs. AG + GG)
rs1398553	GG	204	214	0.91 (0.7 to 1.19)	0.54 (GG vs. AA + AG)
	AG	195	175	1.2 (0.92 to 1.57)	0.20 (AG vs. GG + AA)
	AA	35	45	0.77 (0.49 to 1.21)	0.30 (AA vs. AG + GG)

a - *P* value was not significant after Bonferroni correction.

We further investigated the five SNP (rs4505848, rs11732095, rs6822844, rs4492018 and rs1398553) haplotypes of the *KIAA1109*/*Tenr*/*IL2*/*IL21* gene region. These seven haplotypes of the combined five SNPs of the block have a pooled frequency of 96%. We observed a nonsignificant undertransmission of the AATGG haplotype (*P*=0.19) (Table 3.4.4), which was reported as associated with RA in the Dutch study (Zhernakova et al., 2007). No RR was detected for any haplotype genotype (data not shown).

Table 3.4.4. Family-based association for the seven haplotypes[a]: TDT.

Haplotype[a]	Transmitted	Untransmitted	Transmission[b] (%)	*P*-value
GAGGA	113	112	50	0.95
AAGAG	133	126	52	0.69
AATGG	76	93	45	0.19
AAGGG	110	102	52	0.58
AGGGG	67	67	50	1
GAGGG	81	64	56	0.16
AAGGA	53	43	55	0.31

a - rs4505848 (G/A) – rs11732095 (G/A) – rs6822844 (T/G) – rs4492018 (G/A) – rs1398553 (G/A);
b - Percentage of transmission from heterozygous parents.

There were no differences between families of French origin and other continental Western Europe families, as well as no differences between paternal transmission and maternal transmission for all statistical analyses performed (data not shown).

3.4.5. Discussion

A recent case–control study in the Dutch population has shown association of RA, coeliac disease and T1D with the 4q27 gene region. In that study the rs6822844-T allele was reported to be a perfect proxy for a haplotype that is highly associated with autoimmune diseases with frequencies of 0.13 in cases versus 0.19 in North European controls (Zhernakova et al., 2007). In addition, a study from the WTCCC identified this 4q27 region in a search for risk factors for T1D (WTCCC, 2007). In a follow-up study, some support for this association with T1D was provided (Todd et al., 2007). The last meta-analysis of data from three GWA studies of T1D – testing 305,090 SNPs in 3,561 T1D cases and 4,646 controls of European ancestry –

obtained further support for 4q27 T1D association (P=1.9 x 10^{-8}), indicating that this is a true T1D locus (Cooper et al., 2008). The same region was studied in IBD such as CD and UC. Four SNPs were genotyped in three cohorts concerning 4,782 cases (3,194 UC, 1,588 CD) and 2,616 controls. All four SNPs were strongly associated with UC and moderately associated with CD (Festen et al., 2009). The 4q27 locus was also reported to be associated with coeliac disease (van Heel et al., 2007). Recent evidence is also provided of a role for this region in Ps and PsA (Liu et al., 2008). Finally, a genetic association with SLE and two SNPs located within the IL-21 gene was found in a case–control study (1,318 cases and 1,318 controls) (Sawalha et al., 2008). All of these studies provide evidence that 4q27 seems to be a common locus for multiple forms of autoimmune diseases.

In our family-based study, compared with the Dutch study, we have detected a similar undertransmission of the rs6822844-T allele, the rs6822844-GT genotype and the AATGG haplotype, but without significance. Furthermore, we observed evidence of association between RA and the rs4505848-AG genotype, but no significance was found after Bonferroni correction. These results exclude the 4q27 locus from being a major RA genetic region, but a minor association should be not excluded and needs to be tested in a larger RA trio sample. Furthermore the already cited last RA meta-analysis, suggesting a 4q27 true-positive association, was performed in a large number of individuals. For the rs4572894 SNP tested, there was only a 2% variation between RA and non-RA minor allele frequency (27% versus 29%, respectively) with a P value of 1.1% (Raychaudhuri et al., 2008). The putative effect of the 4q27 locus in RA is therefore very small and difficult to replicate in our RA family sample. In addition, a two-stage GWA study of RA in the Spanish population did not found 4q27 as a RA susceptibility gene region (Julià et al., 2008).

The long region of LD at chromosome 4q27 contains several genes: testis nuclear RNA-binding protein, a gene encoding a protein of unknown function (KIAA1109), and genes encoding the IL-2 and IL-21 cytokines. Testis nuclear RNA-binding protein is expressed primarily in the testis and KIAA1109 transcripts are ubiquitous, hence their roles in autoimmunity are not particularly compelling. Both IL-2 and IL-21 belong to the type 1 cytokine family, share a large degree of homology, and possess pleotropic functions in immune cells (van Heel et al., 2007). These two genes are both plausible functional candidates as genetic modifiers of autoimmunity, and thus have particular interest to RA.

IL-2 exerts its effects on many cell types, the most prominent of which is the T lymphocyte. Accordingly, a major function of IL-2 is to promote proliferation and expansion of both antigen-specific clones of CD4+ and CD8+ T cells as well as to induce production of other

cytokines. In CD4+ T cells, IL-2 plays a nonredundant role in the development of CD4+ CD25+ Treg. Accumulating evidence supports CD4+ $CD25^{high}$ Treg playing an essential role in controlling and preventing autoimmunity (Chistiakov et al., 2008). In addition, the IL2 receptor (CD25) susceptibility locus has recently been reported to be associated with RA (WTCCC, 2007).

IL-21 is involved in both cell-mediated and humoral responses and has a pleiotropic effect on a variety of immune and nonimmune cells. In RA, the synovial fluid and tissue have enhanced inflammatory responses to IL-21 and elevated IL-21 receptor expression. In two animal models - CIA and adjuvant-induced arthritis - treatment with an IL-21-blocking agent ameliorated disease and/or reversed established disease, and also lowered levels of IL-6 and IL-17 (Spolski and Leonard, 2008).

In conclusion, using a family-based approach, we have provided a trend for the association of the KIAA1109/Tenr/IL2/IL21 gene region with RA in European descent populations. Nevertheless, we failed to replicate a significant association of this region in our RA family sample. Further investigation of this region, including detection and testing of all variants, is required to confirm RA association.

3.5. Linkage proof for 6q23 locus, a Rheumatoid Arthritis susceptibility genetic region

Teixeira VH, Mbarek H, Pierlot C, Migliorini P, Balsa A, Westhovens R, Barrera P, Alves H, Vaz C, Fernandes M, Pascual-Salcedo D, Bombardieri S, Dequeker J, Radstake TR, Van Riel P, van de Putte L, Lopes-Vaz A, Bardin T, Prum B, Dieudé P, Cornelis F, Petit-Teixeira E; ECRAF. (**Manuscript in preparation**)

3.5.1. Abstract

The 6q23 locus has recently been identified in the genome wide association study performed by the Wellcome Trust Case Control Consortium (WTCCC). The association of Rheumatoid Arthritis (RA) with the rs6920220 in 6q23 was confirmed in a second British sample. A protective association with the rs10499194 located 3.8 kb away from rs6920220 has been reported in the study of an American sample. These two single nucleotide polymorphisms (SNPs) map between the genes oligodendrocyte transcription factor 3 (*OLIG3*) and tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3*). The aim of our study was to provide the linkage proof for the 6q23 locus-RA association, taking advantage of the European Consortium of Rheumatoid Arthritis Families (ECRAF) resource dedicated to RA linkage studies.

1277 West European individuals from 429 trio families were genotyped for rs10499194 (C/T) and rs6920220 (A/G) SNPs using the standard fluorescence genotyping technique. The linkage analysis for each SNPs and haplotypes were performed using the Transmission Disequilibrium Test (TDT). The OR and 95% C.I. were estimated by using the method of Woolf as modified by Haldane.

The TDT analysis of the haplotypes provided the linkage proof for *TNFAIP3* locus with RA: undertransmission for the T-G haplotype = 43% (P=0.01) and overtransmission for the C-A haplotype = 58% (P=0.01). After autoantibodies stratification, the linkage proof of the "protective" haplotype T-G was found in both RF- and ACPA-positive subgroups (P=0.007 and P=0.04, respectively). We also observed a linkage between the "risk" haplotype C-A and RA in the RF-positive subgroup (P=0.01). In the ACPA-positive subgroup a trend for linkage with RA was observed however without significance (P=0.08).

This study provides the linkage proof for the 6q23 locus as a new RA factor. Functional studies will be required to identify the pathophysiological mechanism underlying the association, which would provide a target for definitive treatment of RA.

3.5.2. Introduction

The WTCCC identified, through a GWA study, nine new putative RA susceptibility loci, in addition to *HLA* and *PTPN22* (WTCCC, 2007). One SNP located in the chromosomal region 6q23, rs6920220, was unequivocally replicated in a validation study and reported to be stronger in patients with ACPA or RF positivity than in the ACPA- or RF-negative subgroup (Thomson et al., 2007). Interestingly, association to the same SNP and a second, independently associated polymorphism in the region (rs10499194) was detected in a GWA study in a RA ACPA-positive US population, confirming the locus as important in RA causation (Plenge et al., 2007b). Increased risk for RA is conferred by the minor allele of rs6920220 and protection by the minor allele of rs10499194. The SNP rs6920220 identified in the WTCCC study is located only 3.8 kb away from marker rs10499194. However, fine mapping of the region followed by regression analysis showed that these two signals are independent, and a haplotype analysis using these two SNPs showed that a two-allele model of risk provided the strongest risk predictor. Construction of a haplotype tree indicated the haplotype tagged by rs1099194 as protective, whereas the haplotype tagged by rs6920220 is the risk haplotype (Plenge et al., 2007b).

Two recent studies in the Spanish population reported an absence of association between these two polymorphisms (rs6920220 and rs13207033, which is a perfect proxy of rs10499194) and RA (Dieguez-Gonzalez et al., 2009; Perdigones et al., 2009). However after autoantibodies stratification they reported, in the first study, an association between the rs13207033 SNP and RA only in the patients with ACPA (Dieguez-Gonzalez et al., 2009) and an association of the rs6920220 with RA in ACPA or RF-positive patients, in the second study (Perdigones et al., 2009). Finally, two meta-analysis performed in this locus confirmed the susceptibility risk of 6q23 region with RA (Raychaudhuri et al., 2008; Patsopoulos and Ioannidis, 2009).

Given that association studies compare the frequencies in patients versus healthy individuals, unknown biases in control frequencies may lead to spurious associations. Family-based association studies therefore remain important to definitively establish association, especially when small effect sizes as well as variability in allele frequency in different populations are observed. With the aim to provide the linkage proof for the 6q23 locus-RA association, we therefore took advantage of one of the largest reported European family resources dedicated to RA family-based studies.

3.5.3. Patients and methods

Study design and study population

DNA was available from 429 white trio families from Western Europe through the ECRAF collection followed by the selection of individuals fulfilling the ACR 1987 criteria for RA (Arnett et al., 1988), according to the rheumatologist in charge of the patient. Each family consisted of one RA patient and both parents. Families with RA patients aged <18 years were excluded. All individuals provided written informed consent and the study was approved by the Ethics Committee of l Kremlin-Bicêtre Hospital (Paris, France).

Genotyping

All samples were genotyped using the standard fluorescence genotyping technique. Centre d'Etude du Polymorphisme Humain DNA samples were co-genotyped with all our samples, with no inconsistencies detected. The genotyping success rate in the 429 trio families was 100% (parents and probands included).

Statistical analysis

Using the previously reported frequencies for rs10499194 C allele in the USA (Plenge et al., 2007b) and rs6920220 A allele in the UK RA patients (Thomson et al., 2007) (24.0% and 26.3%, respectively) and in controls (30.0% and 22.3%, respectively), the power to detect an association was calculated using the method described by Garnier and colleagues (2007).

The HWE was checked in the control group (constituting the nontransmitted parental chromosomes from the trio) prior to analysis.

The linkage analysis for each SNPs and haplotypes relied on the TDT (Spielman et el., 1993). ORs were calculated and 95% CIs were approximated using Woolf's method (1955), with Haldane's correction (1956).

3.5.4. Results

Power calculation

Using the previously reported frequencies for rs10499194-T allele in the USA and rs6920220-A allele in the UK RA patients (24.0% and 26.3%, respectively) and in controls (30.0% and 22.3%, respectively), the association analysis of 429 trio families provides a 88% and 61% power to reach statistical significance (P<0.05), respectively.

Hardy–Weinberg equilibrium check

The two SNPs in the sample investigated were in HWE in the control group.

Test for linkage in RA trio families

A total of 1277 European individuals from 429 trio families were analyzed. Concerning the rs10499194 (C-T) SNP we observed deviation from Mendel's first law, with a nonsignificant undertransmission of 46% of the T allele to the patients in the 429 families (*P*=0.09). We observed a significant overtransmission of 56% of the rs6920220-A allele to RA patients (*P*=0.04) (Table 3.5.1). Since 6q23 has been defined as a susceptibility factor in a group of ACPA-positive patients, we tried to ascertain if there were any differences in genetic frequencies when serological data (RF and ACPA) were taken into account. Concerning the rs10499194 SNP no linkage was found even after auto-antibody stratification. We observed a significant overtransmission of 57% of the rs6920220-A allele to RF-positive patients subgroup (*P*=0.04). However no linkage with RA was found in the subgroup ACPA-positive (Table 3.5.1). Furthermore, no linkage was found between these two SNPs and RA in RF- and ACPA-negative subgroups.

Table 3.5.1. Family-based linkage of the chromosome 6q23 gene region with RA: TDT.

6q23 region SNPs	Allele	Transmitted allele	Untransmitted allele	Transmission[a] (%)	*P*-value
rs10499194 (C/T)	T	172	204	46%	0.09
RF+	T	121	153	44%	0.05
RF-	T	51	51	50%	1
ACPA+	T	80	100	45%	0.14
ACPA-	T	24	36	40%	0.12
rs6920220 (A/G)	A	154	120	56%	0.04
RF+	A	123	93	57%	0.04
RF-	A	31	27	54%	0.59
ACPA+	A	77	60	56%	0.14
ACPA-	A	26	19	58%	0.29

a - Percentage of transmission from heterozygous parents.

We further investigated the two SNP (rs10499194 and rs6920220) haplotypes of the 6q23 gene region. The TDT analysis of the haplotypes provided the linkage proof for *TNFAIP3* locus with RA: undertransmission for the T-G haplotype = 43% (*P*=0.01) and overtransmission for the C-A haplotype = 58% (*P*=0.01). After autoantibodies stratification, the linkage proof of the "protective" haplotype T-G was found in both RF- and ACPA-positive subgroups (*P*=0.007 and *P*=0.04, respectively). We also observed a linkage between the "risk" haplotype C-A and RA in the RF-positive subgroup (*P*=0.01). In the ACPA-positive subgroup a trend for linkage with RA was observed however without significance (*P*=0.08). Furthermore, no linkage was found between these two haplotypes and RA in RF- and ACPA-negative subgroups. All these results were presented in Table 5.3.2.

Table 3.5.2. Family-based association for the haplotypes rs10499194 (C/T) - rs6920220 (A/G): TDT.

Haplotype	Transmitted	Untransmitted	Transmission[a] (%)	*P* value
Global				
C-A	118	83	59	0.01
C-G	175	171	51	0.83
T-A	1	1	50	1
T-G	114	153	43	0.01
RF+				
C-A	94	63	60	0.01
C-G	131	125	51	0.83
T-A	1	1	50	1
T-G	76	113	40	0.007
RF-				
C-A	24	20	55	0.54
C-G	44	46	49	0.43
T-G	38	40	49	0.54

Table 3.5.2. Family-based association for the haplotypes rs10499194 (C/T) - rs6920220 (A/G): TDT (continued).

Haplotype	Transmitted	Untransmitted	Transmission[a] (%)	*P* value
ACPA+				
C-A	62	44	59	0.08
C-G	91	86	51	0.70
T-G	52	75	41	0.04
ACPA-				
C-A	21	13	62	0.17
C-G	27	24	53	0.67
T-G	19	30	39	0.11

a - Percentage of transmission from heterozygous parents.

3.5.5. Discussion

We demonstrated here linkage between RA and the 6q23 locus, by replication in a West European sample. We provided the linkage estimate for the "risk" and "protective" haplotypes for the largest European trio RA family resource published to date, measuring the over-transmission of the C-A haplotype (T = 59%) and the undertransmission of the T-G haplotype (T = 43%) compared with the 50% transmission expected from Mendel's law. We also observed the linkage proof of the "protective" haplotype T-G in both RF- and ACPA-positive subgroups and of the "risk" haplotype C-A and RA in the RF-positive subgroup. These results in our family-based study are consistent with the literature from cases-controls studies in European populations (Patsopoulos and Ioannidis, 2009).

Both associated variants map to an intergenic region of 6q23, between the *OLIG3* and *TNFAIP3* genes. OLIG3 is involved in the development and differentiation of neuronal cells and therefore not an obvious candidate gene for RA (Filippi et al., 2005). Conversely, A20, the product of *TNFAIP3*, is a potent anti-inflammatory protein, since it is required for the termination of both TNF and TLR-induced NF-κB signals (Wertz et al., 2004; Boone et al., 2004). In addition, A20 deficient mice develop severe inflammation, which includes inflammation of the joints (Lee et al., 2000). Therefore, TNFAIP3 seems a robust candidate gene for RA susceptibility.

Interestingly several markers in the *TNFAIP3* region have recently been shown to be associated with the related autoimmune disease, SLE, in two independent studies (Graham et al., 2008; Musone et al., 2008). Additionally, a recent robust study has shown that rs6920220 and rs10499194 are also independently associated with T1D (Fung et al., 2008). Moreover, a GWA study has demonstrated strong association of the *TNFAIP3* with Ps (Nair et al., 2009). These findings confirm the importance of the 6q23 locus. Future studies will be required to ascertain whether RA, SLE and T1D are associated with the same or different causative variants within the 6q23 region, and whether the locus harbours risk alleles for other autoimmune or inflammatory diseases.

In conclusion, this study provides the linkage proof for the 6q23 locus as a new RA factor. Functional studies will be required to identify the pathophysiological mechanism underlying this association.

Chapter 4

Genetic and Expression Studies of Candidate Genes in Rheumatoid Arthritis

4.1. Association and expression study of *PRKCH* Gene in a French Caucasian Population with Rheumatoid Arthritis

Based on: Teixeira VH, Jacq L, Moore J, Lasbleiz S, Hilliquin P, Resende Oliveira CR, Cornelis F, Petit-Teixeira E. (2008) J Clin Immunol. 28(2):115-121.

4.1.1. Abstract

Protein kinase C, eta (*PRKCH*) gene, which encodes the η isozyme of protein kinase C (PKCη), is a good functional candidate for susceptibility to Rheumatoid Arthritis (RA) because this auto-immune disease is apparently caused by autoreactive T cells and PKC plays an important role in signal transduction controlling T cell activation. A previous study has shown that multiple SNPs located in 3 distinct Linkage Disequilibrium (LD) blocks were significantly associated with RA in a case-control Japanese population. Further, the *PRKCH* gene is expressed at high levels in resting T cells and this expression is down-regulated by immune responses suggesting that PKCη is involved in signalling pathways to T cells. The aim of this study was to test the *PRKCH* gene association with or linkage to RA in a family-based study from the French Caucasian population. Moreover, the level of expression of *PRKCH* messenger RNA (mRNA) between patients and controls and the correlation between the genotype and level of expression were studied.

100 French Caucasian RA Trio families (one patient and both parents) were genotyped for +8134C/T, rs767755, rs912620 and rs959728 SNPs by PCR-RFLP and TaqMan®. Association and linkage were analysed using the Transmission Disequilibrium Test (TDT) and the Genotype Relative Risk (GRR). Relative quantification of *PRKCH* mRNA expression was performed from whole blood in 24 RA unrelated patients and 16 controls by Real-Time PCR. The expression level of the *PRKCH* transcript was quantified using the treshold cycle (Ct) method. The correlation of the level of expression and the genotype of 24 RA patients was assessed by the Kruskal-Wallis ANOVA Test.

There is no significant association or linkage between three SNPs (+8134C/T, rs912620 and rs959728) and RA ($P>0.05$). The SNP rs767755 was not analyzed because of Hardy-Weinberg disequilibrium in the control constituted by non-transmitted parental alleles from RA trio families. The *PRKCH* messenger RNA was expressed at higher level in controls than in RA patients. We did not observed a significant correlation between the genotypes of the +8134C/T, rs912620 and rs959728 SNPs and the level of expression in RA patients ($P>0.05$).

This study provides evidence that the *PRKCH* gene is not a RA susceptibility genetic factor in the French Caucasian population. Furthermore, the lower expression of this gene in RA patients comparing to healthy controls suggests that PRKCH could be associated with the patho-physiologic mechanism of RA.

4.1.2. Introduction

During the RA development, autoreactive T cells are activated and recruited to joints mediating the synovial inflammation and finally causing tissue damage and cartilage and bone invasion (Panayi et al., 1992). Previous studies have shown that many autoreactive T cells, expressing high levels of CD45RO, are present in synovial fluid of RA patients (Thomas et al., 1992) demonstrating that these autoreactive T cells are activated and have adopted an effector/memory phenotype (Sallusto et al., 2004). Immunosuppressive drugs or anti-cell antibodies were used to eliminate or inhibit T cells causing an amelioration of the disease (Mima et al., 1995). Hence, it is apparent that autoreactive T cells play important roles in synovial tissue inflammation in RA patients. Though, the biologic mechanism of these autoreactive T cells in RA remains poorly studied.

PRKCH gene, which encodes PKCη, is a good functional candidate for susceptibility to RA because PKC plays an important role in signal transduction controlling T cell activation. The PKC gene family consists of more than 11 members, including *PRKCH*/*PKC*η, and their individual products have been revealed to be involved in different cellular biological functions in various cell types (Dempsey et al., 2000). Recent papers suggest that some isozymes of PKC are involved in critical functions of T cells (Volkov et al., 2001; Baier, 2003).

A previous study reported a significant association ($P<0.05$) for a landmark SNP (rs767755), located in intron 2 of the *PRKCH* gene, with RA in a case-control Japanese population. Subsequent analysis of additional SNPs within this gene revealed multiple SNPs located in 3 distinct LD blocks to be significantly associated with RA. In each LD block the most significant associated SNP was reported (+8134C/T, rs912620 and rs959728). Furthermore, they have shown that *PRKCH* gene was expressed at high levels in resting T cells and this expression was down regulated by immune responses suggesting that PKCη is involved in signalling pathways to T cells (Takata et al., 2007).

In this study, we tested *PRKCH* +8134C/T, rs912620 and rs959728 SNPs for RA association and linkage in 100 French Caucasian trio. Moreover, the level of expression of *PRKCH* mRNA in 24 French Caucasian unrelated RA patients and in 16 French Caucasian healthy controls and the association between haplotypes of the SNPs tested and the level of expression in 24 unrelated RA patients were studied.

4.1.3. Patients and Methods

Patients and healthy controls

The study was approved by the Ethics Committees of Bicêtre and Saint Louis Hospitals (Paris, France) and all subjects provided informed consent. 100 RA trio families (one patient and both healthy parents) with the four grandparents of French Caucasian origin were recruited through a national media campaign. Characteristics of the RA trio families sample were reported in Table 4.1.1.

Table 4.1.1. Characteristics of RA index cases from the investigated sample.

Characteristic	**RA patients (*n* = 100)**
Females (%)	87
Mean age (± standard deviation, SD) at disease onset (years)	32 (± 10)
Mean (± SD) disease duration (years)	18 (± 7)
RA patients with bone erosions (%)	90
RA patients seropositive for rheumatoid factor (%)	81
RA patients seropositive for anti-cycle citrullinated peptides antibodies (%)	78
RA patients carrying at least one *HLA-DRB1*shared epitope allele (%)*	78

* - *DRB1**0101, *DRB1**0102, *DRB1**0401, *DRB1**0404, *DRB1**0405, *DRB1**0408, *DRB1**1001

Among the 24 French Caucasian unrelated RA patients, 18 were women (mean ± SD age at enrolment 53.6 ± 12.1). Depending on the swollen/tender joint count at the time of the study there were 17 severe RA patients (minimum 5 inflamed joints) and 7 mild RA patients (less than 5 active joints). All RA patients have received DMARDs and corticosteroids therapy

before the inclusion in the study. All RA patients satisfied the revised criteria of the ACR (Arnett et al., 1988) according to the rheumatologist in charge of the patient. A rheumatologist university fellow reviewed all clinical data. Between the 16 French Caucasian healthy controls, 11 were women (mean ± SD age at enrolment 47.8 ± 7.5).

Molecular Genotyping Method

Genomic DNA of the 100 French Caucasian RA Trio families and the 24 French Caucasian unrelated RA patients was isolated and purified from fresh peripheral blood leucocytes according to standard protocols. Genotyping of the *PRKCH* +8134C/T polymorphism was performed by the polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) method (Botstein et al., 1980). The designed primers were: sense 5'ATGTGGTTTATTATAGTATTCTACTTT3' and anti-sense 5' TACTCACCTGTTCCTACCTGCA 3'. Primers were tested using the BLAST algorithm to ensure that only the *PRKCH* gene was amplified. PCR amplification was performed on each sample in a 25 µl reaction volume consisting of 10X PCR buffer (Perkin Elmer), 1.25 mM of each dNTP, 1.25 U of Taq Gold DNA polymerase (Perkin Elmer), 3 mM MgCl2, 0.0125 nM of the two primers (Invitrogen) and 50 ng of genomic DNA, diluted to the final volume with H2O on Eppendorf thermocycler. The PCR program was carried out using the following amplification protocol: 37 cycles of denaturation at 96°C for 30 s, with annealing temperature at 59°C for 30 s followed by an elongation step at 72°C for 1 min. One final cycle of the extension was performed at 72°C for 10 min. A 360-bp amplified fragment was digested with *NlaIII* (Ozyme) generating two fragments (187 bp and 139 bp) when the restriction site located at the SNP locus was present (C allele). Two independent investigators assessed genotypes blindly. Genotyping of the *PRKCH* rs912620 and rs959728 SNPs was carried out with a Taqman 5' allelic discrimination assay on an ABI 7500 real time PCR machine (assays: C___7600119_10, C___7600090_10, respectively; Applied Biosystems). Allele-specific probes were labelled with the fluorescent dyes VIC and FAM. PCR reaction was carried out in a total volume of 15 µl with the following amplification protocol: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s, annealing and extension at 60°C for 1 min. Genotyping of each sample was automatically attributed using the SDS software for allelic discrimination. 10% of the samples choose at random were genotyped again for quality control.

***PRKCH* mRNA expression by real–time quantitative reverse transcription-PCR**

Total RNA of the 24 French Caucasian unrelated RA patients and 16 French Caucasian healthy controls from whole blood was extracted using a PAXgene Blood RNA kit (Qiagen). The measure of the RNAs concentration was performed using the RNA RiboGreen dye (Invitrogen). The integrity of the RNAs was analyzed using the Agilent 2100 Bionalyzer (Agilent). Reverse-transcription was performed in a total volume of 20 µl with SuperScript™ III First-Strand Synthesis SuperMix for qRT–PCR (Invitrogen) using 1 µg of each total RNA and random hexamers according to the manufacturer's protocol. The kit includes RNaseOUT™ recombinant ribonuclease inhibitor as an RNase protector. Real-time quantitative RT-PCR analysis was executed on an ABI Prism 7500 machine, using TaqMan Gene Expression Assays probes (Applied Biosystems) for *PRKCH* (Hs00178933_m1) and TaqMan Endogenous Controls probes (Applied Biosystems) for *ACTB* (β-actin), *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) and *B2M* (β-2-microglobulin). Each sample was tested in duplicate and a sample without template was included in each run as a negative control. The expression level of the *PRKCH* transcript was quantified using the threshold cycle (Ct) method and normalized to the amount of *ACTB*, *GAPDH* and *B2M*. Samples showing Ct values > 35 and duplicates with a Ct > 0.3 were re-tested.

Haplotype assignment

The *PRKCH* gene haplotypes obtained taking into account the unphased SNP genotype data of the 24 unrelated RA patients was performed using fastPHASE 1.2 software (Scheet and Stephens, 2006).

Statistical Analysis

Following the hypothesis of an RA association profile of *PRKCH* in a French Caucasian population similar to that observed in Japanese RA patients, we used the reported allelic frequencies of the *PRKCH* +8134C/T SNP in Japanese RA patients (17%) and in controls (22,2%) (Takata et al., 2007) to detect the power of an association using the method described by Garnier and colleagues (2007).

The Hardy-Weinberg equilibrium was checked in the control group (constituted by the non transmitted parental chromosomes from trio) prior to analysis.

The association analysis relied on the TDT (Spielman et el., 1993) and on the GRR (Lathrop, 1983).

Results of relative mRNA expression are presented as the mean ± standard deviation percentage. Statistical analysis of the relative expression of the *PRKCH* in RA patients and healthy controls was performed using the Mann-Whitney test and $P<0.05$ was considered significant.

The association between haplotypes of the SNPs tested and the level of expression in 24 unrelated RA patients was assessed by the Mann-Whitney Test. Data are expressed as the mean ± standard deviation and $P<0.05$ was considered significant.

4.1.4. Results

Power calculation

Using the previously reported allelic frequencies of the *PRKCH* +8134C/T SNP in Japanese RA patients (17%) and in controls (22,2%) (Takata et al., 2007), association analysis of 100 trio families provides a 88,5%power to reach statistical significance ($P<0.05$).

Hardy-Weinberg equilibrium check

The *PRKCH* +8134C/T, rs912620 and rs959728 SNPs were in Hardy-Weinberg equilibrium in the control sample investigated.

Test for linkage and association in RA trio families

We test the *PRKCH* putative susceptibility alleles +8134C, rs912620-T and rs959728-T for which association with RA was the most significant in the case-control Japanese population (Takata et al., 2007).

We did not observe a linkage and association between the +8134C allele and RA: there was an equal transmission of +8134C allele and +8134T allele from heterozygous parents (40 transmitted *vs.* 40 non transmitted, $P=1$) (Table 4.1.2). There was a no significant over-transmission of the rs912620-T allele from heterozygous parents (46 transmitted *vs.* 42 non transmitted, $P=0.67$) (Table 4.1.2). The same result is observed for the rs959728-C allele (22 transmitted *vs.* 17 non transmitted, $P=0.42$) (Table 4.1.2).

Table 4.1.2. Transmission Disequilibrium Test between *PRKCH* +8134C/T, rs912620, rs959728 SNPs and RA.

***PRKCH* SNPs**	**Allele**	**Transmitted**	**Non Transmitted**	**T[a] (%)**	***P*-value**
+8134C/T	C	40	40	50	1
rs912620 (G>T)	G	42	46	47.7	0.67
rs959728 (C>T)	C	17	22	43.6	0.42

[a] - Percentage of transmission from heterozygous parents.

The GRR analysis of the *PRKCH* +8134C/T, rs912620 and rs959728 SNPs showed no significant (P>0.05) association of the homozygous or heterozygous genotypes for susceptibility alleles with RA (Table 4.1.3). The TDT analysis of *PRKCH* +8134C/T-rs912620-rs959728 haplotypes did not reveal any significant association for the seven haplotypes estimated (data not shown).

Table 4.1.3. Genotype Relative Risk for *PRKCH* +8134C/T, rs912620, rs959728 SNPs and RA.

***PRKCH* SNPs**	**Genotypes**	**Lathrop *P*-value (one genotype *vs* the others)**	***P*-value**
+8134C/T	CC	0.63	1
	CT		
	TT	0.44	
rs912620 (G>T)	GG	0.46	0.90
	GT		
	TT	0.89	
rs959728 (C>T)	CC	0.28	0.61
	CT		
	TT	0.58	

Expression analysis

A relative quantification of *PRKCH* mRNA expression was performed in total RNA from whole blood in 24 unrelated RA patients and in 16 healthy controls. *PRKCH* was expressed in RA patients at lower level (-92%; $P<0.0001$) than in healthy controls (Figure 4.1.1).

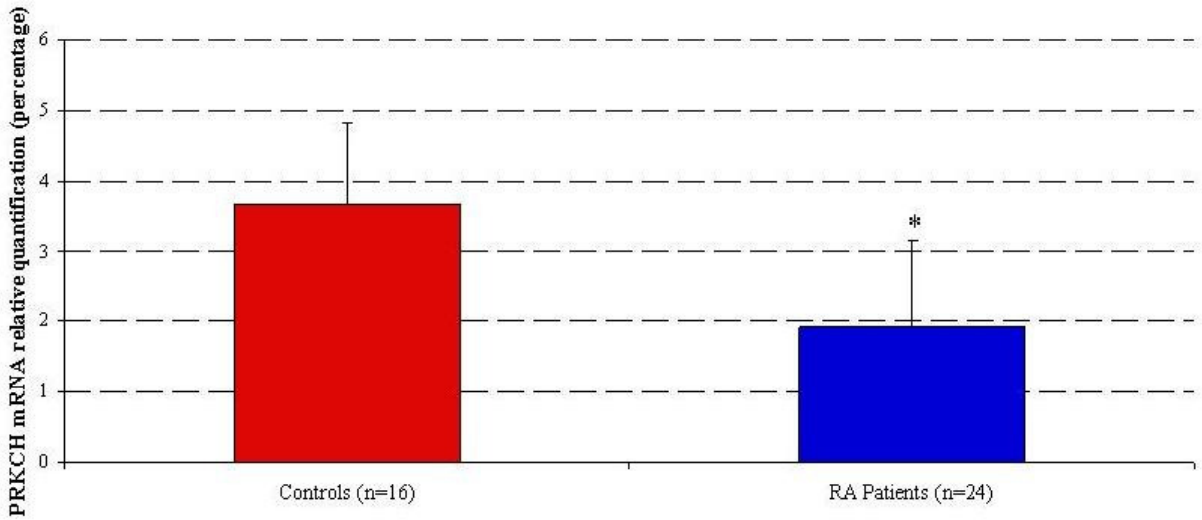

Figure 4.1.1. Levels of *PRKCH* mRNA expression in peripheral blood from RA unrelated patients and healthy controls. Data are presented as the mean ± SD percentage of the *PRKCH* mRNA expression. * = $P<0.0001$ versus Controls, by Mann-Whitney test.

The relative quantification of *PRKCH* mRNA in severe and mild RA patients was reported in Figure 4.1.2. The expression of *PRKCH* in severe RA patients was higher than in mild RA patients ($P=0.008$).

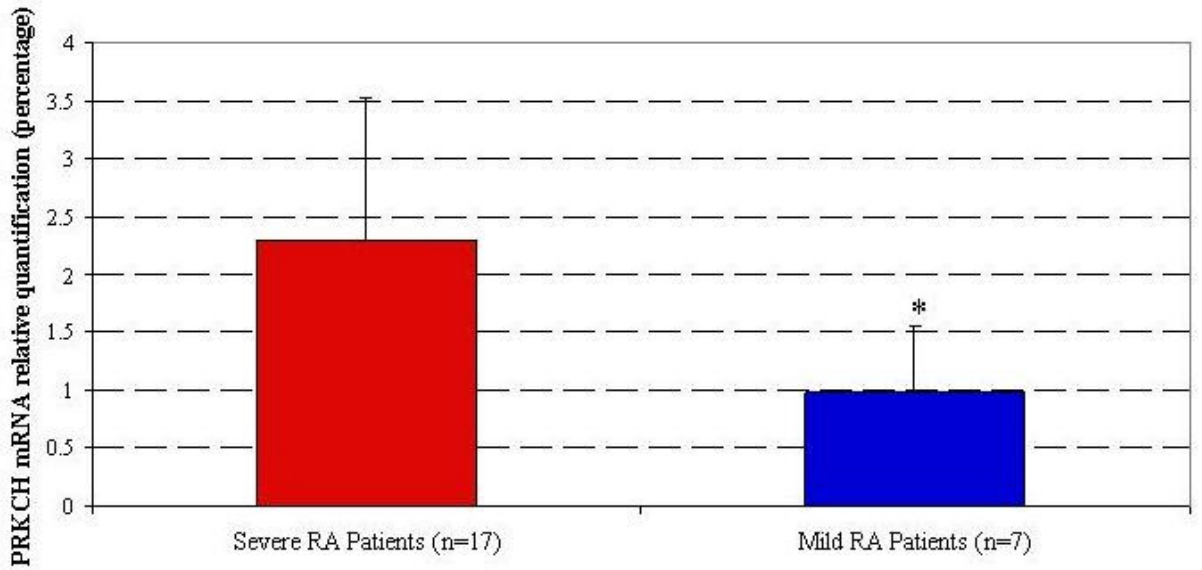

Figure 4.1.2. Levels of *PRKCH* mRNA expression in peripheral blood from severe and mild RA patients. Data are presented as the mean ± SD percentage of the *PRKCH* mRNA expression. * = $P=0.008$ versus severe RA patients, by Mann-Whitney test.

We had select three haplotype combinations: CTT/CTT (haplotype homozygous for the susceptible alleles), CTT/X (haplotype heterozygous for the susceptible alleles) and X/X (haplotype with no susceptible alleles). In the set of 24 unrelated RA patients, there were 4 RA patients with CTT/X haplotype, 20 with X/X and no one with CTT/CTT. Even though the expression of *PRKCH* mRNA was lower in RA patients with CTT/X haplotype comparing to X/X haplotype (Figure 4.1.3), we did not observed a significant association (*P*=0.94).

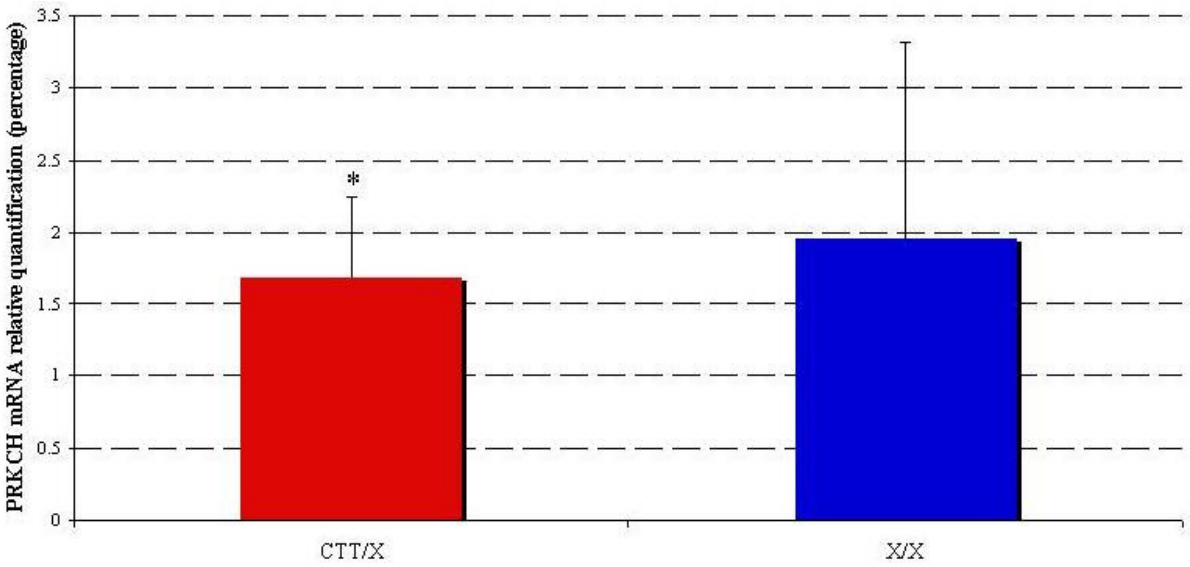

Figure 4.1.3. Association between the level of *PRKCH* mRNA expression and *PRKCH* haplotype (+8134C/T-rs912620-rs95972824) in RA patients. Data are presented as the mean ± SD percentage of the *PRKCH* mRNA expression. * = *P*=0.94 versus X/X haplotypes, by Mann-Whitney test.

4.1.5. Discussion

This study was designed to test the *PRKCH* +8134C/T, rs912620 and rs959728 SNPs for linkage to, and association with, RA in a French Caucasian familial population. In addition, we studied the level of expression of *PRKCH* mRNA in RA patients and in healthy controls and the association between haplotypes of the SNPs tested and the level of expression in RA patients.

Takata and colleagues (2007) described the +8134C, rs912620-T and rs959728-T alleles from SNPs located in 3 different LD blocks as susceptible for RA. We have confirmed that these susceptibility alleles were not in LD in the French Caucasian population (data not shown). In the LD block D described by Takata and colleagues (2007), we have chosen to test the rs959728 SNP which is more associated to RA than rs2230500 SNP (*P*=0.00011 *vs* *P*=0.0016). This functional SNP rs2230500 located in exon 9 (V3741I) was reported to

increase the risk of cerebral infarction (Kubo et al., 2007). Our results showed a clear absence of RA linkage and association in the population investigated for the 3 SNPs tested. In good agreement with the TDT analysis, the GRR revealed a lack of association between the different genotypes and RA. The linkage analysis of *PRKCH* +8134C/T-rs912620-rs959728 haplotypes was not significant as none of the seven haplotypes described were over-transmitted (data not shown). The results were obtained with a particularly robust method, the TDT, that avoid for imperfect population match between patients and controls and permitting the direct test of the Mendel's law. These results allowed us to exclude *PRKCH* as a major significant genetic factor in RA in a French Caucasian population. There were several genetic studies that reported differences in ethnic variations of polymorphisms associated with RA as *PADI4* and *SLC22A4* genes (Tokuhiro et al., 2003; Caponi et al., 2005; Barton et al., 2005). Moreover *PRKCH* locus was not included among the suggested 19 non-HLA regions in the French Caucasian population (Osorio et al., 2004). The last GWA study in a UK Caucasian population had found 9 SNPs that map to loci not associated previously to RA (WTCCC, 2007). However *PRKCH* locus was not present in these regions. Thus, others populations need to be studied to define extensively the involvement of this gene in the genetics of RA.

Even some PKC isozymes have been suggested to be involved in important functions of T cells (Volkov et al., 2001; Baier, 2003) the physiological function of PKCη in T cells has not yet been well documented and the pathway by which *PRKCH* SNPs may influence RA risk remains unknown.

This study is the first to show that *PRKCH* was expressed in RA patients at lower level than in non-RA controls in peripheral blood cells. This result suggests that expression of the *PRKCH* gene is regulated through immune responses. This observation confirms the study of Takata and colleagues (2007) who have shown that *PRKCH* gene is expressed in resting CD4+ cells (T helper/inducer cells) or CD8+ cells (T suppressor/cytotoxic cells) at higher levels than in resting CD19+ cells (B cells) or CD14+ cells (monocytes) and the expression in these cells were significantly down-regulated by activation.

Our data also show the *PRKCH* mRNA highly expressed in severe RA patients comparing to mild RA patients, supporting the increase of expression of *PRKCH* during the progression of the disease from mild to sever RA. PKCη functions have been associated to the cytokine signalling cascade in monocytes and macrophages. Indeed plasma levels of nitric oxide, a mediator of inflammation, were shown to be elevated in patients with severe RA, and a positive association between *PRKCH* and inducible nitric oxide synthase (*iNOS*) expression

was observed in peripheral blood monocytes-derived macrophages from severe RA patients. Furthermore, this co-expression was not present in healthy controls (Pham et al., 2003).

Heale and colleagues (2007) have confirmed these results reporting a PKCη-expression phenotype in monocytes for mild and severe RA patients and not for healthy controls. Moreover, there was a progressive and concordant expression of PKCη and iNOS phenotypes in monocytes from RA patients associated with the severity of the disease.

These studies provided evidence supporting the possible involvement of PKCη in immunologic activities of T cells, monocytes and macrophages, whereas our study highlighted an overall expression of *PRKCH* gene in whole blood from RA patients. The association of *PRKCH* differential expression and the patho-physiologic mechanism in RA should be then further investigated.

The relation between haplotypes and expression level of *PRKCH* gene was not identified, as the expression of *PRKCH* mRNA was lower in RA patients with CTT/X haplotype comparing to X/X haplotype but without significance. Nevertheless, the determination of haplotypes effects should require an extensive examination of expression in different cell subsets and RA states.

In conclusion, we provided evidence against the involvement of the *PRKCH* gene in the genetics of RA in a French Caucasian familial population. However, replication studies in other independent Caucasian populations are required to confirm these results. Our study is the first to show that *PRKCH* was expressed in RA patients at lower level than in non-RA controls in peripheral blood cells. Further investigations in the regulation of *PRKCH* expression in RA are necessary to prove the involvement of PKCη molecular mechanisms in disease susceptibility, more specifically in signalling pathways of RA specific immune response.

4.2. Genetic and expression analysis of *CASP7* Gene in a European Caucasian Population with Rheumatoid Arthritis

Based on: Teixeira VH, Jacq L, Lasbleiz S, Hilliquin P, Oliveira CR, Cornelis F, Petit-Teixeira E, ECRAF. (2008) J Rheumatol. 35(10):1912-1918.

4.2.1. Abstract

An imbalance between cell proliferation and insufficient apoptosis of synovial macrophages, fibroblasts and lymphocytes is one mechanism that might contribute to persistence of RA. Caspase 7 (CASP7) is an executioner caspase crucial for apoptosis. Our aim was to study the possible role of the *CASP7* gene in susceptibility to RA in a European Caucasian population.

CASP7 rs2227309 SNP was genotyped in 197 French RA trio families and in 252 European RA families available for replication using Taqman® allelic discrimination assay. Relative quantification of CASP7 isoforms α and β mRNA expression was performed from whole blood in 25 unrelated RA patients and in 15 healthy controls by real–time qRT-PCR. The genetic analyses for association and linkage were performed using the comparison of allelic frequencies, the Transmission Disequilibrium Test (TDT) and the Genotype Relative Risk (GRR).

We observed, in the first sample, a significant association of rs2227309-AA genotype (*P*=0.03, OR=2.11 95% CI=1.0-4.6) with RA. The second sample did not show any significant association of the AA genotype with RA (*P*=0.6, OR=0.87 95% CI=0.4-1.8). When the two samples were combined no significant association of the AA genotype (*P*=0.3, OR=1.32 95% CI=0.8-2.2) was observed. *CASP7* isoforms α and β mRNA were expressed in RA patients at lower level than in healthy controls (-89%, *P*=0.003 and -47%, *P*=0.01; respectively).

CASP7 rs2227309 SNP is not associated with RA in a European Caucasian population. Nevertheless, *CASP7* isoforms α and β could have an involvement in apoptosis process in RA.

4.2.2. Introduction

An imbalance between cell proliferation and insufficient apoptosis of synovial macrophages, fibroblasts and lymphocytes is one mechanism that might contribute to persistence of RA (Pope, 2002). In fact, the increase of osteoblasts apoptotic cell death may contribute to periarticular bone loss in RA patients while impaired apoptosis of rheumatoid synovial cells appears to cause hyperplasia of the synovial tissues (Stanczyk et al., 2006). Further, different studies in animal arthritis models suggest that an increase of the induction of synovial cells apoptosis ameliorates synovial tissue hyperplasia (Zhang et al., 2005; Peng, 2006). Additionally, the dysregulation of apoptosis is involved in a large variety of human diseases including cancer, autoimmune diseases and neurodegenerative disorders (Kuribayashi et al., 2006).

Apoptosis is a cell suicide program, evolutionarily conserved, by which a cell undergoes an orderly demise. Apoptotic cell death is coordinated in cells by a family of the cysteine-aspartic acid proteases: caspases. In mammals, caspases involved in apoptotic responses are classified into two groups according to their structure and function. The first group is composed by termed initiator caspases (caspase-2, 8, 9, 10) which contain N-terminal adapter domains that allow for their auto-cleavage and the activation of downstream caspases. The second group is composed by effector or executioner caspases (caspase-3, 6, 7) which lack N-terminal adapter domains and are cleaved and activated by initiator caspases (Degterev et al., 2003).

CASP7 gene is located in the chromosomal area 10q25.1-10q25.2. CASP7 exists as an inactive proenzyme, which undergo proteolytic processing at conserved aspartic residues to produce two subunits, a large and small one, that dimerize to form the active enzyme. The precursor of this caspase is cleaved by caspase 3 and 10. Alternative splicing results in four transcript variants, encoding three distinct isoforms [alpha (α), beta (β) and delta (Δ)] the majority of which retain catalytic activity (www.ncbi.nlm.nih.gov). In the procaspase 7 isoforms α and Δ, upon activation *in vivo*, a short N-terminal sequence is removed and an 'IQADSG' site is cleaved generating the active CASP7 isoforms with catalytic activity (Stennicke and Salvensen, 2000). Procaspase β uses the same start codon that the variant α but has a distinct C-terminus compared with the other variants. Thus, the CASP7 isoform β lacks the active site of the enzyme and may act as a dominant inhibitor for active procaspase 7 isoforms (Fernande-Alnemri et al., 1995). *CASP7* gene mutations are present in human malignancies and the inactivating mutations of *CASP7* gene might lead to the loss of its

apoptotic function and contribute to the pathogenesis of human solid cancers (Soung et al., 2003). Additionally, *CASP7* has been proposed like a positional candidate for susceptibility to T1D (Babu et al., 2003).

In the field of genetic factors analysis in RA, a previous study reported a significant association for a rs2227309 (A/G) SNP (allele G *P*=0.001, OR=1.32 [1.11-1.56]; genotype GG *P*=0.0005, OR = 1.47 [1.18-1.83]), located in exon 7 of the *CASP7* gene, with RA in a case-control Spanish Caucasian population. Furthermore, they have shown a statistically significant deviation in the relative expression of the mRNA encoding *CASP7* isoform β *vs* functional isoforms in healthy individuals stratified according to their rs2227309 genotypes (García-Lozano et al., 2007).

The aim of this study was to confirm the role of the *CASP7* gene in susceptibility to RA in a European Caucasian population. For that, we used RA familial material to test *CASP7* rs2227309 SNP for RA association and linkage. Moreover, the expression level of *CASP7* isoforms α and β mRNA in French Caucasian unrelated RA patients and in French Caucasian healthy controls and the relation between genotypes of the SNP tested and the level of expression in these two groups were studied.

4.2.3. Patients and methods

Study populations.

The study was approved by the Ethics Committees of Hôpital Bicêtre and Hôpital Saint Louis (Paris, France) and all subjects provided informed consent. RA families were recruited through a national media campaign. All RA patients satisfied the revised criteria of the ACR (Arnett et al., 1988) according to the rheumatologist in charge of the patient. A rheumatologist university fellow reviewed all clinical data. Sample 1 and 2, used for association and linkage study, consisted of 197 French Caucasian unrelated trio families (one RA patient and both parents) with the four grandparents of French Caucasian origin and of 252 European Caucasian unrelated trio families (Caucasian families from France, Italy, Portugal, Spain, Belgium, and The Netherlands). Characteristics of the RA French Caucasian families (sample 1) and European Caucasian families (sample 2) are reported in Table 4.2.1.

Table 4.2.1. Characteristics of RA index cases from the investigated samples

Characteristic	**Sample 1 (*n* = 197)**	**Sample 2 (*n* = 252)**
Females (%)	88.3	85.3
Mean age (± standard deviation, SD) at disease onset (years)	31.5 (± 9.3)	30 (±9)
Mean (± SD) disease duration (years)	13 (± 8.1)	8.1 (±7)
RA patients with bone erosions (%)	81,5	70.2
RA patients seropositive for rheumatoid factor (%)	71.6	70.6
RA patients seropositive for anti-cyclic citrulinated peptides antibodies (%)	77.6	Not available
RA patients carrying at least one *HLA-DRB1* shared epitope allele (%)*	79.7	Not available

*- *DRB1**0101, *DRB1**0102, *DRB1**0401, *DRB1**0404, *DRB1**0405, *DRB1**0408, *DRB1**1001.

Among the 25 French Caucasian unrelated RA patients used for expression study, 19 were women (mean ± SD age at enrolment 53.4 ± 11.1). Among the 15 French Caucasian healthy controls used for expression study, 11 were women (mean ± SD age at enrolment 46.9 ± 6.6).

Molecular Genotyping Method.

Genomic DNA of the 449 European Caucasian RA trio families and the 25 French Caucasian unrelated RA patients was isolated and purified from fresh peripheral blood leucocytes according to standard protocols. Genotyping of the *CASP7* rs2227309 SNP (A/G) was carried out with a Taqman 5' allelic discrimination assay on an ABI 7500 real time PCR machine (assay: C_500778_10, Applied Biosystems) according to the manufacturer's protocol.

Genotyping of each sample was automatically attributed using specific software for allelic discrimination. Ten percent of the samples chosen at random were genotyped again for quality control.

CASP7 isoforms α and β mRNA expression.

Total RNA from whole blood was extracted in 25 French Caucasian unrelated RA patients and 15 French Caucasian healthy controls using a PAXgene Blood RNA kit (Qiagen). The measure of the RNAs concentration was performed using the RNA RiboGreen dye (Invitrogen). RNAs integrity was analyzed using the Agilent 2100 Bionalyzer (Agilent). Reverse-transcription was performed with SuperScript™ III First-Strand Synthesis SuperMix for qRT–PCR (Invitrogen) according to the manufacturer's protocol. Real-time quantitative RT-PCR analysis was executed on an ABI Prism 7500 machine, using TaqMan Gene Expression Assays probes (Applied Biosystems) for *CASP7* isoform β (Hs01032058_m1) and *CASP7* isoform α (Hs01029847_m1) and TaqMan Endogenous Controls probes (Applied Biosystems) for *ACTB*, *GAPDH* and *B2M*. Each sample was tested in duplicate and a sample without template was included in each run as a negative control. The expression level of the *CASP7* isoforms α and β mRNA was quantified using the threshold cycle (Ct) method and normalized to the amount of *ACTB*, *GAPDH* and *B2M*. Samples showing Ct values > 35 and duplicates with a Ct > 0.3 were re-tested.

Statistical Analysis.

Using the previously reported frequencies of GG genotypes for *CASP7* rs2227309 in Spanish RA patients (56%) and in controls (46.4%) (García-Lozano et al., 2007), power to detect an association was calculated using the method described by Garnier and colleagues (2007).

The Hardy-Weinberg equilibrium was checked in the control group (constituted by the non transmitted parental chromosomes from trio) prior to analysis.

The association analysis relied first on the AFBAC (Thompson, 1995). The linkage analysis relied on the TDT (Spielman et al., 1993). Secondly, we used the GRR method proposed by Lathrop (1983). The OR and 95% C.I. were estimated by using the method of Woolf (1955), as modified by Haldane (1956). The significance of the *P* value was assessed at 5%, leading to replication tests in sample 2.

Results of relative mRNA expression are presented as the mean ± standard deviation percentage. Statistical analysis of the relative quantification of *CASP7* isoforms α and β

mRNA expression in RA patients and healthy controls was performed using the Mann-Whitney test and $P<0.05$ was considered significant. The association between genotypes of the SNP tested and the relative quantification of *CASP7* isoform α *vs* isoform β mRNA expression in 25 unrelated RA patients and in 15 healthy controls was assessed by the Mann-Whitney Test. Data are expressed as the mean ± standard deviation and $P<0.05$ was considered significant.

4.2.4. Results

Power calculation.

Using the previously reported frequencies of GG genotypes for *CASP7* rs2227309 in Spanish RA patients (56%) and in controls (46.4%) (García-Lozano et al., 2007), association analysis of 449 trio families (RA samples 1 + 2) provides a 100% power to reach statistical significance ($P<0.05$).

Hardy-Weinberg equilibrium check.

The *CASP7* rs2227309 SNP in the two samples investigated was in Hardy-Weinberg equilibrium in control group.

Test for linkage and association in RA families

Sample 1. A total of 591 French Caucasian individuals from 197 trio families (one RA case and both parents) were analysed. The rs2227309-A allele frequency was slightly higher in RA index cases than in virtual controls ($P=0.5$) (Table 4.2.2). There was a non significant over-transmission of the rs2227309-A allele from heterozygous parents ($P=0.5$) (Table 4.2.3). The GRR analysis of the *CASP7* rs2227309 showed a significant association of the homozygous AA genotype with RA ($P=0.03$) (Table 4.2.4). The same tests were performed in subgroups stratified for RF positivity, anti-cyclic citrulinated peptide positivity and presence of the *HLA-DRB1* SE and no significant association/linkage were detected (data not shown).

Sample 2. The significant association observed for *CASP7* rs2227309-AA genotype in the sample 1 led to a replication test in the sample 2 with the hypothesis of an AA genotype association with RA. A total of 756 European Caucasian individuals from 252 trio families were analysed. 113 families were from French origin and 139 were from continental Western European countries. In this sample, we did not observed a trend for association and linkage (AFBAC, $P=0.6$; TDT, $P=0.6$) (Table 4.2.2 and 4.2.3) of the allele A with RA. The GRR did

not shown a significant association of the AA genotype with RA (*P*=0.6) (Table 4.2.4). The same tests were performed in the 36 Spanish trio families from Sample 2 and no significant association/linkage were detected (data not shown).

Samples 1 + 2. The combination of the two samples, authorized by the absence of any significant clinical difference between them, showed any significant association and linkage of the allele A with RA (Table 4.2.2 and 4.2.3). The GRR test did not shown a significant association of the *CASP7* rs2227309-AA genotype with RA (*P*=0.3) (Table 4.2.4).

Table 4.2.2. Affected family-based controls analysis in RA trio families

***CASP7* rs2227309 (A/G) SNP**	**Allele**	**RA cases**	**Controls**	***P*-value**
a) RA Sample 1 (*n* = 197)	A	0.26	0.24	0.5
	G	0.74	0.76	
b) RA Sample 2 (*n* = 252)	A	0.26	0.27	0.6
	G	0.74	0.73	
c) RA Sample 1 + 2 (*n* = 449)	A	0.26	0.26	1
	G	0.74	0.74	

Table 4.2.3. Transmission Disequilibrium Test in RA trio families

***CASP7* rs2227309 (A/G) SNP**	**Allele**	**Transmitted**	**Non Transmitted**	**T[a] (%)**	***P*-value**
a) RA Sample 1	A	76	68	52.8	0.5
(*n* = 197)	G	68	76	47.2	
b) RA Sample 2	A	96	104	48	0.6
(*n* = 252)	G	104	96	52	
c) RA Sample 1 + 2	A	172	172	50	1
(*n* = 449)	G	172	172	50	

Table 4.2.4. **Genotype Relative Risk analysis in RA trio families**

***CASP7* rs2227309 (A/G) SNP**	**Genotypes**	**RA Cases**	**Controls**	**Odd Ratio**	**95% CI**	**Lathrop *P*-value (one genotype *vs* the others)**
a) RA Sample 1	AA	20	10	2.11	1.0-4.6	0.03 (AA *vs* AG + GG)
(*n* = 197)	AG	62	74	0.76		
	GG	115	113	1.04	0.7-1.6	0.94 (GG *vs* AA + AG)
b) RA Sample 2	AA	15	17	0.87	0.4-1.8	0.5 (AA *vs* AG + GG)
(*n* = 252)	AG	99	103	0.94		
	GG	138	132	1.1	0.8-1.6	0.7 (GG *vs* AA + AG)
c) RA Sample	AA	35	27	1.32	0.8-2.2	0.4 (AA *vs* AG + GG)
1 + 2 (*n* = 449)	AG	161	177	0.86		
	GG	253	245	1.07	0.8-1.4	0.7 (GG *vs* AA + AG)

Expression analysis.

A relative quantification of *CASP7* isoforms α and β mRNA expression was performed in total RNA isolated from whole blood of 25 unrelated RA patients and 15 healthy controls. *CASP7* isoforms α and β mRNA were expressed in RA patients at lower level than in healthy controls (-89%, *P*=0.003 and -47%, *P*=0.01, respectively) (Figure 4.2.1). The expression level of isoform α mRNA in each group was higher than the expression level of isoform β mRNA (Figure 4.2.1) and the mRNA expression ratio (isoform α *vs* isoform β) in healthy controls was higher comparing to RA patients (+32%).

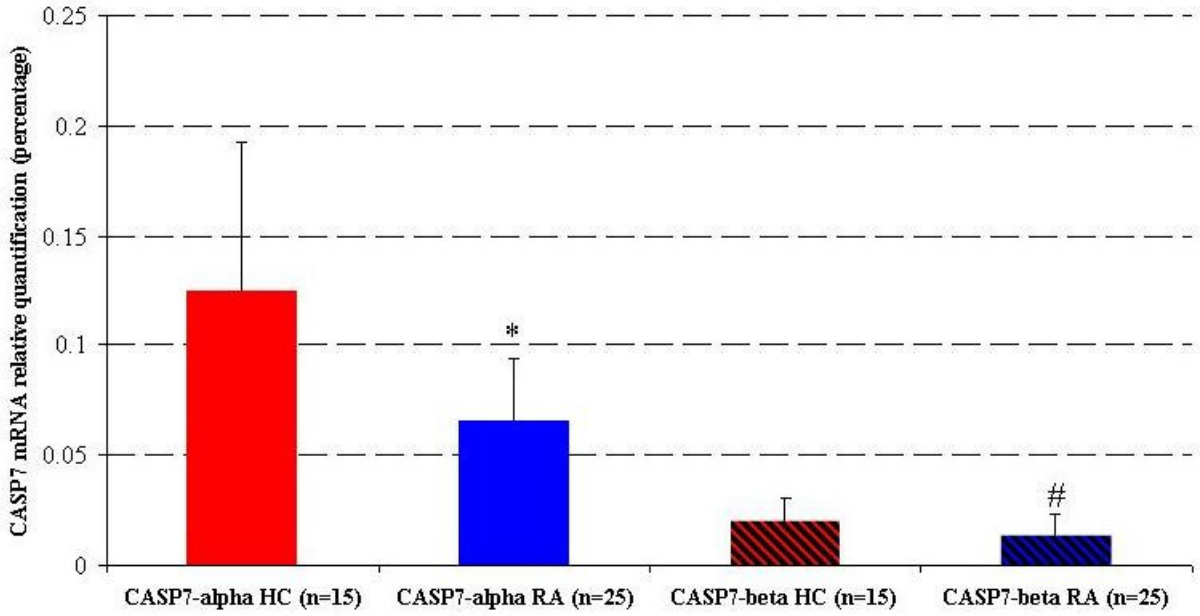

Figure 4.2.1. Levels of *CASP7* isoforms α and β mRNA expression in peripheral blood from RA unrelated patients (RA) and healthy controls (HC). Data are presented as the mean ± SD percentage of the *CASP7* isoforms α and β mRNA expression. *: P<0.0001 *vs* Controls and #: *P*=0.01 *vs* Controls by Mann-Whitney test.

A non statistically significant deviation was observed when comparing mRNA expression ratio (isoform α *vs* isoform β) in RA patients (Figure 4.2.2.A) or in healthy control samples (Figure 4.2.2.B) stratified according to their rs2227309 genotypes (AA *vs* AG, AA *vs* GG, AG *vs* GG, *P*>0.05).

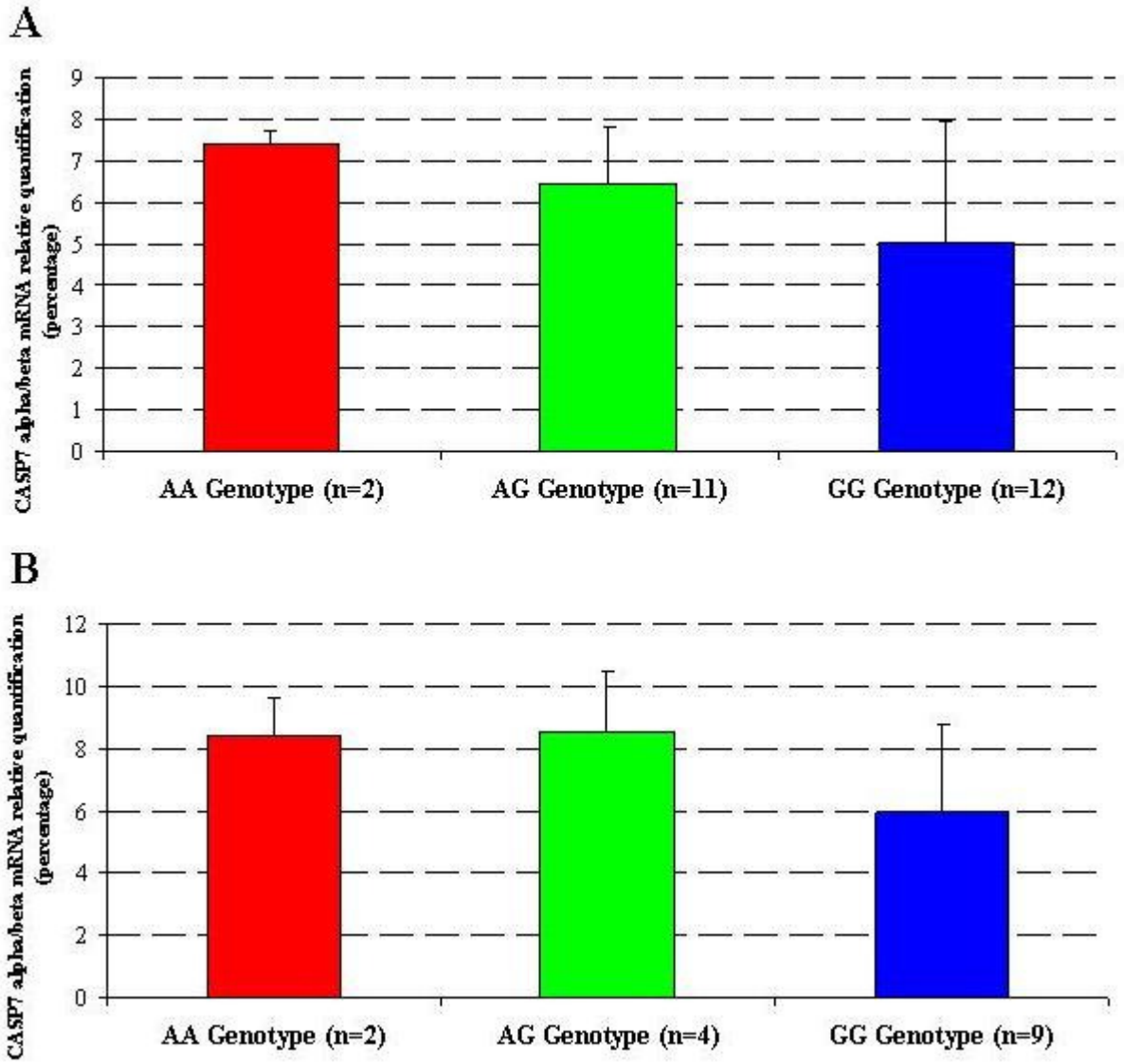

Figure 4.2.2. - Levels of *CASP7* isoform α *vs* isoform β mRNA expression in RA patients (A) and in healthy controls (B) stratified according to their rs2227309 genotypes. Data are presented as the mean ± SD percentage of the *CASP7* isoform α *vs* isoform β mRNA expression in RA patients and in healthy controls (AA *vs* AG, AA *vs* GG, AG *vs* GG: $P>0.05$ by Mann-Whitney test).

4.2.5. Discussion

CASP7 is crucial for apoptosis and contributes to some mitochondrial events such as the loss of mitochondrial membrane potential and the release of proapoptotic factors as cytochrome c and apoptosis-inducing factor (Lakhani et al., 2006). CASP7 is an executioner caspase that requires cleavage to turn into the active isoforms α or Δ (Stennicke and Salvensen, 2000). The CASP7 isoform β which is not cleaved may act as a dominant inhibitor of the active forms (Fernandes-Alnemri et al., 1995). Deregulation of apoptosis synovial cells was one of the mechanisms suspected to be involved in RA physiopathology (Pope, 2002).

A previous study reported a significant association (P<0.05) for rs2227309-GG genotype of the *CASP7* gene with RA in a case-control Spanish Caucasian population (García-Lozano et al., 2007). This SNP produces a K249R change in the isoform β and a synonymous change in the isoform α. Furthermore, this polymorphism is not located in the active site of the caspase7 protein. Our results showed an over-transmission of the rs2227309-A allele from heterozygous parents and a significant association of the homozygous rs2227309-AA genotype with RA in the sample 1. This significant association led to a replication test in the sample 2 with the hypothesis of an AA genotype association with RA. In this sample, we did not observed neither a trend for association and linkage of the A allele nor a significant association of the AA genotype with RA. Moreover, the combination of the two samples did not shown any significant association of the *CASP7* rs2227309-AA genotype with RA. This family-based analysis avoids for imperfect population match between patients and controls, permitting the direct test of the Mendel's law. The allele frequencies that we described in RA patients (A=26% and G=74%) were the same as the one observed in reported RA Spanish patients (García-Lozano et al., 2007). However, the allele frequency inferred from parental non transmitted chromosomes (A=26% and G=74%) was different to that observed in Spanish controls (A=31.6% and G=68.4%) (García-Lozano et al., 2007). Altogether, our results allowed to exclude *CASP7* rs2227309 SNP as a major significant genetic factor in RA in a European Caucasian population. To exclude *CASP7* gene association with RA, other tagSNPs should be tested. Anyway, *CASP7* locus was not included among the 19 suggested non-HLA regions in the French Caucasian population linked to RA (Osorio et al., 2004). Furthermore, the last GWA study in a UK Caucasian population found 9 SNPs that map to loci not associated previously to RA (WTCCC, 2007) and *CASP7* locus was not present in these regions.

This study is the first to show one statistically significant difference in the expression of *CASP7* isoforms α and β mRNA between RA patients and healthy controls in peripheral blood cells. When we compared the expression of these two isoforms in healthy controls or in RA patients we observed a higher mRNA expression of the α isoform comparing to the β isoform. Furthermore, the decrease of mRNA *CASP7* isoform α expression in RA patients compared to healthy controls was more significant than observed for *CASP7* isoform β mRNA expression. Thus, in RA patients, peripheral blood cells could have a lower apoptotic activity based on the lower relative quantity of the functional isoform α comparing to healthy controls. As the decrease of isoform β mRNA expression in RA patients was less significant

than the decreased observed for isoform α, isoform β may act as a dominant inhibitor of active forms. Thus, peripheral blood cells from RA patients could have a lower apoptotic activity based on the higher proportion of the non functional isoform (β isoform) comparing to the active one (α isoform). Several reports demonstrated that impaired apoptosis is a characteristic of several autoimmune diseases and administration of factors that stimulate apoptosis decrease inflammation (Zhang et al., 2005; Mahoney and Rosen, 2005; Catrina et al., 2005; Stanczyk et al., 2006; Peng, 2006). Furthermore, in mice, the knockout of CASP7 decreases the apoptotic process (Lakhani et al., 2006). Nevertheless, to complete our observed data, further investigations should be done in specific cells such as T and B lymphocytes, monocytes, macrophages and synoviocytes.

We can not exclude the effects of the MTX in the apoptotic process of RA patients because in our study, 92% of RA patients have received MTX treatment. However, the mechanism of MTX action in autoimmune diseases remains unclear. An influence of MTX on apoptosis in PBMC has been demonstrated only under non-physiological conditions, for example after stimulation with mitogens (Genestier et al., 1998; Johnston et al., 2005). Employing stimulation with conventional antigens, others studies have failed to demonstrate an apoptotic effect of MTX on PBMC, and instead attributed the effect of MTX on T cells in suppression of T cell activation and reduced expression of T cell adhesion molecules (Johnston et al., 2005). Finally, a recent study has reported that MTX inhibits proliferation in *Candida albicans* (CA)- and tetanus toxoid (TT)-stimulated CD4+ T cells, and induces apoptosis in a subset of stimulated CD4+ T cells. They have suggested that stimulation of T cells with the conventional antigens CA and TT more probably resembles stimulation with self-antigens occurring in autoimmune diseases, including RA, and the anti-proliferative and pro-apoptotic effects of MTX demonstrated reflect the actions of this drug in the treatment of rheumatic diseases (Nielsen et al., 2007). Further investigations should be done in RA patients without MTX treatment to clarify this point and to precise the exact mechanism of MTX in RA.

In conclusion, our findings provide evidence against the involvement of the *CASP7* rs2227309 SNP in the genetics of RA in a European Caucasian familial population. Our study is the first to show that *CASP7* isoform α (functional isoform) and isoform β (non functional isoform) mRNA were expressed in RA patients at lower level than in healthy controls in peripheral blood cells. Further investigations in the regulation of *CASP7* expression in RA are required to prove the involvement of *CASP7* in disease susceptibility, more specifically in signalling pathways of RA apoptosis.

Chapter 5

Transcriptome Analysis describing new Immunity and Defense Genes in Peripheral Blood Mononuclear Cells of Rheumatoid Arthritis Patients

Based on: Teixeira VH, Olaso R, Martin-Magniette ML, Lasbleiz S, Jacq L, Oliveira CR, Hilliquin P, Gut I, Cornelis F, Petit-Teixeira E. (2009) PLoS One. Aug 27;4(8):e6803

5.1. Abstract

Large-scale gene expression profiling of pheripheral blood mononuclesar cells (PBMCs) from RA patients could provide a molecular description that reflects the contribution of diverse cellular responses associated with this disease. The aim of our study was to identify peripheral blood gene expression profiles for Rheumatoid Arthritis (RA) patients, using Illumina technology, to gain insights into RA molecular mechanisms.

The Illumina Human-6v2 Expression BeadChips were used for a complete genome-wide transcript profiling of PBMCs from 18 RA patients and 15 controls. Differential analysis *per* gene was performed with one-way analysis of variance (ANOVA) and *P* values were adjusted to control the False Discovery Rate (FDR<5%). Genes differentially expressed at significant level between patients and controls were analyzed using Gene Ontology (GO) in the PANTHER database to identify biological processes. A differentially expression of 339 Reference Sequence genes (238 down-regulated and 101 up-regulated) between the two groups was observed. We identified a remarkably elevated expression of a spectrum of genes involved in Immunity and Defense in PBMCs of RA patients compared to controls. This result is confirmed by GO analysis, suggesting that these genes could be activated systemically in RA. No significant down-regulated ontology groups were found. Microarray data were validated by real time PCR in a set of nine genes showing a high degree of correlation.

Our study highlighted several new genes that could contribute in the identification of innovative clinical biomarkers for diagnostic procedures and therapeutic interventions.

5.2. Introduction

The multifactorial nature of RA provides high disease heterogeneity with specific combinations of a genetic background and environmental factors that influence the susceptibility, severity and outcome of the disease (Firestein, 2003). RA heterogeneity is demonstrated by the presence of distinct autoantibody specificities, like RF and ACPA in the serum (Waaler, 1940; Schellekens et al., 1998), the differential responsiveness to treatment (Lipsky et al., 2000; Edwards et al., 2004b), and the variability in clinical presentation. In addition, several gene expression profiling studies of synovial tissues and PBMCs from RA patients showed marked variation in gene expression profiles that allowed to identify distinct molecular disease mechanisms involved in RA pathology (van der Pow Kraan et al., 2007; Toonen et al., 2008). The relative contribution of the different mechanisms may vary among patients and in different stages of disease. Thus, the broad goals of expression profiling in RA are to (i) improve our understanding of the pathogenic mechanisms underlying RA, (ii) identify new drugs targets, (iii) assess activity of the disease, (iv) predict future outcomes, such as responsiveness therapy, overall disease severity, and organ specific risk and (v) develop new diagnostic tests (Baechler et al., 2006). PBMCs gene expression profiling allows both pathogenetic and pathophysiological processes identification as demonstrated in several types of diseases: cancer (Alizadeh et al., 2000), asthma (Brutsche et al., 2001), SLE (Mandel and Achiron, 2006), cardiovascular diseases (Henriksen and Kotelevtsev, 2002) and psychiatric disorders (Colangelo et al., 2002). Pathogenetic processes are primarily associated with the cause of a disease. Then, microarrays could lead to the identification of abnormal genes and gene activities that may not be only limited to PBMCs, but could occur in cells of pathological tissue as well. In contrast, pathophysiological changes in lymphocytic gene expression are considered an essentially normal reaction of the immune system to a pathological stimulus. Therefore, pathophysiological gene profiles may be shared in a variety of diseases, whereas pathogenetic gene expression is expected to be disease specific (Gladkevich et al., 2005). The differences in expression profiles provide opportunities to stratify RA patients based on molecular criteria that may require different treatment strategies. Considering several comparative studies Illumina and other microarray technologies have similar performances (Barnes et al., 2005; MAQCC, 2006; Maouche et al., 2008). However, these studies showed that each approach was able to detect specific genes, meaning an increase in knowledge by each platform. To complete previous studies on RA with other microarrays (Toonen et al., 2007), we applied Illumina large-scale gene

expression profiling in PBMCs of RA patients to potentially gain insights into molecular mechanism of this disease. We identified new genes involved in different functional Immunity and Defense related mechanisms as pro-inflammation, anti-microbial activity, cellular stress and immunomodulatory functions in RA.

5.3. Patients and methods

Study population.

The study and all protocols presented here were approved by the Ethics Committees of Bicêtre and Saint Louis Hospitals (Paris, France) and all study participants provided written informed consent. All RA patients satisfied the revised criteria of the ACR (Arnett et al., 1988) according to the rheumatologist in charge of the patient. A rheumatologist university fellow reviewed all clinical data. Characteristics of the 18 RA French Caucasian Patients are reported in Table 5.1. Among the control group consisted of 15 RA French Caucasian healthy individuals, 11 were females (mean ± Standard Deviation (SD) age at enrolment 56.9 ± 6.6). In all comparisons mentioned, the groups were age and sex-matched.

Table 5.1. Clinical and demographic characteristics of the RA patients**.**

Clinical features patients	**RA Patients (*n*=18)**
Mean age (years)	60
Women (%)	72.3
Caucasian (%)	100
RF-positive (%)	88.9
ACPA-positive (%)	90.9 (out of 11 RA patients)
Mean disease duration (years)	8.6
Erosions (%)	63.2
Disease Activity Score 28 (DAS28) mean	5.22
Disease-Modifying Anti-Rheumatic Drugs use (%)	100
Anti-TNF therapy	0

Isolation of total RNA.

Peripheral blood was drawn in PAXgene RNA isolation tubes (PreAnalytix) from 18 patients and 15 controls. Total RNA was isolated from PBMCs using the PAXgene RNA isolation kit (PreAnalytix). Total RNA yield (ng) was determined spectrophotometrically using the NanoDrop ND-1000 (Wilmington). Total RNA profiles were recorded using a Bioanalyzer 2100 (Agilent). RNA integrity numbers were determined and the mean value was 8.07+/- 0.51 SD and a Coefficient of Variation (CV) of 6.4%.

Probe synthesis, hybridization and detection.

cRNA was synthesized, amplified and purified using the Illumina TotalPrep RNA Amplification Kit (Ambion Inc.) following manufacturer recommendations. Briefly, 200 ng of RNA was reverse transcribed. After second strand synthesis, the cDNA was transcribed in vitro and cRNA labelled with biotin-16-UTP. Labelled probe hybridization to Illumina BeadChips human-6v2 was carried out using Illumina's BeadChip 6v2 protocol. These beadchips contain 48,701 unique 50-mer oligonucleotides in total, with hybridization to each probe assessed at ~30 different beads on average. 22,403 probes (46%) are targeted at Reference Sequence (RefSeq) (Pruitt et al., 2007) transcripts and the remaining 26,298 probes (54%) are for other transcripts, generally less well characterized (including predicted transcripts).

Beadchips were scanned on the Illumina BeadArray 500GX Reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). Illumina BeadStudio software (version 1.5.0.34) was used for preliminary data analysis. To assess quality metrics of each run, several quality control procedures were implemented. Total RNA control samples were analyzed with each run. The Illumina BeadStudio software was used to view control summary reports, scatter plots of the total RNA control results from different days and scatter plots of daily run samples. The scatter plots compared control against control or sample against sample and calculated a correlation coefficient (Figure 5.1).

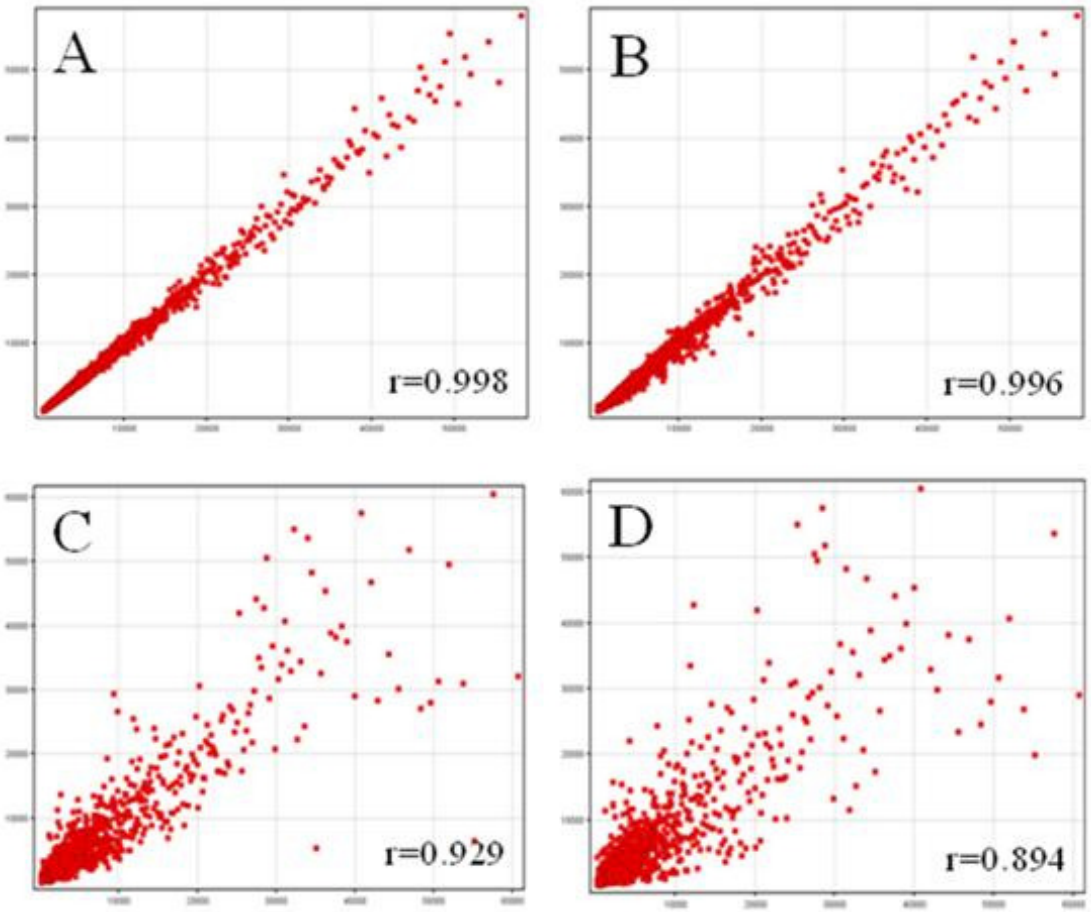

Figure 5.1. Scatter plots representation of signal intensities. Scatter plot of technical replicates: (A) same reference RNA undergoing two different hybridization or (B) same reference RNA from two different labeling runs. Typical scatter plots of data obtained from two patients (C) or a patient and a control (D). Pearson correlation coefficient is indicated in each scatter plot.

Viewing the scatter plots determined whether controls across different days varied in quality, indicating a reduction in assay performance, and highlighted those samples that were of lower quality. The control summary report is generated by the BeadStudio software, which evaluates the performance of the built-in controls of the BeadChips across particular runs. This allows the user to look for variations in signal intensity, hybridization signal, background signal and the background to noise ratio for all samples analyzed in that run. Data are expressed as log2 ratios of fluorescence intensities of the experimental and the common reference sample. The Illumina data were then normalized using the 'normalize quantiles' function in the BeadStudio Software.

All microarray data reported in this study is described in accordance with MIAME guidelines and have been deposited in the National Center for Biotechnology Information Gene

Expression Omnibus (GEO, http://www.pantherdb.org) public repository, and they are accessible through GEO accession (GSE15573).

Real-Time PCR

Total RNA was reverse transcribed using Superscript III and oligo(dT) primers (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was carried out using the SYBR-green master mix (Applied BioSystems) in an Mx 3005P thermocycler (Agilent). PCR conditions were 95 ^{o}C for 10 min, followed by 40 cycles of 95 ^{o}C for 15 sec and, 60 ^{o}C for 1 min. At the end of the amplification reaction, melting curve analyses were performed to confirm the specificity as well as the integrity of the PCR products by the presence of a single peak. Gene-specific primers were designed inside or nearby the microarray sequence targeted, using Primer Express Software (PE Applied Biosystems). Primers sequences of all genes analyzed are provided in Table 5.2. Absence of cross contamination and primer dimer was checked on genomic DNA and water. From a list of 8 housekeeping genes, we chose *HMBS* and *ALDOA* which meet the criteria of less variation between samples and compatible expression level with the studied genes. The geometric mean of housekeeping genes expression was used to normalize the expression of genes of interest (Vandesompele et al., 2002). Standard curves were generated from assays made with serial dilutions of reference cDNA to calculate PCR efficiencies (100% +/- 15%, with r^2 >=0.997). Ct samples were transformed into quantity values using the formula $(1+\text{Efficiency})^{Ct}$. Only means of triplicate with a CV of less than 10 % were analyzed. Inter-plate variation was below 8 %.

Table 5.2. Primers sequences of the nine genes analyzed by real-time PCR

Gene Symbol	Accession number	Forward primer	Reverse primer
DNMT1	NM_001379	AGCCCGAGAGAGTGCCTCA	GGCAGAACTAGTCCTTAGCAGCTT
IL2RB	NM_000878	TTCATCATCTTAGTGTACTTGCTGATCA	TCTGGACGTCTCCTCCATGC
IRF1	NM_002198	GGGTACCTACTCAATGAACCTGGA	TGTGAAGACACGCTGTAGACTCAG
LY96	NM_015364	CGAGGATCTGATGACGATTACTCTT	ATTGTTGTATTCACAGTCTCTCCCTTC
ORM1	NM_000607	CACCACCTACCTGAATGTCCAG	AGTTCTTCTCATCGTTCACGTCAA
ORM2	NM_000608	AGAATGGGACCGTCTCCAGAT	TCTTCTCATCGTCCAGGTAGGAAC
RPL31	NM_000993	CTTTCCTTCTCCCACAATCCTTC	TTCTTCTCGCCACCCTTCTTT
RUNX3	NM_004350	AGGCTCACTCAGCACCACAAG	GAATGGGTTCAGTTCCGAGGT
S100A12	NM_005621	AAAGGAGCTTGCAAACACCATC	CAGGCCTTGGAATATTTCATCAA
ALDOA	NM_000034	CTGTCACTGGGATCACCTTCCT	AGGTTGATGGACGCCTCCT
HMBS	NM_000190	ACCAAGGAGCTTGAACATGC	GAAAGACAACAGCATCATGAG

Statistical analysis.

Statistical analysis on microarray data was performed using one-way analysis of variance (ANOVA) per gene where the normalized signals are explained by the patient status. One contrast was built to determine an expression difference between controls and RA patients (Gentleman and Carey, 2002). Since the number of individuals is large, the residual variance was used to calculate the statistic test (Irizarry et al., 2003). The raw *P* values were adjusted by the Benjamini-Hochberg (1995) procedure, which controls the False Discovery Rate (FDR). For the contrast, a gene is considered differentially expressed if the Benjamini-Hochberg-corrected *P* value is less than 0.05.

Genes that were expressed at significantly different levels between patients and controls were analyzed by supervised hierarchical clustering (uncentered correlation, complete linkage) (Eisen et al., 1998) to visualize the correlation of co-expressed genes in Treeview (available at http://rana.lbl.gov/EisenSoftware.html).

For an interpretation of the biological processes that are represented by the genes that show a significantly different level of expression in RA patients compared to the controls, we applied Gene Ontology analysis in the PANTHER database at http://www.pantherdb.org (Applied Biosystems) (Mi et al., 2005). PANTHER uses the binomial statistics tool to compare our gene list to a reference list (NCBI: Homo sapiens genes) determining the statistically significant over- or under- representation of PANTHER biological process (Cho and Campbell, 2000). After, for each biological process in PANTHER, the genes associated with that term are evaluated according to the likelihood that their fold changes were drawn randomly from the overall distribution of fold changes. The Mann-Whitney U Test (Wilcoxon Rank-Sum Test) is used to determine the *P* value that, say, if specific biological process genes have random fold changes relative to the overall list of values that was input. A significant *P* value indicates that the distribution (fold change) for this category is non-random and different from the overall distribution (Clark et al., 2003). In both statistical tests, processes with a *P* value < 0.05 were considered significant after Bonferroni correction which was applied to adjust for multiple comparisons. Correlations between two set of data were measured using Pearson coefficient.

5.4. Results

Gene expression profiling in PMBCs of RA patients.

Genome-wide transcriptional profiles of PBMCs from 18 RA patients and 15 age and sex-matched controls were measured on microarrays that contain 48,701 unique 50-mer oligonucleotides in total, with a mean ~30 hybridizations *per* sequence. Data were analyzed using one-way analysis of variance (ANOVA). Using this test with a false discovery rate (FDR) of 5% we identified 380 transcripts with significant expression. The proportion of detected transcripts was substantially higher among RefSeq genes (91%) than non-RefSeq genes (9%), reflecting the greater degree of knowledge and certainty about the existence of RefSeq transcripts. Four genes, represented more than once in this list, were averaged from sequences with the same Unigene identifier. Significant difference in expression level between the two groups was observed for 339 RefSeq genes. Among them, 238 were downregulated (Table 5.3) and 101 were upregulated (Table 5.4). The significant gene expression differences between RA patients and controls were visualized in a cluster diagram (Figure 5.2).

Table 5.3. List of 238 downregulated genes differentially expressed between RA patients and controls.

Gene Symbol	Definition
AASDHPPT	Homo sapiens aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase (AASDHPPT), m
ACTG1	Homo sapiens actin, gamma 1 (ACTG1), mRNA.
ACYP1	Homo sapiens acylphosphatase 1, erythrocyte (common) type (ACYP1), transcript variant 1, mRNA.
ADFP	Homo sapiens adipose differentiation-related protein (ADFP), mRNA.
AFF4	Homo sapiens AF4/FMR2 family, member 4 (AFF4), mRNA.
AKR7A2	Homo sapiens aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase) (AKR7A2), mRNA.
ALDH9A1	Homo sapiens aldehyde dehydrogenase 9 family, member A1 (ALDH9A1), mRNA.
ANK3	Homo sapiens ankyrin 3, node of Ranvier (ankyrin G) (ANK3), transcript variant 1, mRNA.
ANXA5	Homo sapiens annexin A5 (ANXA5), mRNA.
ANXA7	Homo sapiens annexin A7 (ANXA7), transcript variant 2, mRNA.
AP4B1	Homo sapiens adaptor-related protein complex 4, beta 1 subunit (AP4B1), mRNA.
APEH	Homo sapiens N-acylaminoacyl-peptide hydrolase (APEH), mRNA.
ARHGAP17	Homo sapiens Rho GTPase activating protein 17 (ARHGAP17), transcript variant 2, mRNA.
ATIC	Homo sapiens 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC
BET1L	Homo sapiens blocked early in transport 1 homolog (S. cerevisiae)-like (BET1L), transcript variant 2, mRNA
BHLHB2	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.
BRWD2	Homo sapiens bromodomain and WD repeat domain containing 2 (BRWD2), mRNA.
C10orf61	Homo sapiens chromosome 10 open reading frame 61 (C10orf61), transcript variant 1, mRNA.
C12orf65	Homo sapiens chromosome 12 open reading frame 65 (C12orf65), mRNA.
C13orf23	Homo sapiens chromosome 13 open reading frame 23 (C13orf23), transcript variant 1, mRNA.
C14orf43	Homo sapiens chromosome 14 open reading frame 43 (C14orf43), transcript variant 1, mRNA.
C16orf58	Homo sapiens chromosome 16 open reading frame 58 (C16orf58), mRNA.
C16orf80	Homo sapiens chromosome 16 open reading frame 80 (C16orf80), mRNA.
C17orf69	Homo sapiens chromosome 17 open reading frame 69 (C17orf69), mRNA.
C20orf11	Homo sapiens chromosome 20 open reading frame 11 (C20orf11), mRNA.
C20orf72	Homo sapiens chromosome 20 open reading frame 72 (C20orf72), mRNA.
C3orf37	Homo sapiens chromosome 3 open reading frame 37 (C3orf37), transcript variant 2, mRNA.
C4orf14	Homo sapiens chromosome 4 open reading frame 14 (C4orf14), mRNA.
C5orf25	Homo sapiens chromosome 5 open reading frame 25 (C5orf25), mRNA.
C6orf136	Homo sapiens chromosome 6 open reading frame 136 (C6orf136), mRNA.
C8orf55	Homo sapiens chromosome 8 open reading frame 55 (C8orf55), mRNA.
CALM3	Homo sapiens calmodulin 3 (phosphorylase kinase, delta) (CALM3), mRNA.
CARD11	Homo sapiens caspase recruitment domain family, member 11 (CARD11), mRNA.
CCDC64	Homo sapiens coiled-coil domain containing 64 (CCDC64), mRNA.
CCDC92	Homo sapiens coiled-coil domain containing 92 (CCDC92), mRNA.
CCNC	Homo sapiens cyclin C (CCNC), transcript variant 1, mRNA.
CD74	Homo sapiens CD74 molecule, major histocompatibility complex, class II invariant chain (CD74), transcript v
CD81	Homo sapiens CD81 molecule (CD81), mRNA.
CD96	Homo sapiens CD96 molecule (CD96), transcript variant 1, mRNA.
CDC37	Homo sapiens cell division cycle 37 homolog (S. cerevisiae) (CDC37), mRNA.
CLASP2	Homo sapiens cytoplasmic linker associated protein 2 (CLASP2), mRNA.
CLEC2D	Homo sapiens C-type lectin domain family 2, member D (CLEC2D), transcript variant 1, mRNA.
CMAH	Homo sapiens cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminate mo (CMAH) on chromosome 6.
CNO	Homo sapiens cappuccino homolog (mouse) (CNO), mRNA.
COPS7B	Homo sapiens COP9 constitutive photomorphogenic homolog subunit 7B (Arabidopsis) (COPS7B), mRNA.
CRKL	Homo sapiens v-crk sarcoma virus CT10 oncogene homolog (avian)-like (CRKL), mRNA.
CRLF3	Homo sapiens cytokine receptor-like factor 3 (CRLF3), mRNA.
CRY1	Homo sapiens cryptochrome 1 (photolyase-like) (CRY1), mRNA.
CXCR3	Homo sapiens chemokine (C-X-C motif) receptor 3 (CXCR3), mRNA.
CXorf45	Homo sapiens chromosome X open reading frame 45 (CXorf45), transcript variant 1, mRNA.
DAGLA	Homo sapiens diacylglycerol lipase, alpha (DAGLA), mRNA.
DAZAP1	Homo sapiens DAZ associated protein 1 (DAZAP1), transcript variant 1, mRNA.

DAZAP2	Homo sapiens DAZ associated protein 2 (DAZAP2), mRNA.
DCK	Homo sapiens deoxycytidine kinase (DCK), mRNA.
DCTD	Homo sapiens dCMP deaminase (DCTD), transcript variant 2, mRNA.
DDX24	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 24 (DDX24), mRNA.
DDX47	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 47 (DDX47), transcript variant 1, mRNA.
DDX54	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 54 (DDX54), mRNA.
DFFB	Homo sapiens DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase) (DFFB), mRNA
DGUOK	Homo sapiens deoxyguanosine kinase (DGUOK), nuclear gene encoding mitochondrial protein, transcript var
DNAJA3	Homo sapiens DnaJ (Hsp40) homolog, subfamily A, member 3 (DNAJA3), mRNA.
DNMT1	Homo sapiens DNA (cytosine-5-)-methyltransferase 1 (DNMT1), mRNA.
DOCK9	Homo sapiens dedicator of cytokinesis 9 (DOCK9), mRNA.
DSCR3	Homo sapiens Down syndrome critical region gene 3 (DSCR3), mRNA.
E4F1	Homo sapiens E4F transcription factor 1 (E4F1), mRNA.
EDEM1	Homo sapiens ER degradation enhancer, mannosidase alpha-like 1 (EDEM1), mRNA.
EEF2	Homo sapiens eukaryotic translation elongation factor 2 (EEF2), mRNA.
EEF2K	Homo sapiens eukaryotic elongation factor-2 kinase (EEF2K), mRNA.
EIF2B1	Homo sapiens eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa (EIF2B1), mRNA.
EIF4A3	Homo sapiens eukaryotic translation initiation factor 4A, isoform 3 (EIF4A3), mRNA.
EP400	Homo sapiens E1A binding protein p400 (EP400), mRNA.
ETS1	Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1), mRNA.
EVL	Homo sapiens Enah/Vasp-like (EVL), mRNA.
FAM110A	Homo sapiens family with sequence similarity 110, member A (FAM110A), transcript variant 2, mRNA.
FAM120A	Homo sapiens family with sequence similarity 120A (FAM120A), mRNA.
FAM43A	Homo sapiens family with sequence similarity 43, member A (FAM43A), mRNA.
FBXL12	Homo sapiens F-box and leucine-rich repeat protein 12 (FBXL12), mRNA.
FBXO21	Homo sapiens F-box protein 21 (FBXO21), transcript variant 2, mRNA.
FKSG44	Homo sapiens FKSG44 gene (FKSG44), mRNA.
FLJ12716	Homo sapiens FLJ12716 protein (FLJ12716), transcript variant 1, mRNA.
FNBP1	Homo sapiens formin binding protein 1 (FNBP1), mRNA.
FOXJ3	Homo sapiens forkhead box J3 (FOXJ3), mRNA.
GATA2	Homo sapiens GATA binding protein 2 (GATA2), mRNA.
GHITM	Homo sapiens growth hormone inducible transmembrane protein (GHITM), mRNA.
GIMAP6	Homo sapiens GTPase, IMAP family member 6 (GIMAP6), transcript variant 3, mRNA.
GLTSCR1	Homo sapiens glioma tumor suppressor candidate region gene 1 (GLTSCR1), mRNA.
GMDS	Homo sapiens GDP-mannose 4,6-dehydratase (GMDS), mRNA.
GNB1	Homo sapiens guanine nucleotide binding protein (G protein), beta polypeptide 1 (GNB1), mRNA.
GNE	Homo sapiens glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (GNE), mRNA.
GPR172A	Homo sapiens G protein-coupled receptor 172A (GPR172A), mRNA.
HADH	Homo sapiens hydroxyacyl-Coenzyme A dehydrogenase (HADH), nuclear gene encoding mitochondrial prote
HDAC1	Homo sapiens histone deacetylase 1 (HDAC1), mRNA.
HEATR2	Homo sapiens HEAT repeat containing 2 (HEATR2), mRNA. XM_935824 XM_935825
HELB	Homo sapiens helicase (DNA) B (HELB), mRNA.
HERC1	Homo sapiens hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like d (HERC1), mRNA.
HNRPAB	Homo sapiens heterogeneous nuclear ribonucleoprotein A/B (HNRPAB), transcript variant 2, mRNA.
HNRPD	Homo sapiens heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa) (transcript variant 3, mRNA.
HNRPDL	Homo sapiens heterogeneous nuclear ribonucleoprotein D-like (HNRPDL), transcript variant 3, transcribed R
HNRPH1	Homo sapiens heterogeneous nuclear ribonucleoprotein H1 (H) (HNRPH1), mRNA.
HNRPR	Homo sapiens heterogeneous nuclear ribonucleoprotein R (HNRPR), mRNA.
HPS6	Homo sapiens Hermansky-Pudlak syndrome 6 (HPS6), mRNA.
IARS	Homo sapiens isoleucyl-tRNA synthetase (IARS), transcript variant short, mRNA.
IKBKB	Homo sapiens inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB), mRNA.
IL10RA	Homo sapiens interleukin 10 receptor, alpha (IL10RA), mRNA.
IL2RB	Homo sapiens interleukin 2 receptor, beta (IL2RB), mRNA.
ILF3	Homo sapiens interleukin enhancer binding factor 3, 90kDa (ILF3), transcript variant 2, mRNA.
IMP3	Homo sapiens IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast) (IMP3), mRNA.
INTS9	Homo sapiens integrator complex subunit 9 (INTS9), mRNA.
IRAK2	Homo sapiens interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA.
ITGB1	Homo sapiens integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)

	transcript variant 1D, mRNA.
JTV1	Homo sapiens JTV1 gene (JTV1), mRNA.
KCTD5	Homo sapiens potassium channel tetramerisation domain containing 5 (KCTD5), mRNA.
KIAA0182	Homo sapiens KIAA0182 (KIAA0182), mRNA.
KIAA0355	Homo sapiens KIAA0355 (KIAA0355), mRNA.
KIAA1542	Homo sapiens CTD-binding SR-like protein rA9 (KIAA1542), mRNA.
KLF10	Homo sapiens Kruppel-like factor 10 (KLF10), transcript variant 1, mRNA.
KLF13	Homo sapiens Kruppel-like factor 13 (KLF13), mRNA.
KLF2	Homo sapiens Kruppel-like factor 2 (lung) (KLF2), mRNA.
LARP1	Homo sapiens La ribonucleoprotein domain family, member 1 (LARP1), transcript variant 2, mRNA.
LCP2	Homo sapiens lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa) (LCP2), r
LEO1	Homo sapiens Leo1, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae) (LEO1), mRNA.
MAGED1	Homo sapiens melanoma antigen family D, 1 (MAGED1), transcript variant 3, mRNA.
MAT2B	Homo sapiens methionine adenosyltransferase II, beta (MAT2B), transcript variant 1, mRNA.
MDFIC	Homo sapiens MyoD family inhibitor domain containing (MDFIC), mRNA.
MED29	Homo sapiens mediator complex subunit 29 (MED29), mRNA.
METAP1	Homo sapiens methionyl aminopeptidase 1 (METAP1), mRNA.
MFNG	Homo sapiens MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase (MFNG), mRNA.
MIF4GD	Homo sapiens MIF4G domain containing (MIF4GD), mRNA.
MLLT10	Homo sapiens myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, transcript variant 1, mRNA.
MS4A7	Homo sapiens membrane-spanning 4-domains, subfamily A, member 7 (MS4A7), transcript variant 3, mRNA
MTMR12	Homo sapiens myotubularin related protein 12 (MTMR12), mRNA.
MXD4	Homo sapiens MAX dimerization protein 4 (MXD4), mRNA.
NKTR	Homo sapiens natural killer-tumor recognition sequence (NKTR), mRNA.
NUDT9	Homo sapiens nudix (nucleoside diphosphate linked moiety X)-type motif 9 (NUDT9), transcript variant 3, m
NUP62	Homo sapiens nucleoporin 62kDa (NUP62), transcript variant 2, mRNA.
NUP93	Homo sapiens nucleoporin 93kDa (NUP93), mRNA.
OPN3	Homo sapiens opsin 3 (encephalopsin, panopsin) (OPN3), mRNA.
ORAOV1	Homo sapiens oral cancer overexpressed 1 (ORAOV1), mRNA.
PACSIN1	Homo sapiens protein kinase C and casein kinase substrate in neurons 1 (PACSIN1), mRNA.
PAFAH1B1	Homo sapiens platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa (PAFAH1B1), mRN
PARP1	Homo sapiens poly (ADP-ribose) polymerase family, member 1 (PARP1), mRNA.
PAXIP1	Homo sapiens PAX interacting (with transcription-activation domain) protein 1 (PAXIP1), mRNA.
PCBP1	Homo sapiens poly(rC) binding protein 1 (PCBP1), mRNA.
PDHA1	Homo sapiens pyruvate dehydrogenase (lipoamide) alpha 1 (PDHA1), mRNA.
PDHB	Homo sapiens pyruvate dehydrogenase (lipoamide) beta (PDHB), mRNA.
PGRMC2	Homo sapiens progesterone receptor membrane component 2 (PGRMC2), mRNA.
PHACTR4	Homo sapiens phosphatase and actin regulator 4 (PHACTR4), transcript variant 1, mRNA.
PHF17	Homo sapiens PHD finger protein 17 (PHF17), transcript variant S, mRNA.
POFUT1	Homo sapiens protein O-fucosyltransferase 1 (POFUT1), transcript variant 1, mRNA.
POLS	Homo sapiens polymerase (DNA directed) sigma (POLS), mRNA.
PRKCH	Homo sapiens protein kinase C, eta (PRKCH), mRNA.
PRNP	Homo sapiens prion protein (p27-30) (Creutzfeldt-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fa insomnia) (PRNP), transcript variant 3, mRNA.
PRPSAP1	Homo sapiens phosphoribosyl pyrophosphate synthetase-associated protein 1 (PRPSAP1), mRNA.
PTBP1	Homo sapiens polypyrimidine tract binding protein 1 (PTBP1), transcript variant 1, mRNA.
PUS1	Homo sapiens pseudouridylate synthase 1 (PUS1), transcript variant 2, mRNA.
PYCR2	Homo sapiens pyrroline-5-carboxylate reductase family, member 2 (PYCR2), mRNA.
QSOX2	Homo sapiens quiescin Q6 sulfhydryl oxidase 2 (QSOX2), mRNA.
RAB11FIP3	Homo sapiens RAB11 family interacting protein 3 (class II) (RAB11FIP3), mRNA.
RAB9A	Homo sapiens RAB9A, member RAS oncogene family (RAB9A), mRNA.
RAD17	Homo sapiens RAD17 homolog (S. pombe) (RAD17), transcript variant 3, mRNA.
RBL2	Homo sapiens retinoblastoma-like 2 (p130) (RBL2), mRNA.
RCC2	Homo sapiens regulator of chromosome condensation 2 (RCC2), mRNA.
RFTN1	Homo sapiens raftlin, lipid raft linker 1 (RFTN1), mRNA.
RNF144	Homo sapiens ring finger protein 144 (RNF144), mRNA.
RNF214	Homo sapiens ring finger protein 214 (RNF214), transcript variant 1, mRNA.
RNF34	Homo sapiens ring finger protein 34 (RNF34), transcript variant 2, mRNA.
RNPS1	Homo sapiens RNA binding protein S1, serine-rich domain (RNPS1), transcript variant 2, mRNA.

RPA1	Homo sapiens replication protein A1, 70kDa (RPA1), mRNA.
RPLP2	Homo sapiens ribosomal protein, large, P2 (RPLP2), mRNA.
RTN1	Homo sapiens reticulon 1 (RTN1), transcript variant 1, mRNA.
RUNX3	Homo sapiens runt-related transcription factor 3 (RUNX3), transcript variant 2, mRNA.
SAMD3	Homo sapiens sterile alpha motif domain containing 3 (SAMD3), transcript variant 1, mRNA.
SBF1	Homo sapiens SET binding factor 1 (SBF1), transcript variant 1, mRNA.
SEPT9	Homo sapiens septin 9 (SEPT9), mRNA.
SFRS14	Homo sapiens splicing factor, arginine/serine-rich 14 (SFRS14), transcript variant 2, mRNA.
SFRS2B	Homo sapiens splicing factor, arginine/serine-rich 2B (SFRS2B), mRNA.
SFRS5	Homo sapiens splicing factor, arginine/serine-rich 5 (SFRS5), transcript variant 2, mRNA.
SH2B3	Homo sapiens SH2B adaptor protein 3 (SH2B3), mRNA.
SHMT1	Homo sapiens serine hydroxymethyltransferase 1 (soluble) (SHMT1), transcript variant 1, mRNA.
SIAHBP1	Homo sapiens fuse-binding protein-interacting repressor (SIAHBP1), transcript variant 2, mRNA.
SLBP	Homo sapiens stem-loop (histone) binding protein (SLBP), mRNA.
SLC16A11	Homo sapiens solute carrier family 16, member 11 (monocarboxylic acid transporter 11) (SLC16A11), mRNA
SLC23A2	Homo sapiens solute carrier family 23 (nucleobase transporters), member 2 (SLC23A2), transcript variant 2, r
SLC25A3	Homo sapiens solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3 (SLC25A3), nucle mitochondrial protein, transcript variant 3, mRNA.
SLC25A5	Homo sapiens solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 (SL
SMAD7	Homo sapiens SMAD family member 7 (SMAD7), mRNA.
SOX13	Homo sapiens SRY (sex determining region Y)-box 13 (SOX13), mRNA.
SPG7	Homo sapiens spastic paraplegia 7 (pure and complicated autosomal recessive) (SPG7), nuclear gene encodin protein, transcript variant 1, mRNA.
SPOCK2	Homo sapiens sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2 (SPOCK2), mRNA.
SPRYD5	Homo sapiens SPRY domain containing 5 (SPRYD5), mRNA.
SRF	Homo sapiens serum response factor (c-fos serum response element-binding transcription factor) (SRF), mRN
SRRM1	Homo sapiens serine/arginine repetitive matrix 1 (SRRM1), mRNA.
SRRM1L	PREDICTED: Homo sapiens serine/arginine repetitive matrix 1-like (SRRM1L), mRNA.
STK38	Homo sapiens serine/threonine kinase 38 (STK38), mRNA.
STX2	Homo sapiens syntaxin 2 (STX2), transcript variant 2, mRNA.
SUMO3	Homo sapiens SMT3 suppressor of mif two 3 homolog 3 (S. cerevisiae) (SUMO3), mRNA.
SYPL1	Homo sapiens synaptophysin-like 1 (SYPL1), transcript variant 1, mRNA.
TAF1L	Homo sapiens TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 210kDa-like (T
TEX261	Homo sapiens testis expressed 261 (TEX261), mRNA.
TFG	Homo sapiens TRK-fused gene (TFG), transcript variant 2, mRNA.
TH1L	Homo sapiens TH1-like (Drosophila) (TH1L), transcript variant 2, mRNA.
THAP11	Homo sapiens THAP domain containing 11 (THAP11), mRNA.
THRAP4	Homo sapiens thyroid hormone receptor associated protein 4 (THRAP4), transcript variant 1, mRNA.
TJAP1	Homo sapiens tight junction associated protein 1 (peripheral) (TJAP1), mRNA.
TMEM109	Homo sapiens transmembrane protein 109 (TMEM109), mRNA.
TMEM43	Homo sapiens transmembrane protein 43 (TMEM43), mRNA.
TMEM5	Homo sapiens transmembrane protein 5 (TMEM5), mRNA.
TMEM87A	Homo sapiens transmembrane protein 87A (TMEM87A), mRNA.
TNFSF5IP1	Homo sapiens tumor necrosis factor superfamily, member 5-induced protein 1 (TNFSF5IP1), mRNA.
TNPO1	Homo sapiens transportin 1 (TNPO1), transcript variant 2, mRNA.
TRIAD3	Homo sapiens TRIAD3 protein (TRIAD3), transcript variant 1, mRNA.
TRRAP	Homo sapiens transformation/transcription domain-associated protein (TRRAP), mRNA.
TSHZ1	Homo sapiens teashirt zinc finger homeobox 1 (TSHZ1), mRNA.
TSPYL1	Homo sapiens TSPY-like 1 (TSPYL1), mRNA.
TTF2	Homo sapiens transcription termination factor, RNA polymerase II (TTF2), mRNA.
U2AF2	Homo sapiens U2 small nuclear RNA auxiliary factor 2 (U2AF2), transcript variant 1, mRNA.
UBAC2	Homo sapiens UBA domain containing 2 (UBAC2), mRNA.
UBE2Q1	Homo sapiens ubiquitin-conjugating enzyme E2Q (putative) 1 (UBE2Q1), mRNA.
UBXD8	Homo sapiens UBX domain containing 8 (UBXD8), mRNA.
UVRAG	Homo sapiens UV radiation resistance associated gene (UVRAG), mRNA.
WDR33	Homo sapiens WD repeat domain 33 (WDR33), transcript variant 3, mRNA.
WDR37	Homo sapiens WD repeat domain 37 (WDR37), mRNA.
WDR4	Homo sapiens WD repeat domain 4 (WDR4), transcript variant 2, mRNA.
WDR59	Homo sapiens WD repeat domain 59 (WDR59), mRNA.
WDR67	Homo sapiens WD repeat domain 67 (WDR67), mRNA.

WRNIP1	Homo sapiens Werner helicase interacting protein 1 (WRNIP1), transcript variant 1, mRNA.
XPNPEP1	Homo sapiens X-prolyl aminopeptidase (aminopeptidase P) 1, soluble (XPNPEP1), mRNA.
YEATS2	Homo sapiens YEATS domain containing 2 (YEATS2), mRNA.
ZC3H7A	Homo sapiens zinc finger CCCH-type containing 7A (ZC3H7A), mRNA.
ZCCHC14	Homo sapiens zinc finger, CCHC domain containing 14 (ZCCHC14), mRNA.
ZFAND5	Homo sapiens zinc finger, AN1-type domain 5 (ZFAND5), mRNA.
ZFP36	Homo sapiens zinc finger protein 36, C3H type, homolog (mouse) (ZFP36), mRNA.
ZMYND8	Homo sapiens zinc finger, MYND-type containing 8 (ZMYND8), transcript variant 1, mRNA.
ZNF207	Homo sapiens zinc finger protein 207 (ZNF207), transcript variant 2, mRNA.
ZNF212	Homo sapiens zinc finger protein 212 (ZNF212), mRNA.
ZNF330	Homo sapiens zinc finger protein 330 (ZNF330), mRNA.
ZNF664	Homo sapiens zinc finger protein 664 (ZNF664), mRNA.
ZNF696	Homo sapiens zinc finger protein 696 (ZNF696), mRNA.

Table 5.4. List of 101 upregulated genes differentially expressed between RA patients and controls.

Gene Symbol	Definition	Transcript Identifier
ABCA1	Homo sapiens ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), mRNA.	NM_005502.2
ABCA7	Homo sapiens ATP-binding cassette, sub-family A (ABC1), member 7 (ABCA7), mRNA.	NM_019112.3
AIF1	Homo sapiens allograft inflammatory factor 1 (AIF1), transcript variant 3, mRNA.	NM_001623.3
ANKRD22	Homo sapiens ankyrin repeat domain 22 (ANKRD22), mRNA.	NM_144590.2
ARG1	Homo sapiens arginase, liver (ARG1), mRNA.	NM_000045.2
ATG10	Homo sapiens ATG10 autophagy related 10 homolog (S. cerevisiae) (ATG10), mRNA.	NM_031482.3
ATP5J	Homo sapiens ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6 (ATP5J), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	NM_001003703.1
ATP6V0E1	Homo sapiens ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e1 (ATP6V0E1), mRNA.	NM_003945.3
ATP6V1D	Homo sapiens ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D (ATP6V1D), mRNA.	NM_015994.2
BAG4	Homo sapiens BCL2-associated athanogene 4 (BAG4), mRNA.	NM_004874.2
BLOC1S1	Homo sapiens biogenesis of lysosome-related organelles complex-1, subunit 1 (BLOC1S1), mRNA.	NM_001487.1
BUD31	Homo sapiens BUD31 homolog (S. cerevisiae) (BUD31), mRNA.	NM_003910.2
C14orf2	Homo sapiens chromosome 14 open reading frame 2 (C14orf2), mRNA.	NM_004894.1
C16orf7	Homo sapiens chromosome 16 open reading frame 7 (C16orf7), mRNA.	NM_004913.2
C19orf59	Homo sapiens chromosome 19 open reading frame 59 (C19orf59), mRNA.	NM_174918.2
C5orf32	Homo sapiens chromosome 5 open reading frame 32 (C5orf32), mRNA.	NM_032412.3
CAMP	Homo sapiens cathelicidin antimicrobial peptide (CAMP), mRNA.	NM_004345.3
CBS	Homo sapiens cystathionine-beta-synthase (CBS), mRNA.	NM_000071.1
CBX3	Homo sapiens chromobox homolog 3 (HP1 gamma homolog, Drosophila) (CBX3), transcript variant 2, mRNA.	NM_016587.2
CCDC72	Homo sapiens coiled-coil domain containing 72 (CCDC72), mRNA.	NM_015933.3
CITED4	Homo sapiens Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 (CITED4), mRNA.	NM_133467.2
CKLF	Homo sapiens chemokine-like factor (CKLF), transcript variant 5, mRNA.	NM_001040138.1
CNOT1	Homo sapiens CCR4-NOT transcription complex, subunit 1 (CNOT1), transcript variant 2, mRNA.	NM_206999.1
COX6A1	Homo sapiens cytochrome c oxidase subunit VIa polypeptide 1 (COX6A1), nuclear gene encoding mitochondrial protein, mRNA.	NM_004373.2
COX7A2	Homo sapiens cytochrome c oxidase subunit VIIa polypeptide 2 (liver) (COX7A2), mRNA.	NM_001865.2
COX7C	Homo sapiens cytochrome c oxidase subunit VIIc (COX7C), nuclear gene encoding mitochondrial protein, mRNA.	NM_001867.2
CPEB3	Homo sapiens cytoplasmic polyadenylation element binding protein 3 (CPEB3), mRNA.	NM_014912.3
DDEF1	Homo sapiens development and differentiation enhancing factor 1 (DDEF1), mRNA.	NM_018482.2
DDIT3	Homo sapiens DNA-damage-inducible transcript 3 (DDIT3), mRNA.	NM_004083.4
DKFZp761E198	Homo sapiens DKFZp761E198 protein (DKFZp761E198), mRNA.	NM_138368.3
ERH	Homo sapiens enhancer of rudimentary homolog (Drosophila) (ERH), mRNA.	NM_004450.1
F5	Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA.	NM_000130.4

FBN2	Homo sapiens fibrillin 2 (congenital contractural arachnodactyly) (FBN2), mRNA.	NM_001999.3
GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA.	NM_002046.3
GDPD3	Homo sapiens glycerophosphodiester phosphodiesterase domain containing 3 (GDPD3), mRNA.	NM_024307.2
GLRX	Homo sapiens glutaredoxin (thioltransferase) (GLRX), mRNA.	NM_002064.1
GMFG	Homo sapiens glia maturation factor, gamma (GMFG), mRNA.	NM_004877.1
GTF2B	Homo sapiens general transcription factor IIB (GTF2B), mRNA.	NM_001514.3
HK3	Homo sapiens hexokinase 3 (white cell) (HK3), nuclear gene encoding mitochondrial protein, mRNA.	NM_002115.1
HNMT	Homo sapiens histamine N-methyltransferase (HNMT), transcript variant 2, mRNA.	NM_001024074.1
ING4	Homo sapiens inhibitor of growth family, member 4 (ING4), mRNA.	NM_016162.2
JARID1B	Homo sapiens jumonji, AT rich interactive domain 1B (JARID1B), mRNA.	NM_006618.3
KCTD20	Homo sapiens potassium channel tetramerisation domain containing 20 (KCTD20), mRNA.	NM_173562.3
KIAA1530	Homo sapiens KIAA1530 protein (KIAA1530), mRNA.	NM_020894.1
LHFPL2	Homo sapiens lipoma HMGIC fusion partner-like 2 (LHFPL2), mRNA.	NM_005779.2
LOC432369	Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit pseudogene 2 (LOC432369) on chromosome 13.	NR_002162.1
LRRFIP1	Homo sapiens leucine rich repeat (in FLII) interacting protein 1 (LRRFIP1), mRNA.	NM_004735.2
LSM10	Homo sapiens LSM10, U7 small nuclear RNA associated (LSM10), mRNA.	NM_032881.1
LSM3	Homo sapiens LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae) (LSM3), mRNA.	NM_014463.1
LY96	Homo sapiens lymphocyte antigen 96 (LY96), mRNA.	NM_015364.2
MRPL33	Homo sapiens mitochondrial ribosomal protein L33 (MRPL33), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	NM_004891.3
MRPS18C	Homo sapiens mitochondrial ribosomal protein S18C (MRPS18C), nuclear gene encoding mitochondrial protein, mRNA.	NM_016067.1
MSL3L1	Homo sapiens male-specific lethal 3-like 1 (Drosophila) (MSL3L1), transcript variant 1, mRNA.	NM_078629.1
MXD3	Homo sapiens MAX dimerization protein 3 (MXD3), mRNA.	NM_031300.2
MYL6	Homo sapiens myosin, light chain 6, alkali, smooth muscle and non-muscle (MYL6), transcript variant 1, mRNA.	NM_021019.3
MYL6B	Homo sapiens myosin, light chain 6B, alkali, smooth muscle and non-muscle (MYL6B), mRNA.	NM_002475.3
NBEAL2	Homo sapiens neurobeachin-like 2 (NBEAL2), mRNA.	NM_015175.1
NCF4	Homo sapiens neutrophil cytosolic factor 4, 40kDa (NCF4), transcript variant 2, mRNA.	NM_013416.2
NDUFA1	Homo sapiens NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, nuclear gene encoding mitochondrial protein, mRNA.	NM_004541.2
NDUFB3	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa (NDUFB3), mRNA.	NM_002491.1
NFAT5	Homo sapiens nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5), transcript variant 5, mRNA.	NM_173215.1
NRD1	Homo sapiens nardilysin (N-arginine dibasic convertase) (NRD1), mRNA.	NM_002525.1
NUFIP2	Homo sapiens nuclear fragile X mental retardation protein interacting protein 2 (NUFIP2), mRNA.	NM_020772.1
NUP214	Homo sapiens nucleoporin 214kDa (NUP214), mRNA.	NM_005085.2
ORM1	Homo sapiens orosomucoid 1 (ORM1), mRNA.	NM_000607.1
ORM2	Homo sapiens orosomucoid 2 (ORM2), mRNA.	NM_000608.2
PGLYRP1	Homo sapiens peptidoglycan recognition protein 1 (PGLYRP1), mRNA.	NM_005091.1
PHF20L1	Homo sapiens PHD finger protein 20-like 1 (PHF20L1), transcript variant 2, mRNA.	NM_032205.3

POLE4	Homo sapiens polymerase (DNA-directed), epsilon 4 (p12 subunit) (POLE4), mRNA.	NM_019896.2
PPP2R3C	Homo sapiens protein phosphatase 2 (formerly 2A), regulatory subunit B", gamma (PPP2R3C), mRNA.	NM_017917.2
PTGES3	Homo sapiens prostaglandin E synthase 3 (cytosolic) (PTGES3), mRNA.	NM_006601.4
PTRH2	Homo sapiens peptidyl-tRNA hydrolase 2 (PTRH2), nuclear gene encoding mitochondrial protein, mRNA.	NM_016077.3
RAB24	Homo sapiens RAB24, member RAS oncogene family (RAB24), transcript variant 2, mRNA.	NM_130781.1
RBP7	Homo sapiens retinol binding protein 7, cellular (RBP7), mRNA.	NM_052960.1
RNASE2	Homo sapiens ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) (RNASE2), mRNA.	NM_002934.2
RNASE3	Homo sapiens ribonuclease, RNase A family, 3 (eosinophil cationic protein) (RNASE3), mRNA.	NM_002935.2
RRAGD	Homo sapiens Ras-related GTP binding D (RRAGD), mRNA.	NM_021244.3
S100A12	Homo sapiens S100 calcium binding protein A12 (S100A12), mRNA.	NM_005621.1
S100A8	Homo sapiens S100 calcium binding protein A8 (S100A8), mRNA.	NM_002964.3
S100A9	Homo sapiens S100 calcium binding protein A9 (S100A9), mRNA.	NM_002965.3
SAMD4B	Homo sapiens sterile alpha motif domain containing 4B (SAMD4B), mRNA.	NM_018028.2
SCNM1	Homo sapiens sodium channel modifier 1 (SCNM1), mRNA.	NM_024041.2
SF3B14	Homo sapiens splicing factor 3B, 14 kDa subunit (SF3B14), mRNA.	NM_016047.3
SLC11A1	Homo sapiens solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (SLC11A1), mRNA.	NM_000578.3
SLC22A17	Homo sapiens solute carrier family 22 (organic cation transporter), member 17 (SLC22A17), transcript variant 2, mRNA.	NM_016609.3
SLPI	Homo sapiens secretory leukocyte peptidase inhibitor (SLPI), mRNA.	NM_003064.2
SRPK1	Homo sapiens SFRS protein kinase 1 (SRPK1), mRNA.	NM_003137.3
SSH1	Homo sapiens slingshot homolog 1 (Drosophila) (SSH1), mRNA.	NM_018984.2
STX10	Homo sapiens syntaxin 10 (STX10), mRNA.	NM_003765.1
STX6	Homo sapiens syntaxin 6 (STX6), mRNA.	NM_005819.4
SULT2B1	Homo sapiens sulfotransferase family, cytosolic, 2B, member 1 (SULT2B1), transcript variant 2, mRNA.	NM_177973.1
TCEB2	Homo sapiens transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B) (TCEB2), transcript variant 1, mRNA.	NM_007108.2
THEM2	Homo sapiens thioesterase superfamily member 2 (THEM2), mRNA.	NM_018473.2
TLR5	Homo sapiens toll-like receptor 5 (TLR5), mRNA.	NM_003268.4
TMCC3	Homo sapiens transmembrane and coiled-coil domain family 3 (TMCC3), mRNA.	NM_020698.1
TMEM97	Homo sapiens transmembrane protein 97 (TMEM97), mRNA.	NM_014573.2
TNPO1	Homo sapiens transportin 1 (TNPO1), transcript variant 2, mRNA.	NM_153188.2
TXN	Homo sapiens thioredoxin (TXN), mRNA.	NM_003329.2
UBL5	Homo sapiens ubiquitin-like 5 (UBL5), transcript variant 2, mRNA.	NM_001048241.1
VPS25	Homo sapiens vacuolar protein sorting 25 homolog (S. cerevisiae) (VPS25), mRNA.	NM_032353.2
ZNF148	Homo sapiens zinc finger protein 148 (ZNF148), mRNA.	NM_021964.2

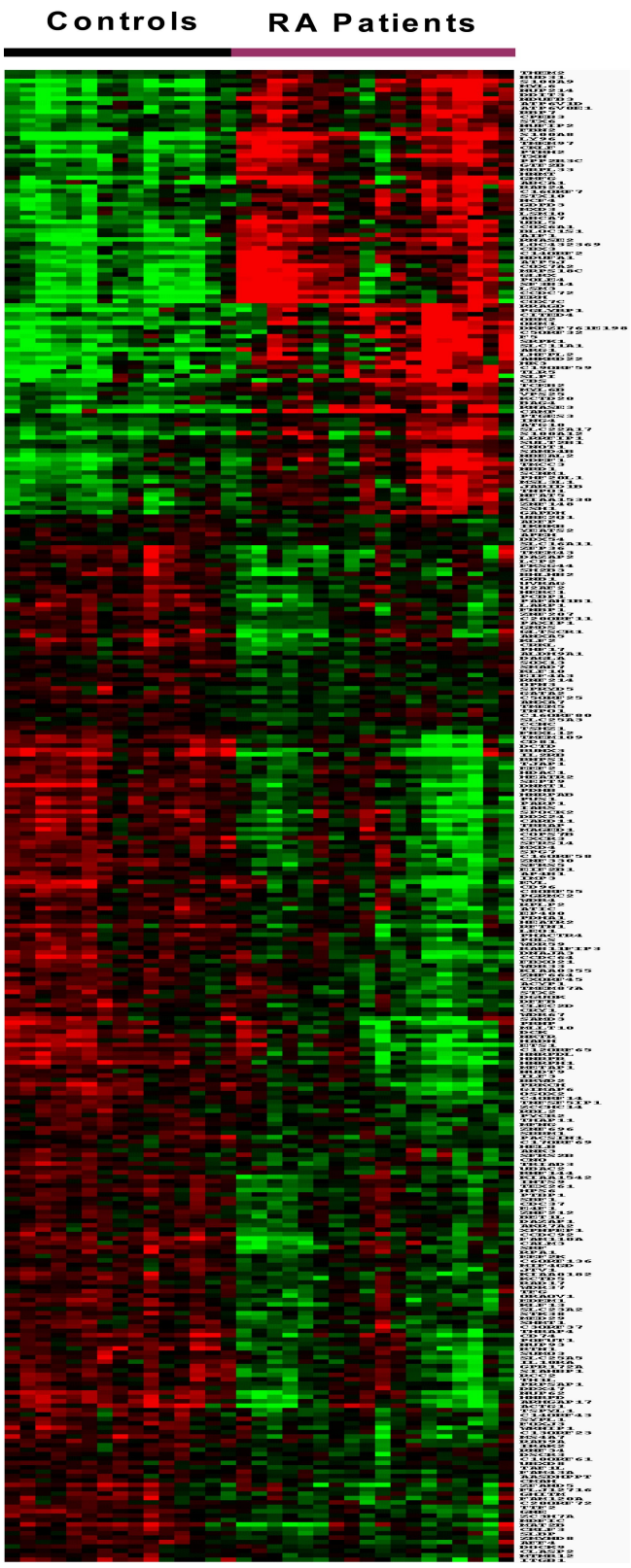

Figure 5.2. Cluster diagram of the expression of 339 significantly expressed genes in 18 RA patients and 15 controls. Genes are organized by hierarchical clustering based on overall similarity in expression patterns. Red represents relative expression greater than the median expression level across all samples, and green represents an expression level lower than the median. Black indicates intermediate expression.

Genes upregulated in RA

To categorize the up-regulated 101 genes into functional biological groups we used the PANTHER database (described in Methods section). We observed an elevated expression of a spectrum of genes involved in Immunity and Defense, nucleoside, nucleotide and nucleic acid metabolism, signal transduction, protein metabolism and modification, mRNA transcription, transport and developmental processes in the peripheral blood of RA patients compared to controls. Compared to a NCBI Homo sapiens reference list, the differentially up-regulated genes list revealed three biological processes significantly over represented (P<0.05) (Figure 5.3.A). Then, the fold change of the genes associated to each biological process was compared to the overall distribution of fold changes. Immunity and Defense was the only significant functional biological process after Bonferroni correction (P=0.03) (Figure 5.3.B).

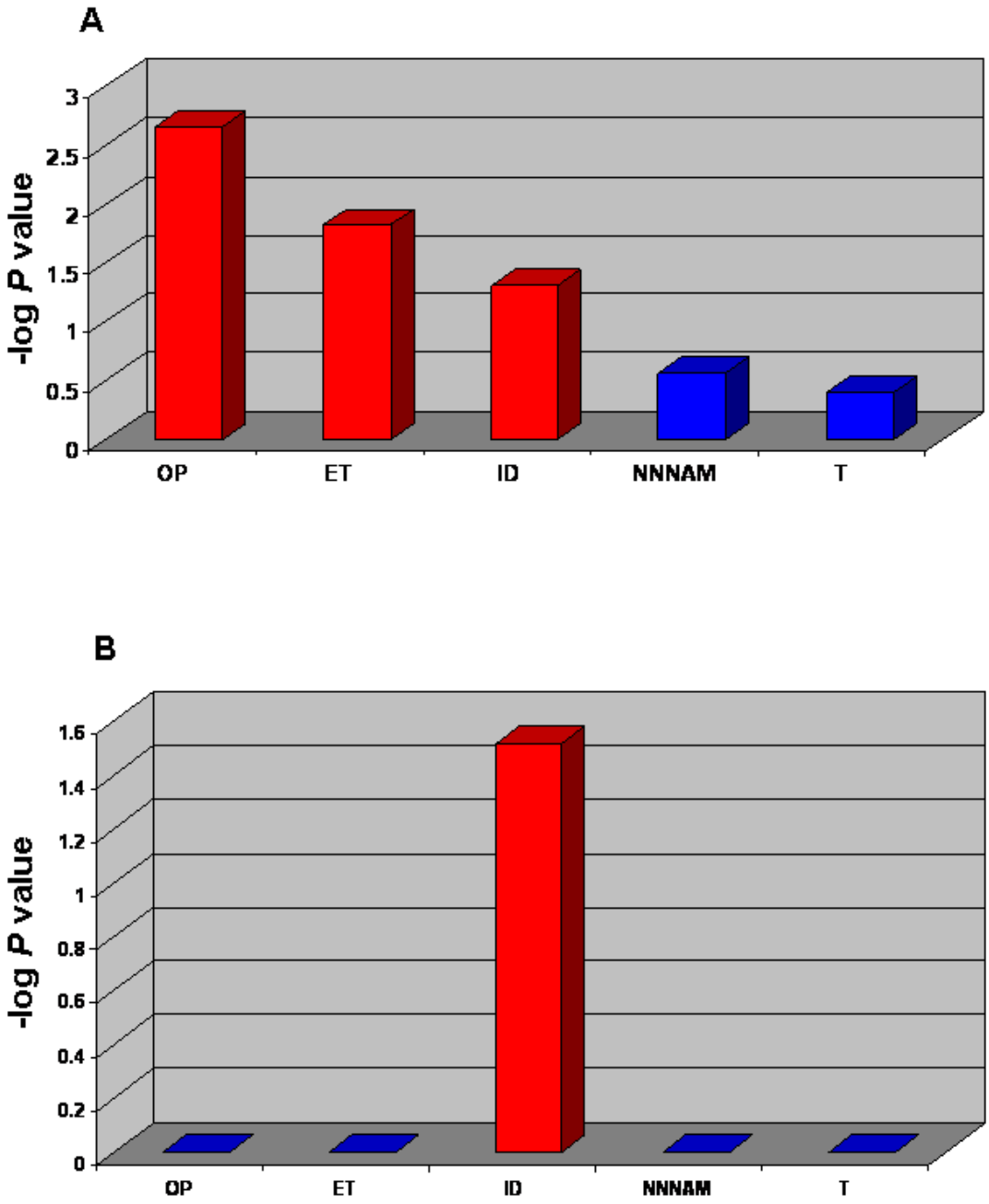

Figure 5.3. Gene ontology analysis of the most representative biological processes represented by the 101 up-regulated genes in RA patients. Gene Ontology (GO) analysis in the PANTHER database was applied for an interpretation of the biological processes that are represented by the genes showing a higher significantly different expression level in RA patients compared to the controls. A and B - Biological processes with a P value < 0.05 (red bars) were considered significant after Bonferroni correction. The GO analysis P-value was plotted on the y axis versus biological processes on the x axis. A - The binomial statistics tool was used to compare our gene list to a reference list (NCBI: Homo sapiens genes) determining the statistically significant (P value) over- or under- representation of PANTHER biological process. B - The Mann-Whitney U Test was used to determine significant P values which indicate that the distribution (fold change) for each biological process is non-random and different from the overall distribution. OP - Oxidative Phosphorylation; ET - Electron Transport; ID - Immunity and Defense; NNNAM - Nucleoside, Nucleotide and Nucleic Acid Metabolism; T – Transport.

This cluster of genes involved in Immunity and Defense process contains the S100 family proteins S100 calcium-binding protein A8 (*S100A8*), *S1000A9* and *S100A12*, the orosomucoid family proteins *ORM1* and *ORM2*, as well as other inflammatory mediators like lymphocyte antigen 96 (*LY96*), cathelicidin antimicrobial peptide (*CAMP*), thioredoxin (*TXN*), allograft inflammatory factor 1 (*AIF1*), nuclear factor of activated T cells 5 (*NFAT5*), *F5* (coagulation factor V), *SLC11A1* (solute carrier family 11, member 1) and *PGLYRP1* (peptidoglycan recognition protein 1). These genes are characterized in Table 5.5.

Table 5.5. Differentially up-regulated transcripts linked to Immunity and Defense biological process in RA patients.

Gene Bank	Name	Genome Location	GeneID (NCBI)
S100A8	S100 calcium binding protein A8	1q21	6279
S100A9	S100 calcium binding protein A9	1q21	6280
S100A12	S100 calcium binding protein A12	1q21	6283
AIF1	Allograft inflammatory factor 1	6p21.3	199
TXN	Thioredoxin	9q31	7295
NFAT5	Nuclear factor of activated T cells 5, tonicity-responsive	16q22.1	10725
CAMP (LL37)	Cathelicidin antimicrobial peptide	3p21.3	820
LY96 (MD-2)	Lymphocyte antigen 96	8q21.11	23643
ORM1	Orosomucoid 1	9q31-q32	5004
ORM2	Orosomucoid 2	9q32	5005
SLC11A1 (NRAMP1)	Solute carrier family 11, member 1	2q35	6556
PGLYRP1	Peptidoglycan recognition protein 1	19q13.2-q13.3	8993
F5	Coagulation factor V	1q23	2153

Genes downregulated in RA.

Compared to the same NCBI Homo sapiens reference list, the mainstream of the genes that showed a lower expression in RA patients are linked to different biological processes such as nucleoside, nucleotide and nucleic acid metabolism, mRNA transcription and regulation, cell cycle, intracellular protein traffic. A small number of down-regulated genes were also involved in oncogenesis like runt-related transcription factor 3 (*RUNX3*), SMAD family member 7 (*SMAD7*), PHD finger protein 17 (*PHF17*) as well as interleukin-1 receptor-associated kinase 2 (*IRAK2*), interleukin 2 receptor, beta (*IL2RB*), *CD96* and SH2B adaptor protein 3 (*SH2B3*) related to immune functions. Therefore, in PANTHER database classification we found four significant functional biological processes in our 238 down-regulated genes list. However, no significant biological process was found (after Bonferroni correction) when we evaluate all the down-regulated genes associated to biological processes, according with their fold changes drawn randomly from the overall distribution of fold changes (data not shown).

Real time PCR validation.

In all samples, we confirmed the expression of five up-regulated genes (*LY96*, *S100A12*, *ORM2*, *ORM1*, *RPL31)* and four down regulated genes (*IL2RB*, *DNMT1*, *RUNX3* and *IRF1*) in RA patients, by real-time PCR. From microarray and real time PCR data, we calculated the RA patients/controls ratio for each genes expression. The qPCR expression data of the nine genes showed a high correlation with the microarray expression data (r = 0.937) (Figure 5.4).

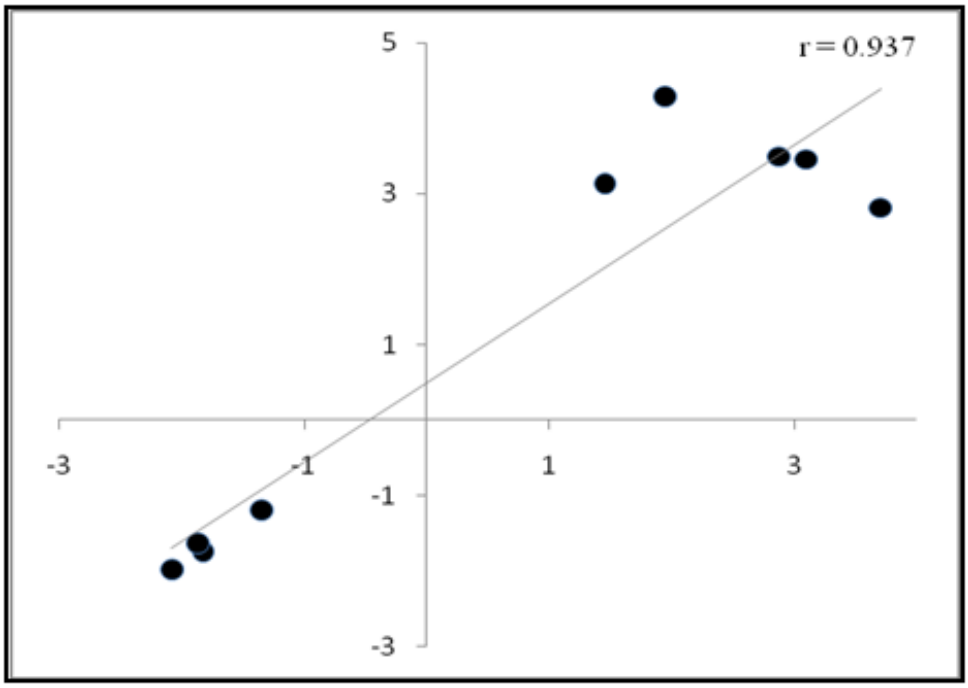

Figure 5.4. Correlation between microarray and real time PCR data. The scatter plot compares mean expression of RA patients/controls ratio for nine genes. Each point represents the RA patients/controls ratio from the microarray (y axis) and real time PCR (x axis). Pearson correlation coefficient is indicated in the scatter plot.

5.5. Discussion

Microarray technology has been used to discriminate differences in gene expression profiles in tissues and PBMCs. Both synovial tissue and PBMCs have been used to evaluate differences in the gene expression profiles in RA (van der Pow Kraan et al., 2007; Toonen et al., 2008). If expression-based profiling is to be of practical importance, sample accessibility becomes crucial. In this context, peripheral mononuclear cells are key sentinels of host defence, being used to identify novel disease mediators, disease variants and treatment responses (Toonen et al., 2008; Yamagata et al., 2006).

Transcriptome studies using Illumina and other technologies showed that each approach was able to detect specific genes, meaning an increase in knowledge by each platform (Barnes et al., 2005; MAQCC, 2006; Maouche et al., 2008). To complete previous studies on RA with Affymetrix or double colour microarrays, we decided to use Illumina technology. Our study did not confirm a specific expression for the genes regulated by interferon type I, as described in a RA large-scale expression profiling (van der Pow Kraan et al., 2007) (data not shown). Our analysis revealed only one significantly increased biological mechanism: Immunity and Defense. This process was already highlighted by other studies in RA, as several genes that we described (*S100A8*, *S1000A9*, *S100A12* and *AIF1*). Additionnally, our

study identified new genes like *LY96/MD-2*, *NFAT5*, *TXN*, *CAMP/LL37*, *ORM1*, *ORM2*, *SLC11A1*, *PGLYRP1* and *F5*. These genes are involved in different functional Immunity and Defense related mechanisms as pro-inflammation, anti-microbial activity, oxidative and osmotic cellular stress and immunomodulatory functions (Figure 5.5).

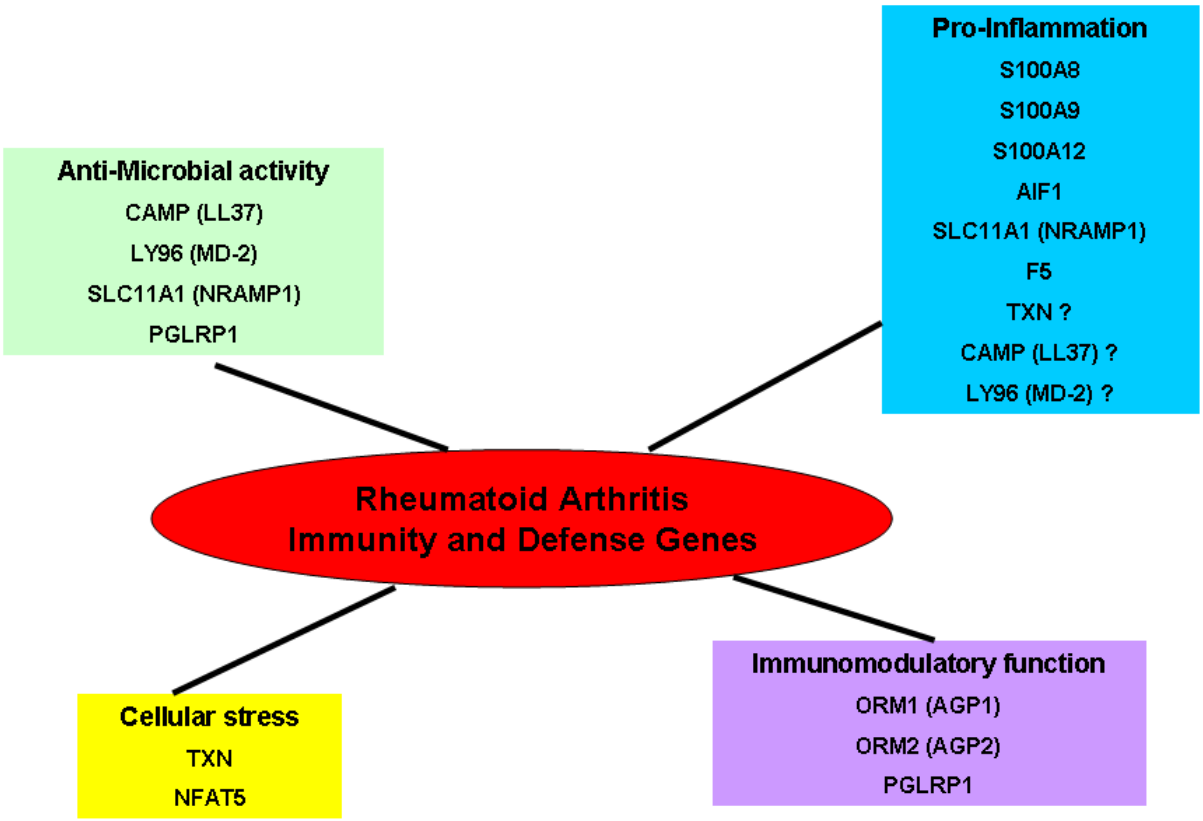

Figure 5.5. Biological functions of Immunity and Defense genes highlighted in our RA study. The genes differentially expressed in Immunity and Defense process are stratified in four functional related mechanisms: pro-inflammation, anti-microbial activity, oxidative and osmotic cellular stress and immunomodulatory functions.

The S100 calcium-binding proteins (S100A8, S100A9, and S100A12) are characterized by strong prevalence in cells of myeloid origin. Activated phagocytes expressing S100A8 and S100A9 proteins are among the first cells infiltrating inflammatory lesions in the synovium (Foell and Roth, 2004). The expression of S100A8 and S100A9 was found to be strongest at the cartilage–pannus junction, which is the prime site of cartilage destruction and bone erosion in arthritis (Youssef et al., 1999). S100A12 is strongly expressed in inflamed synovial tissue, whereas it is nearly undetectable in synovia of control subjects or patients after successful treatment (Foell et al., 2003). Further studies showed increased levels of S100A8/S100A9 and S100A12 concentrations in synovial fluid (SF) and serum in RA patients (Frosch et al., 2000; Kane et al., 2003; Rouleau et al., 2003). In addition, S100A9,

S100A8 and S100A12 levels were associated with body mass index, presence of ACPA and RF and presence of ACPA, respectively (Chen et al., 2009). Furthermore, several expression profiling studies in PBMCs showed a highly expression of S100A8, S100A9 and S100A12 in RA patients compared to controls (Bovin et al., 2004; Batliwalla et al., 2005; van der Pow Kraan et al., 2007). Therefore, pro-inflammatory S100 proteins are attractive therapeutic targets for immune interventions in the treatment of RA.

Human allograft inflammatory factor-1 (AIF-1) is a Ca^{2+}-binding EF-hand protein encoded within the MHC class III region of chromosome 6. AIF-1 is produced by macrophages and lymphocytes, and its synthesis is mediated by several cytokines, such as IFN-γ (Utans et al., 1995). Kimura and colleagues (2007) have demonstrated that AIF-1 is expressed in synovial and mononuclear cells in RA synovial tissue and increases proliferation of cultured synoviocytes and IL-6 production by these synoviocytes and PBMCs. A recent study, showed an increased expression of *AIF1* mRNA in RA patients PMBCs compared with controls as well as in synovial macrophages in the lining layer of all the inflamed RA synovial membranes compared with non-inflamed OA controls (Harney et al., 2008). AIF-1 plays therefore an important role in the pathogenesis of RA by affecting key processes such as the activation of synovial cell proliferation and the inflammatory cytokine cascade including IL-6 in joints and may represent a new molecular target in RA therapy.

The LY96 (MD-2) acts as an extracellular adaptor protein in the activation of TLR4 by the LPS of Gram negative bacteria, a well-known inducer of the innate immune response (Viriyakosol et al., 2001). Knockout studies in mice have demonstrated that MD-2 is indispensable for LPS responses (Nagai et al., 2002). Multiple evidences points to the potential importance of TLR signaling in RA pathogenesis by means of the presence of different TLR ligands and functional TLR receptors in inflamed joints from patients with RA (Brentano et al., 2005). TLR4 mRNA is highly expressed in the synovium at early stages of RA as well as at later stages of the disease. *In vitro* stimulation of the RA synovial fibroblasts with TLR4 ligand (LPS) produces a wide range of proinflammatory cytokines, chemokines, and tissue destructive enzymes (Ospelt et al., 2008). Recently, the potential value of TLR4 or signals derived from this receptor as therapeutic targets has become clearer—mainly as a result of studies in animal models of joint inflammation but also in human RA (O'Neill, 2008). Interestingly, in our study, mRNA *TLR4* was up-regulated in RA patients compared to controls but not significantly. Thus, the TLR4-MD2-LPS complex could be involved in the activation of synovial fibroblasts and contribute to the development of synovial inflammation and joint destruction.

NFAT5, the primordial member of the NFAT family, is expressed by almost all cells and is activated in response to osmotic stress. In lymphocytes, NFAT5 controls the osmotic stress-induced expression of several cytokines, including tumour-necrosis factor (TNF) and LTβ. *NFAT5*-deficient mice have impaired T-cell function under hyperosmotic conditions and decreased cellularity of the thymus and spleen (Macian, 2005). In RA, *NFAT5* mRNA is expressed in proliferating RA-SFs but not in nonproliferating RA-SFs. Furthermore, *NFAT5* mRNA is expressed in RA synovium - but not in normal individuals - as well as at sites of bone destruction. *NFAT5* could be then related not only with proliferation but also with the activation and invasion of RA-SFs *in vivo* (Masuda et al., 2002).

Oxidative stress to essential cell components caused by oxygen free radicals is generally considered as a serious mechanism in RA pathogenicity (Filippin et al., 2008). Thioredoxin (TXN), a cellular reducing catalyst induced by oxidative stress, is involved in the stimulation of the DNA-binding activity of NF-κB transcription factor (Matthews et al., 1992). Increased cytokine production driven by NF-κB can enhance expression of vascular adhesion molecules that attract leucocytes into the joint, as well as matrix MMPs that help to degrade the ECM (Filippin et al., 2008). TXN concentrations were found significantly elevated in SF and serum of RA patients. The positive correlation between the SF TXN and the serum C Reactive Protein in the absence of a high concentration of SF TNF-α may indicate that TXN is involved in the prolongation and persistence of the RA inflammation, because high concentrations of TXN could stimulate NF-κB activation in the presence of the otherwise insufficient concentration of TNF-α (Yoshida et al., 1999). Thus, TXN monitoring in RA patients could provide useful information regarding the extent of oxidative stress. Furthermore, truncated thioredoxin (Txn80) stimulates monocytes/macrophages to induce IL-12, implying that it is involved in immune inflammatory reactions directing Th1 immunity and IFN-γ production (Pekkari et al., 2001). Moreover, Kim and colleagues (2008) have demonstrated that human TXN is a novel target gene induced by IFN-γ. RA is a Th1-driven disease which has IFN-γ as characteristic Th1 cytokine and subsequently the *TXN* mRNA up-regulation in PBMCs could suggest an involvement in RA IFN-γ pathway.

CAMP (LL37) is an antimicrobial peptide which has a broad of antimicrobial activity. LL-37 has the potential to participate in the innate immune response both by killing bacteria and by recruiting a cellular immune response (Gallo et al., 2002). Gilliet and Lande (2008) recently found that LL37, overexpressed in Ps, is the key mediator of plasmacytoid dendritic cells (pDCs) activation in PS. LL37 converts nonstimulatory self-DNA into a potent trigger of pDCs to produce IFN. pDCs respond to self-DNA if coupled with an antimicrobial peptide,

suggesting that modified self-DNA drives autoimmunity in Ps by activating TLR9. In RA, recent studies have described pDCs as perpetuators of synovial inflammation and modulators of B cell responses in the synovial tissue (Lebre and Tak, 2009). The mRNA up-regulation of the LL37 gene found in RA patients comparing to controls in our study could be due to an immunological response to infectious agents such as bacteria and viruses. Moreover the LL37 over-expression could be implicated in a pDCs-dependent mechanism involved in the perpetuation of RA inflammation through the abolition of self-tolerance and subsequent emergence of self-reactive lymphocytes.

Human alpha-1-acid glycoprotein (AGP)—also called orosomucoid—is a 37-kDa molecule consisting of a heavily glycosylated single polypeptide chain. Alpha-1-acid glycoprotein 1 (AGP1) and alpha-1-acid glycoprotein 2 (AGP2), coded by *ORM1* and *ORM2* genes respectively, are positive acute phase proteins (Yuasa et al., 1997; Ceciliani and Pocacqua, 2007). AGPs plasma concentration may increase several fold during acute phase reactions such as inflammation or chronic disease. AGPs have a strong immunomodulatory function (Hochepied et al., 2003). It was shown that under pathological conditions not only the total concentration of AGPs but its glycosylation pattern may be altered (Ceciliani and Pocacqua, 2007). Smith and colleagues have demonstrated that the AGPs populations in the serum and synovial fluid of RA patients are distinct in terms of glycosylation pattern. This discovery has direct functional significance since only the serum AGPs population is capable of blocking leucocyte adhesion (Smith et al., 2002). Furthermore, Haston et al. have shown that AGPs can influence MMP-13 activity. It is hypothesized that AGPs may form part of a negative feedback mechanism which is inadequate to prevent disease progression in RA. These processes may exacerbate the increased turnover of collagen characteristic of the disease (Haston et al., 2003). These results suggest an interesting role for AGP in RA pathogenesis.

SLC11A1 (formerly called *NRAMP1*) is a gene that is important in macrophage-mediated natural resistance to a variety of intracellular pathogens. Exogenous and endogenous agents that mediate inflammation by activating the macrophage can cause NRAMP1 translocation to the membrane of the phagolysosome, where it serves as a cation transporter. The significant increase in iron deposition observed in the synovial membrane of RA patients, and foam cells in atherosclerotic lesions, could be attributable to NRAMP1 (Awomoyi, 2007). In human RA synovium NRAMP1 was detected in macrophages and neutrophils in the linning and subinitimal zone, as well as in inflammatory infiltrates, but was absent in fibroblasts (Telfer and Brock, 2002). NRAMP1 has also pleiotropic effects on macrophage function, including

upregulation of chemokine/cytokine gene, TNFα, IL-1β, inducible nitric oxide synthase (iNOS), MHC expression as well as tumoricidal and antimicrobial activity. These effects are involved in resistance to infection and may also be involved in induction and maintenance of autoimmune disease (Chu et al., 1991; Blackwell, 1996). TNFα and IL-1β play important roles in inflammation and tissue destruction of RA (Miossec et al., 1986; Chu et al., 1991). Bacterial or viral infection may play a role in triggering the development of RA and TNFα and iNOS are key players in enhanced antimicrobial activity of activated macrophages (Blackwell, 1996).

PGRPs are innate immunity proteins, recognizing bacterial PG, and acting in antibacterial immunity. PGRP-1 seems to belong to the innate immune arm of effectors molecules, such as antimicrobial peptides and C-type lectins, among others. This protein was shown to be almost exclusively present as a soluble protein in the granules of polymorphonuclear leucocytes (PMN) (Boneca, 2009). Saha and colleagues (2009) examined the immunomodulating activities of the PGRPs in a PG-induced arthritis mice model. They showed that a systemic injection of PG or muramyldipeptide (MDP) induces an acute arthritis of the joints of the feet in BalbC mice. PG-induced arthritis PGLYRP-1$^{-/-}$ mice, had a MDP-induced activation of proinflammatory genes than WT mice. Moreover, PGLYRP-1$^{-/-}$ mice have longer-lasting MDP-induced arthritis than WT mice. The anti-inflammatory function of PGLYRP-1 manifests itself only in the later stages of MDP-induced arthritis, which is consistent with the local release of PGLYRP-1 from PMN granules after PMNs' arrival into the mice foot. These data point to that PGRP-1 could have a specialized but nevertheless significant role in signalling events like arthritis in mammals.

The multi-step coagulation complex system is activated by tissue factor (TF), which is exposed to blood. In this process, factor 5 (FV) is cleaved and activates factor Va (FVa). After several proteins interactions, the prothrombinase complex (FXa–FVa) converts prothrombin to thrombin which is generated in a large amount. Thrombin activates FV and FVIII and platelets converting fibrinogen to a fibrin clot (Dahlbäck and Villoutreix, 2005). Accumulation of fibrin in the RA synovium exceeds that in control tissue by a wide margin and represents one of the most striking pathologic features of rheumatoid synovitis. For some time, this fibrin deposition has been considered to be a serious contributor to permanent damage by maintaining a vicious circle of inflammation (Barnhart et al., 1967; Weinberg et al., 1991). In the extravascular coagulation at the arthritic synovial joint sequential activity of factor Xa (in the presence of cofactor Va) and of thrombin leads to fibrin deposition in the joint (Busso and Hamilton, 2002). Thus, mRNA *F5* up-regulation in RA patients could

enhance the production of factor V that under cleavage produces factor Va. This Va increase could lead to the augmentation of thrombin and consequently fibrin in RA joints.

In conclusion, our study highlighted several new genes (*LY96/MD-2, NFAT5*, *TXN, CAMP/LL37, ORM1*, *ORM2*, *SLC11A1*, *PGLYRP1* and *F5)* in PBMCs of RA patients that could contribute in the identification of innovative clinical biomarkers for diagnostic procedures and therapeutic interventions. Nevertheless, comparative analysis with another disease involving an inflammatory process could clarify the relation between the expression profiling and the pathophysiological processes specifically involved in RA.

Chapter 6

Associations between genetic factors, tobacco smoking and autoantibodies in familial and sporadic Rheumatoid Arthritis

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6.1. Abstract

The objective of this study was to investigate the association between genes (*HLA-DRB1* and *PTPN22*) and tobacco smoking, separately as well as combined, and serological markers of Rheumatoid Arthritis (RA) in a French population with RA.

274 patients with RA with half of them belonging to RA multicase families were genotyped for *HLA-DRB1* allele and for *PTPN22*-1858 polymorphism. Reumatoid factor (RF) and antibodies to citrullinated protein antigen (ACPA) were determined by ELISA method. The search for association relied on χ^2 test and Odd ratio (OR) with 95% confident interval (CI) calculation. The interaction study relied on the departure-from-additivity-based method.

The presence of at least one *HLA* shared epitope (SE) allele was associated with ACPA presence (82.5% vs. 68.4%, *P*=0.02), particularly with *HLA-DRB1**0401 allele (28.0% vs. 16.4%, *P*=0.01). Tobacco exposure was associated with ACPA, but only in presence of SE. A tendency toward an interaction was found between tobacco, the presence of at least one *HLA-DRB1**0401 allele and ACPA (attributable proportion due to interaction = +0.24 (-20.21+0.76)). The cumulative dose of cigarette smoking was correlated with ACPA titres (r=0.19, *P*=0.04). The presence of both SE and 1858T alleles was associated with a higher, but not significantly different, risk for ACPA presence than for each separately. No association was found between *PTPN22*-1858T allele and tobacco smoking for autoantibody positivity.

Our findings suggest an association between SE alleles and tobacco smoking for ACPA positivity and a tendency toward an interaction between the *HLA-DRB1**0401 allele and smoking for ACPA positivity in this sample of RA.

6.2. Introduction

RA pathogenesis is multifactorial, involving both genetic and environmental factors. Although the association of some *HLA-DRB1* alleles with RA was reported nearly three decades ago, the underlying biological mechanism of this association remains unknown. The presence of the RAA sequence at positions 72-74 of the HLA-DR b chain molecule, for all *HLA-DRB1* alleles known to be associated with RA, led to the SE hypothesis (Gregersen et al., 1987). The modelisation of the SE component in RA has recently been successful (du Montcel et al., 2005; Michou et al., 2006).

RF production in RA is generally associated with a more severe phenotype of the disease. RF production has been associated with carriage of SE alleles. Furthermore, several publications showed that the presence of ACPA together with SE allele carriage was associated with a very high RR for future development of RA (van Gaalen et al., 2004; Berglin et al., 2004). These reports finally led to the hypothesis that SE alleles contribute to the RA only by the development of ACPA and do not independently constitute a risk factor of RA (Huizinga et al., 2005; de Vries et al., 2005; van der Helm-van Mil et al., 2007). Moreover, the combination of the *PTPN22*-1858T variant, another genetic factor RF positive RA-associated and RA-linked, and ACPA was recently reported to give a much higher RR for developing RA than the combination of the *PTPN22*-1858T variant and *HLA* SE (Johansson et al., 2005).

Numerous environmental risk factors have been studied in RA, but tobacco smoking is to date the only well-established environmental risk factor (Pedersen et al., 2006). A gene–environment interaction between tobacco smoking and SE alleles was reported to increase the risk of RF-positive RA (Stolt et al., 2003; Padyukov et al., 2004). More recently, a model has been proposed in which smoking, in the context of SE alleles may trigger specific immune reactions to citrullinated proteins (Klareskog et al., 2006). However, smoking seems to be a risk factor for ACPA production restricted to patients with RA who carry SE alleles (Linn-Rasker et al., 2006). No association was reported between *PTPN22*-1858T variant and tobacco exposure in RA.

Here, we tested the hypothesis of an association between genes (*HLA-DRB1* and *PTPN22*) and tobacco smoking, separately as well as combined, and serological markers of RA in a French population with familial and sporadic RA.

6.3. Patients and Methods

Patients

We studied 274 patients with RA of French Caucasian origin as defined for each of the four grand-parents, 196 patients being index cases of trio families, ie, one patient with RA and both parents and 78 patients being index cases of affected sibling pair families. The diagnosis of RA fulfilled the 1987 ACR criteria (Arnett et al., 1988). All individuals provided informed written consent and the study was approved by the Bicêtre Hospital ethics committee. In this sample of patients with RA, 89% were females, 47% belonged to multicase families with RA, and at the time of serum collection, the mean age of RA onset was 34 (SD 11) years and the mean disease duration was 12 (SD 9) years. Erosions were present in 82% of the patients and rheumatoid nodules in 19%.

Genotyping

Blood samples were collected for DNA extraction and genotyping. *HLA-DRB1* typing was performed with the polymerase chain reaction sequence specific primer method using the Dynal Classic SSP DR low resolution and the Dynal Classic high resolution SSP for subtyping of *HLA-DRB1**01, *04, *11, *13 and *15 alleles (Dynal Biotech). SE alleles were *HLA-DRB1**0101, *0102, *0401, *0404, *0405, *0408 and *1001. Alleles were then classified into three groups, S2, S3P and L, according to their 70-74 amino acid sequence, as previously reported (du Montcel et al., 2005; Michou et al., 2006). Genotyping of the *PTPN22*-1858T/C variant was performed by polymerase chain reaction–restriction fragment length polymorphism, as previously reported (Dieudé et al., 2005) This T variant eliminated a restriction site for RsaI enzyme, and genotypes were secondly checked by a polymerase chain reaction–restriction fragment length polymorphism using the XcmI enzyme for which a restriction site was created when the T allele was present.

Autoantibody status

Autoantibody status IgM RF was provided by ELISA method (QUANTA, Lite RF IgM, INOVA diagnostics), and the ACPA status was provided by an anti-CCP antibody ELISA (Immunoscan RA, Euro-Diagnostic). Both ELISA tests were performed on the same serum sample according to the manufacturer's instructions.

Tobacco exposure

Tobacco exposure Information about tobacco exposure was collected by using a questionnaire sent to each patient with RA (Stolt et al., 2003). The following questions about smoking were asked: (1) Do you smoke? (2) If not, did you ever smoke? (3) In which year did you start smoking? (4) In which year did you stop smoking? (5) What sort of tobacco did you smoke: cigarettes, cigars, pipes? (6) Average number of cigarettes smoked per day: 1-5, 6-9, 10-19, ≥20 cigarettes? (7) Duration in years of smoking exposure: <10, 10-19, ≥20 years? Unanswered questionnaires were completed by telephone. Patients with RA who were smokers at the year of the serum collection were considered as current smokers, those who reported that they were smokers and stopped smoking before the year of serum collection were defined as ex-smokers. Current smokers and ex-smokers were defined as ever smokers, and patients with RA who reported they had never smoked were defined as never smokers. The cumulative dose of cigarettes smoked was then expressed as pack-year, one pack year being the equivalent of 20 cigarettes smoked per day for 1 year.

Statistical analysis

Statistical analysis to search for association between exposure to genetic factors (SE alleles and/or *PTPN22*-1858T allele) and/ or tobacco smoking with RF or ACPA positivity, relied on χ^2 or Fisher's exact test when appropriate, OR and 95% CI with 2x2 or 2x3 tables. In order to search for biological interaction, we used the departure from-additivity-based method. Attributable proportion (AP) due to interaction was determined by $Ap = (R_{11}-R_{10}-R_{01}+R_{00})/R_{11}$; AP and 95% CI were calculated with the SYSTAT program (Hosmer and Lemeshow, 1992; Rothman and Greenland, 1998). The correlation study between the cumulative dose of cigarette smoking expressed in pack-years and the ACPA titres relied on a correlation test. Finally, the search for an association between exposure to genetic factors (SE alleles and/or *PTPN22*-1858T allele) and/or tobacco smoking with RF or ACPA positivity was performed with the same statistical tests as the global sample, in the subgroup of familial RA and in the subgroup of sporadic RA.

6.4. Results

Rheumatoid arthritis sample characteristics

A total of 218 (79.6%) patients with RA carried at least one SE allele within 88 patients homozygous for SE alleles, whereas 89 (32.6%) patients with RA carried at least one T allele

of *PTPN22*-1858 variant within nine patients homozygous for the *PTPN22*-1858T allele. A total of 190 (69%) patients with RA were RF positive and 217 (79%) patients had ACPA, and 172 (63%) were both RF and ACPA positive. Among the 243 (89%) patients with RA for whom the information about tobacco exposure was known, 122 (50%) were ever smokers, 43 (35%) being current smokers and 79 (65%) being ex-smokers. There were significantly more men in the subgroup of patients exposed to tobacco (18.0% vs. 1.6%, $P=2x10^{25}$).

Association between autoantibody status and SE alleles

RF and SE alleles

The presence of at least one SE allele in the genotype was not associated with RF-positive RA [81.0% RF positive vs. 76.0% RF negative, OR=1.3 (0.7 to 2.5)]. The hierarchy of the genotype risk in the RF-positive subgroup was different from that previously reported in the literature, S2/S2 being the most at risk genotype followed by S2/S3P and S3P/S3P genotypes. The distribution of *HLA-DRB1* alleles was similar in RF-positive and -negative patients with RA.

ACPA and SE alleles

The presence of at least one SE allele in the genotype was significantly associated with ACPA positive [82.5% in ACPA positive vs. 68.4% in ACPA negative, OR=2.2 (1.2 to 4.2)], particularly for the subgroup of patients homozygous for SE [OR=5.2 (2.0 to 13.6)] (Table 6.1). The hierarchy of the genotype risk in the ACPA positive subgroup was similar to that previously reported in the literature, although 95% CI largely overlapped (Table 6.1). The distribution of *HLA-DRB1* alleles was similar in patients with RA who were ACPA positive and negative, except for *HLA-DRB1**0401 allele [28.0% of *HLA-DRB1**0401 alleles in ACPA positive vs. 16.4% in ACPA negative, OR=1.9 (1.1 to 3.3)].

Table 6.1. Association between ACPA and SE alleles.

	ACPA +	ACPA -	OR (95% CI)
SE/SE	81	7	5.2 (2.0-13.6)
SE/X	98	32	1.1 (0.7-2.9)
X/X	38	18	1 (-)
S2/S3P	54	4	5.8 (1.8-18.6)
S2/S2	14	2	2.8 (0.6-13.6)
S3P/S3P	13	1	4.3 (0.5-35.6)
S2/L	46	11	1.9 (0.8-4.6)
S3P/L	52	21	1.2 (0.5-2.5)
L/L	38	18	1 (-)

S2 = *HLA-DRB1**0401, *1303; S3P = *HLA-DRB1**0101, *0102, *0404, *0408, *1001; L = all other *HLA-DRB1* alleles.

Association between autoantibody status, SE alleles and cigarette smoking

No effect of tobacco exposure on RF status was observed. Tobacco exposure was significantly associated with ACPA occurrence, but only in the presence of SE alleles [OR=2.9 (1.2 to 7.4)] (Table 6.2.A). We found a negative, but not statistically significant interaction between those factors, AP = -0.83 (-1.75, +0.09). We identified an association between tobacco exposure and *HLA-DRB1**0401 allele (*0401) for the presence of ACPA (Table 6.2.B), and a tendency toward a positive but not statistically significant interaction between those factors, AP = +0.24 (-0.21, +0.76).

Table 6.2. OR and 95% CI for developing ACPA in the presence of tobacco exposure (TE) and SE alleles.

(A) TE	SE	ACPA+	ACPA-	OR (95% CI)
-	-	15	11	1 (-)
+	-	21	5	2.9 (0.8-10.1)
-	+	78	17	3.3 (1.3-8.5)
+	+	77	19	2.9 (1.2-7.4)
(B) TE	***0401**	**ACPA+**	**ACPA-**	**OR (95% CI)**
-	-	40	18	1 (-)
+	-	51	18	1.3 (0.6-2.7)
-	+	52	10	2.3 (0.9-5.5)
+	+	48	6	3.4 (1.2-9.4)

Association between ACPA status and cigarette smoking

Tobacco exposure was not associated with RF positivity nor with ACPA positivity. However, the risk for ACPA positivity increased with the number of years of smoking (Table 6.3) and was statistically significant at ≥20 years of tobacco exposure [OR=3.7 (1.1 to 12.8)].

Table 6.3. ORs with 95% CI for developing ACPA antibodies in rheumatoid arthritis for ever-smokers compared with never-smokers by duration of smoking.

No. of years of smoking	ACPA+	ACPA-	OR (95% CI)
< 10	27	11	0.7 (0.3-1.6)
10-19	29	10	0.8 (0.4-2.0)
≥ 20	42	3	3.7 (1.1-12.8)
Never smokers	93	28	1 (–)

The risk for ACPA also increased with the number of cigarettes smoked per day, rising from less than 1 for <10 cigarettes per day to 3.3 when ≥20 cigarettes were smoked (Table 6.4).

Table 6.4. ORs with 95% CI for developing ACPA in RA for ever-smokers compared with never-smokers by intensity of smoking.

No. of cigarettes smoked a day	ACPA+	ACPA-	OR (95% CI)
1 to 5	32	10	0.9 (0.4-2.1)
6 to 9	17	6	0.8 (0.3-2.3)
10 to 19	33	7	1.4 (0.5-3.4)
≥ 20	16	1	3.3 (0.4-26.4)
Never smokers	93	28	1 (-)

Finally, the risk for ACPA increased up to 4.2 for a cumulative dose of cigarettes smoked ≥20 pack-years (Table 6.5). Indeed, we observed a correlation between the cumulative dose of smoked cigarettes expressed in pack-years, and ACPA antibody titres (r=0.19, *P*=0.04).

Table 6.5. OR with 95% CI for developing ACPA in RA for ever-smokers compared with never-smokers by cumulative dose of smoking

No. of pack-years	ACPA+	ACPA-	OR (95% CI)
< 10	52	19	0.8 (0.4-1.6)
10 to 19	26	4	1.8 (0.6-5.6)
≥ 20	20	1	4.2 (0.5-32.4)
Never smokers	93	28	1 (-)

Association between autoantibodies, tobacco exposure, SE and *PTPN22*-1858C/T genotype

We did not find any association between *PTPN22*-1858T allele and tobacco exposure neither for RF nor for ACPA positivity. We observed that the presence of both genetic factors, ie, SE and *PTPN22*-1858T alleles, was associated with a higher, but not statistically significant, risk to develop ACPA [OR=2.9 (1.2 to 7.1)] than for each genetic factor separately (Table 6.6.A). A negative but not statistically significant interaction between those factors was observed, AP = -0.27 (-1.08, +0.54). Considering only the *HLA-DRB1**0401 allele among the SE alleles, the risk for ACPA was similar in the presence of both genetic factors (*HLA-DRB1**0401 and *PTPN22*-1858T alleles) or in the presence of *HLA-DRB1**0401 allele alone, as 95% CI largely overlapped (Table 6.6.B), and a negative but not statistically significant interaction was observed, AP = -0.15 (-0.91, +0.60).

Table 6.6. OR and 95% CI for developing ACPA in the presence of SE alleles and/or 1858T allele of *PTPN22* gene.

(A) SE	1858T	ACPA +	ACPA-	OR (95% CI)
-	-	26	15	1 (-)
+	-	116	27	2.5 (1.2-5.3)
-	+	12	3	2.1 (0.5-8.6)
+	+	62	12	2.9 (1.2-7.1)
(B) *0401	**1858T**	**ACPA +**	**ACPA-**	**OR (95% CI)**
-	-	73	31	1 (-)
+	-	69	11	2.6 (1.2-5.5)
-	+	34	9	1.5 (0.7-3.6)
+	+	40	6	2.7 (1.0-6.9)

The search for association between genes, tobacco smoking and autoantibody positivity remained not significantly different in the subgroup of familial RA (78 affected sibling pairs families and 51 trio families with at least one first- or second-degree relative affected by RA). In the 145 trio families without any family history of RA, the presence of at least one SE allele in the genotype was significantly associated with ACPA positivity [OR=2.7 (1.1 to 6.2)], particularly for the subgroup of patients homozygous for SE [OR=14.5 (1.7 to 120.2)], and the presence of both genetic factors, ie, SE and *PTPN22*-1858T alleles, was associated with a higher, but not statistically significant, risk to develop ACPA [OR=4.8 (1.4 to 16.2)] than for each genetic factor separately.

6.5. Discussion

In this study, we aimed at evaluating the association between the two RA genetic factors (SE and *PTPN22*-1858T alleles), and/ or tobacco exposure and autoantibody (RF and ACPA) positivity in a French population with familial and sporadic RA. We failed to identify any association between RF and SE, nor between RF and tobacco smoking. We observed that the

presence of at least one SE allele was associated with ACPA presence (82.5% vs. 68.4%, *P*=0.02), particularly with *HLA-DRB1**0401 allele (28.0% vs. 16.4%, *P*=0.01). Tobacco exposure was significantly associated with ACPA, but only in the presence of SE. The hierarchy of *HLA-DRB1* genotype risk was respected in the ACPA-positive subgroup, probably because of the high prevalence of the *HLA-DRB1** 0401 allele in this population. A tendency toward a positive but not statistically significant interaction was observed between tobacco, the presence of at least one *HLA-DRB1** 0401 allele and ACPA (AP=+0.24 (-0.21, +0.76)). The risk for ACPA positivity in RA index cases increased as the number of cigarettes smoked per day increased and as the number of years of smoking increased. The cumulative dose of cigarette smoking was correlated with ACPA titres (r=0.19, *P*=0.04). The presence of both SE and *PTPN22*-1858T alleles was associated with a higher, but not significantly different, risk to develop ACPA than for each genetic factor separately. But this increased risk disappeared when considering only the *HLA-DRB1**0401 allele within the SE. No association was found between *PTPN22*-1858T allele and tobacco smoking for autoantibody positivity. Here, we found an association between the ACPA positivity, tobacco exposure and SE alleles, particularly with the *HLA-DRB1**0401 allele. Moreover the ACPA titres were correlated with the intensity of the tobacco exposure, suggesting a strong effect of this environmental factor on autoantibody production, through a gene-environment association. This gene-environment association was not observed for *PTPN22*-1858T allele. However, the *PTPN22* gene encodes a protein that is not involved in antigene recognition and this gene has a minor effect on RA susceptibility in comparison with SE; a larger sample size should be required to observe such a gene-environment association. Surprisingly, we failed to identify any association between RF and ACPA presence and the *PTPN22*-1858 T allele. Indeed, Dieudé and colleagues (2005) reported linkage to and association with this allele and the RF positivity in trio families. This difference may be explained by the fact that, in this study, we pooled these trio families and the affected sibling pairs families in an exposed-not exposed study. Furthermore RF status was determined by an ELISA test for IgM on a serum sample collected at the inclusion in the genetic study, whereas in the previous study, RF was considered as positive when at least one RF-positive result (determined by latex fixation, or Waaler-Rose assay or by laser nephelometry) was observed during the disease course. Although the sample size was limited in this study, our findings of association between SE, ACPA and tobacco exposure were similar to those already reported in the literature (Stolt et al., 2003). Recently, gene-gene and gene-environment interactions in RA were compared in three large case-control studies (Klareskog et al., 2006). This article reported an interaction between SE and *PTPN22*-1858T

alleles for developing ACPA-positive RA, and the absence of an interaction between smoking and the *PTPN22*-1858T allele. The association between tobacco exposure, ACPA and *HLA-DRB1**0401 allele is interesting as the citrullination of peptides such as vimentin selectively increased their binding to HLA-DR molecules containing the SE motif (Klareskog et al., 2006). Indeed, Hill and colleagues (2003b) reported that *HLA-DRB1**0401 transgenic mice had a stronger immune response to citrullinated peptides than to native arginine containing peptides.

In this study, we did not find any association between tobacco exposure, SE alleles and RF positivity although this association was previously reported in the literature. This observation could be due to the long RA duration in this sample and the possible disappearance of the RF during the evolution of the disease, and maybe to the fact that we chose to test only IgM RF and not IgA RF. Moreover the sample size is rather small to study the interaction between genetic factors and environmental factors, such as tobacco smoking. Replication studies with larger sample size of unselected patients with RA should be required before clinical application. Finally, it should be of great interest to go further by performing immunological studies to investigate the functional interaction between tobacco exposure, ACPA and *HLA-DRB1**0401 allele, and to determine which component of the tobacco smoke should be responsible for such an autoimmune reaction.

In conclusion, our findings suggest an association between SE alleles and tobacco smoking for ACPA positivity and a tendency toward an interaction between the *HLA-DRB1**0401 allele and smoking in the development of ACPA positivity in this French population with familial and sporadic RA.

Chapter 7

Conclusions/Perspectives

Rheumatoid Arthritis is a chronic inflammatory polyarthritis that can affect any synovial-lined diarthrodial joint. The typical natural history of RA is one of progressive articular damage leading to joint deformities and disability. The development of autoimmune multifactorial diseases like RA depends on the interaction between genetic background and a number of environmental factors.

Although the etiology remains unsolved, genetic factors have a substantial impact on susceptibility to RA. Multiple loci contribute to the genetic risk for this disease. The *HLA* locus is the most important of these and accounts for 30% to 50% of overall genetic susceptibility to RA. Outside the *HLA* locus, the strongest association identified to date is with a polymorphism in *PTPN22* gene. Recent studies have identified five additional RA risk loci (*TRAF1*-C5, 6q23, *STAT4,* 4q27 and *CTLA4*).

However, the biological mechanism associated to these genetic risk factors remains unknown. Therefore, the research of new RA genetic factors associated is crucial to an improved understanding of the disease physiopathology and to search new therapeutic targets.

In addition, several gene expression profiling studies of PBMCs from RA patients showed marked variation in gene expression profiles that allowed identifying distinct molecular disease mechanisms involved in RA pathology. Thus, this approach can improve the understanding of the pathogenic mechanisms underlying RA.

Numerous environmental risk factors have been studied in RA, but tobacco smoking is to date the only well-established environmental risk factor. Smoking was shown in several studies to be a risk factor for the rheumatoid factor-positive or ACPA-positive subset of rheumatoid arthritis and to have no or a very minor effect on the autoantibody-negative subset. Furthermore, a major environment interaction was noted between *HLA-DR* risk alleles and smoking in patients who were positive for RF or ACPA. Consequently, these data from genetic epidemiological studies need biological explanations for the combined effects of genetic and environmental risk factors.

The aim of this thesis was the identification and characterization of candidate genes in Rheumatoid Arthritis using both different genetics and molecular approaches represented in Figure 8.1.

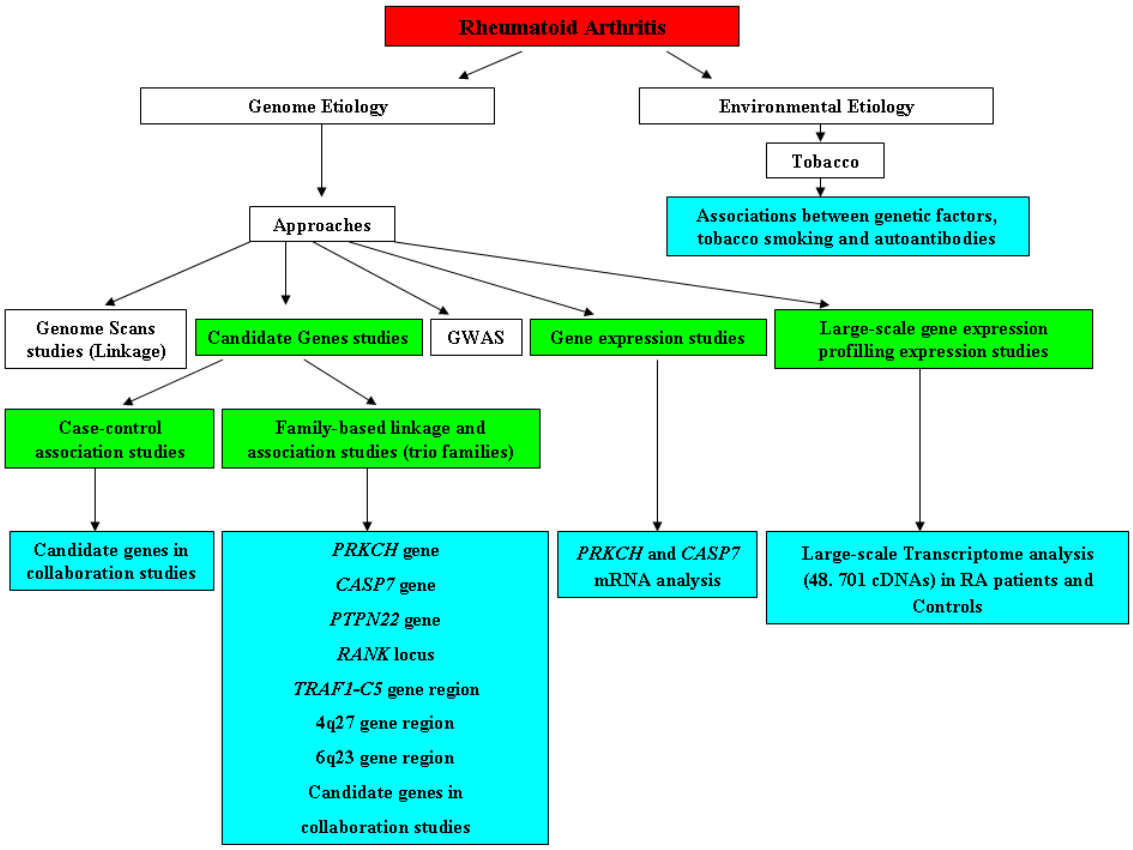

Figure 8.1. Genetics and molecular approaches used to identify and characterize factors linked to RA genome and environmental etiology. Green squares - Genetics and molecular approaches; Blue squares – Purpose of RA studies.

The genetic approach used in this thesis was based in candidate gene family-based association and linkage studies using RA trio families from French and West European origin. Given that case-controls association studies compare the frequencies in patients versus healthy individuals, unknown biases in control frequencies may lead to spurious associations. Thus, family-based association studies remain important to definitively establish association and linkage, especially when small effect sizes as well as variability in allele frequency in different populations are observed. Furthermore, trio families contain additional information on haplotype phase compared to unrelated individuals, in the form of constraints imposed by the rules of Mendelian inheritance.

Using this genetic approach, we have confirmed the association and linkage of *TRAF1-C5* and 6q23 genes regions, and demonstrated a trend for the association and linkage of the *KIAA1109/Tenr/IL2/IL21* gene region with RA in European descent populations. Furthermore, we provided evidence against the involvement of the *PRKCH* gene and the *PTPN22*–1123G allele in RA genetic susceptibility in the French Caucasian population. In addition, we also failed to identify an association and linkage of *CASP7*, *RANK* and *RANKL* genes with RA in a European Caucasian population.

Several collaboration studies were also performed using familial and case-control approaches to search new susceptibility genes in RA and other AID in different ethnic populations as *PTPN22*, *IRF5* and MHC class I polypeptide-related sequence A (*MICA*) genes analysis in Tunisia and Germany populations, respectively.

In a second approach, expression studies were carried out to complete *PRKCH* and *CASP7* genetic analysis. We have showed that *PRKCH* mRNA was expressed in RA patients at lower level than in healthy controls in peripheral blood cells. In the CASP7 study, we demonstrated that functional isoform and non functional isoform mRNAs were expressed in RA patients at lower level than in healthy controls in PBMCs. These results lead to highlight the interest of expression studies in RA and further study correlation between genotypes and expression phenotype.

Concerning the third approach, we performed a large-scale gene expression profiling study performed in RA patients and healthy controls highlighting several new genes (*LY96*/*MD-2, NFAT5*, *TXN, CAMP*/*LL37, ORM1*, *ORM2*, *SLC11A1*, *PGLYRP1* and *F5)* up-regulated in PBMCs of RA patients. These new genes are involved in different functional immunity and defense related mechanisms as pro-inflammation, anti-microbial activity, cellular stress and immunomodulatory functions in RA. Thus, we contributed to gain insights into RA molecular mechanisms.

The fourth approach was focused on genetic, biological and environmental factors interactions in RA patients. We identified an association between *HLA* SE alleles and tobacco smoking for ACPA positivity and a tendency toward an interaction between the *HLA-DRB1**0401 allele and smoking in the development of ACPA positivity in French population with familial and sporadic RA. Therefore, we have confirmed the role of tobacco smoking in RA etiology in the population studied.

All the factors studied and identified in the RA genome and environmental etiology are related to biological processes linked to RA pathology as represented in Figure 8.2. Consequently, all these complementary approaches that allowed the identification of new genes and gene-autoantibodies-environment interactions contribute to a better understanding of RA disease mechanisms and could lead to the identification of innovative clinical biomarkers for diagnostic procedures and therapeutic interventions.

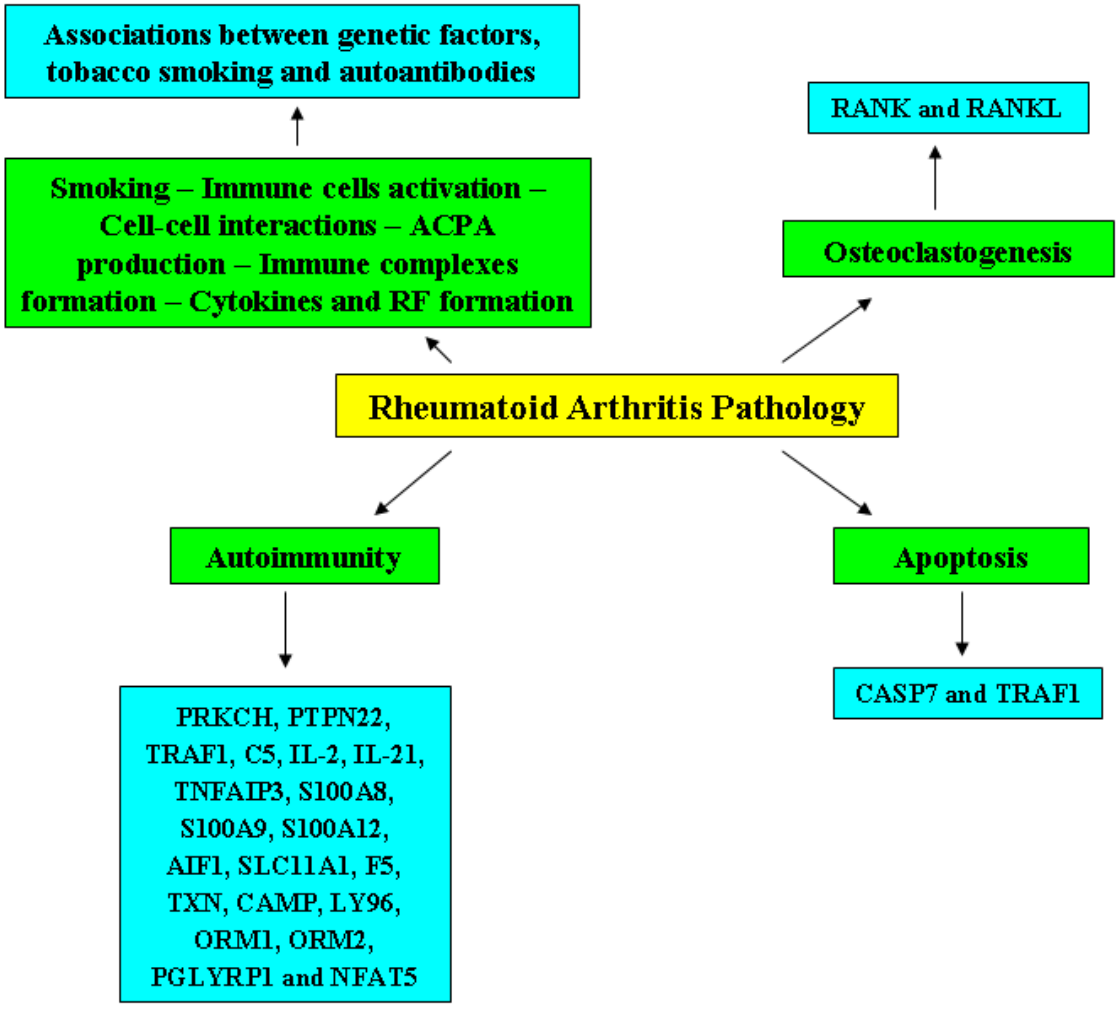

Figure 8.2. Relationship between genome and environmental factors studied and biological processes linked to RA pathology. Green squares – Biological processes linked to RA pathology; Blue squares – RA genome and environmental factors studied.

Perspectives

The consistent observation from successful GWAS was that the effect sizes of RA new loci are low (OR<1.2) and that the combined effect of all known loci can account for only a fraction of the genetic risk of disease. This may be because very large numbers of as yet undefined loci explain complex traits, but may also be described by a significant underestimation of risk for new loci. Therefore, re-sequencing and fine mapping studies may be essential in order to identify all risk/protective alleles at a locus and only once the effect of combinations of these are assessed can the true risk for a given locus be ascribed.

Even though family-based association studies remain crucial to definitively establish RA association and linkage, the number of RA trio families that we have used was restraint to detect and confirm new RA genetic variants which have a very small putative effect in case-

control studies (candidate genes and/or GWA approaches). Once the sample size will be increased, a GWA and linkage studies should be done in RA trio families to identify new RA susceptibility factors and to complete our previous data on already identified genes. Therefore, the number of RA trio families could be increased by the mean of new collect and/or collaborative consortiums. Finally, DNA, RNA and serum samples were not obtained for each RA patient. This should be achieved to allow different approaches (genetic, gene expression and functional studies) to test susceptibility factors with the best homogeneity.

Other approaches can be used to identify new susceptibility factors linked to this disease. The analysis of multiplex RA families could be an important tool to reach this objective. We have begun the analysis of several RA French families, with at least one other autoimmune-disease (AID) in the family among Vitiligo, RA, T1D, Hashimoto thyroiditis, GD, pernicious anemia, SLE and Addison disease. We studied the prevalence of RA autoantibodies (ACPA and RF) and we investigated the presence of *HLA-DRB1* SE, *PTPN22*-1858T and *TRAF1-C5* RA-associated genes. Linkage analysis is planned in the most powerful families to search new loci linked to RA and/or common to several AID.

Additionally, a genome-wide analysis of copy number variation in RA patients could provide important data for the understanding of RA genome structure. Genetic variation in the human genome takes many forms, ranging from large, microscopically visible chromosome anomalies to single nucleotide changes. Recently, multiple studies have discovered an abundance of submicroscopic copy number variation of DNA segments ranging from kilobases to megabases in size (Redon et al., 2006). Deletions, insertions, duplications and complex multi-site variants (Fredman et al., 2004), collectively termed copy number variations (CNVs) or copy number polymorphisms (CNPs), are found in all humans (Feuk et al., 2006) and other mammals examined (Freeman et al., 2006). CNV is a DNA segment that is 1 kb or larger and present at variable copy number in comparison with a reference genome. A CNV can be simple in structure, such as tandem duplication, or may involve complex gains or losses of homologous sequences at multiple sites in the genome (Feuk et al., 2006). Redon and colleagues (2006) found that 285 out of 1,961 (14.5%) genes in the OMIM morbid map overlapped with CNVs and observed numerous examples of possible relevance to both Mendelian and complex diseases.

Finally, the last approach could be the search of genetic factors that influence the expression level of individual transcripts. Phenotypic differences among individuals are partly the result of quantitative differences in transcript abundance. Although environmental stimuli may influence the location, timing, and/or level of transcription of specific genes, genetic

differences among individuals are also known to have a significant role. Transcript levels may be thought of as quantitative endophenotypes that can be subjected to statistical genetic analyses in an effort to localize and identify the underlying genetic factors, an approach that is sometimes referred to as genetical genomics (Jansen and Nap, 2001). The genetic factors that influence the expression level of individual transcripts are referred to as expression quantitative trait loci (eQTLs) and have been extensively described in human lymphocytes by Göring and colleagues (2007). This approach could be a useful resource for the discovery of the genetic factors that influence complex diseases in humans, such as RA.

Chapter 8

Annexes

Publications in Peer-Reviewed Scientific Journals – number 9 to number 15

Research article

Open Access

The *ITGAV* rs3738919-C allele is associated with rheumatoid arthritis in the European Caucasian population: a family-based study

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Abstract

The integrin αvβ3, whose αv subunit is encoded by the *ITGAV* gene, plays a key role in angiogenesis. Hyperangiogenesis is involved in rheumatoid arthritis (RA) and the *ITGAV* gene is located in 2q31, one of the suggested RA susceptibility loci. Our aim was to test the *ITGAV* gene for association and linkage to RA in a family-based study from the European Caucasian population.

Two single nucleotide polymorphisms were genotyped by PCR-restriction fragment length polymorphism in 100 French Caucasian RA trio families (one RA patient and both parents), 100 other French families and 265 European families available for replication. The genetic analyses for association and linkage were performed using the comparison of allelic frequencies (affected family-based controls), the transmission disequilibrium test, and the genotype relative risk.

We observed a significant RA association for the C allele of rs3738919 in the first sample (affected family-based controls, RA index cases 66.5% versus controls 56.7%; $P = 0.04$). The second sample showed the same trend, and the third sample again showed a significant RA association. When all sets were combined, the association was confirmed (affected family-based controls, RA index cases 64.6% versus controls 58.1%; $P = 0.005$). The rs3738919-C allele was also linked to RA (transmission disequilibrium test, 56.5% versus50% of transmission; $P = 0.009$) and the C-allele-containing genotype was more frequent in RA index cases than in controls (RA index cases 372 versus controls 339; $P = 0.002$, odds ratio = 1.94, 95% confidence interval = 1.3–2.9).
The rs3738919-C allele of the *ITGAV* gene is associated with RA in the European Caucasian population, suggesting *ITGAV* as a new minor RA susceptibility gene.

AFBAC = affected family-based controls; bp = base pair; GRR = genotype relative risk; PCR = polymerase chain reaction; RA = rheumatoid arthritis; SNP = single nucleotide polymorphism; TDT = transmission disequilibrium test.

Introduction

Rheumatoid arthritis (RA) is the most common human systemic autoimmune disease (0.8% prevalence in the European Caucasian population), affecting women preferentially [1]. The disease is characterized by a chronic inflammation of the synovial tissues leading to the formation of the rheumatoid pannus, which erodes adjacent cartilage and bone, causing subsequent joint destruction. One hallmark of the pannus is hyperangiogenesis [2].

Previous studies have indicated that the risk of developing the disease in siblings of affected individuals is 2–17 times higher than in the general population, suggesting the importance of genetic factors [1]. Two RA genes have so far been established and confirmed using familial material, *HLA-DRB1* and *PTPN22* [3,4], but they account only for a part of the RA genetic component. The dense genome scan realized in our laboratory suggested 19 non-*HLA* regions in the French Caucasian population [5] and one of these, 2q31, contains the *ITGAV* gene (alias *CD51*, *αv*), which encodes the αv subunit of the integrin family. This family is composed of at least 24 heterodimeric combinations of 18 α subunits and nine β subunits. These transmembranous receptors are expressed at the surface of numerous cells (endothelial cells, macrophages, monocytes, osteoclasts, platelets) and recognize the RGD sequence (Arg–Gly–Asp) of many ligands (such as vitronectin, fibronectin, osteopontin, sialoprotein, thrombospondin, fibrinogen, von Willebrand factor, tenascin, agrin, matrix metalloproteinases, and prothrombin) [6]. The integrins are involved in several functions including adhesion of activated endothelial cells with the extracellular matrix, proliferation, migration, and differentiation signals of vascular cells [6].

The αvβ3 integrin is well documented to play a key role in angiogenesis, and the *ITGAV* knockout animal model is lethal *in utero* for 80% with a presence of large vascular anomalies [7,8].

Angiogenesis also plays a key role in RA when the synovial membrane becomes hyperplasic and destroys the cartilage. We can observe an excess of blood cells (macrophages, T lymphocytes) in the synovial membrane and fluid, and some αvβ3 ligands (that is, fibrinogen or osteopontin) are abundant in the RA synovial fluid [7]. Moreover, some proangiogenic mediators (that is, vascular endothelial growth factor) are overexpressed in RA synovial membrane and serum [9,10].

In addition, several αvβ3 antagonists and angiogenesis inhibitors have been successfully tested on RA animal models [11-14]. The αvβ3 integrin could therefore become a new therapeutic target in RA, and some clinical studies have already begun [15].

Our aim was to use RA familial material to test two intronic *ITGAV* single nucleotide polymorphisms (SNPs) for RA association and linkage in the European Caucasian population.

Materials and methods

All subjects provided informed consent, and the ethics committee of Hôpital Bicêtre (Kremlin-Bicêtre, Assistance Publique-Hôpitaux de Paris, France) approved the study. RA families were recruited through a national media campaign followed by selection of individuals who fulfilled the 1987 American College of Rheumatology criteria for RA according to the physicians in charge of the patients [16]. A rheumatologist university fellow reviewed all clinical data.

Sample 1

Sample 1 (Table 1) constituted the DNA from 100 French Caucasian unrelated trio families (one RA patient and both parents) with the four grandparents of French Caucasian origin. Among these 100 RA patients, 87 were women; their mean age at disease onset was 32 years. In total, 81 patients were rheumatoid factor positive, 78 patients carried at least one *HLA-DRB1* 'shared epitope' susceptibility allele (DRB1*0101, DRB1*0102, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0408, DRB1*1001) [17] and 90 patients presented erosion.

Table 1

Characteristics of rheumatoid arthritis (RA) index cases from the investigated samples

	Sample 1 ($n = 100$)	Sample 2 ($n = 100$)	Sample 3 ($n = 265$)
Females (%)	87	90	86
Mean age of disease onset (years) (±standard deviation)	32 (±10)	31 (±6)	30 (±9)
Mean disease duration (years) (±standard deviation)	18 (±7)	16 (±8)	8 (±7)
RA patients with bone erosions (%)	90	79	72
RA patients seropositive for rheumatoid factor (%)	81	76	73
RA patients carrying at least one *HLA-DRB1* shared epitope allele (%)	78	80	Not available

n, number of cases.

Sample 2

Sample 2 (Table 1) was made up of the DNA from another 100 French Caucasian unrelated trio families with the same characteristics as sample 1. Among these 100 RA patients, 90 patients were women; their mean age at disease onset was 31 years. In all, 76 patients were rheumatoid factor positive, 80 patients carried at least one *HLA-DRB1* shared epitope and 79 patients had an erosive disease.

Sample 3

Sample 3 (Table 1) contained the DNA from 265 European Caucasian unrelated trio families with the same characteristics as sample 1, except for a shorter mean disease duration and a different ethnic origin (Caucasian families from France, Italy, Portugal, Spain, Belgium, and The Netherlands).

Genotyping

DNA was isolated and purified from whole blood according to standard protocols [18]. Two intronic SNPs were selected at the 5' and 3' ends of the gene with a minor allele frequency >25% for European population databases. Moreover the presence of a restriction site for one of the alleles was required (SNP1, rs3768777; SNP2, rs3738919 [19,20]). Genotyping was performed by the PCR followed by restriction fragment length polymorphism method [21].

The designed primers were: sense, 5'-AAGTTGCCAACGTTCCGCGTTGCA-3' and antisense, 5'-GTAGTAGAAGATGGTCCTATCCACG-3' for SNP1; and sense, 5'-ATTTCCAGGTGGAACTTCTTTTGGA-3' and antisense, 5'-TCACAATTCAGATTTTTGCCACTGG-3' for SNP2.

PCR amplification of SNP1 and SNP2 was performed on each sample in a 25 μl reaction volume consisting of 10 U PCR buffer (Perkin Elmer, Boston, MA, USA), 1.25 mM each dNTP, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 3 mM $MgCl_2$, 0.0125 nM of the two primers and 50 ng genomic DNA, diluted to the final volume with H_2O on an Eppendorf thermocycler using a hot start procedure. The PCR program was carried out using a first denaturation cycle of 94°C for 10 minutes followed by 37 cycles of denaturation at 94°C for 40 seconds, with an annealing temperature at 67°C for 30 seconds followed by an elongation step at 72°C for 1 minute. One final cycle of the extension was performed at 72°C for 2 minutes.

For SNP1, a 341-bp amplified fragment was digested with *Nla*III, generating two fragments when the restriction site was present (A allele). For SNP2, the resulting 501-bp fragment was digested with *Alu*I, generating three fragments for the C allele (126 bp, 161 bp and 214 bp), and two fragments for the A allele (permanent restriction site allowing one to validate the restriction protocol; 161 bp and 340 bp). Genotypes were assessed blindly by two independent investigators (LJ and CP). CEPH controls (1347-02 and 884-15) and 40 patients chosen at random were genotyped for quality control. All genotype data will be available online [22].

Power calculation

Using the European population minor allele frequency of 29% and 35% for SNP1 and SNP2, respectively, a sample size of 100 patients and 100 controls, and the arc sinus transformation method described by Garnier and colleagues [23], we had 80% power to detect an association ($P < 0.05$) if the difference in allelic frequencies between patients and controls was at least 11% for SNP1 and 12.2% for SNP2.

Statistical analysis

Prior to association tests, we checked the Hardy–Weinberg equilibrium in 'virtual controls' (constituted by parental untransmitted alleles to RA index cases).

The association and linkage between each polymorphism and RA was examined by three different methods: the affected family-based controls (AFBAC) method was used to compare transmitted and untransmitted allelic frequencies across all families, the transmission disequilibrium test (TDT) was used to detect linkage through preferential transmission of one allele to the affected subjects, and the genotype relative risk (GRR) test was used to compare the genotypic distribution in patients and controls [24-26]. The significance of the *P* value was assessed at 5%, leading to replication tests in sample 2 and, in the case of relevant results, in the larger sample 3.

Results

Hardy–Weinberg equilibrium

Hardy–Weinberg equilibrium in the virtual controls was respected for SNP1 and SNP2 in sample 1 and in the replication samples (data not shown).

Test for association and linkage in sample 1

We observed neither significant association nor linkage between SNP1 and RA in sample 1. For SNP2, we observed a significant association for the C allele and a strong trend for a RA linkage (AFBAC, RA index cases 66.5% versus controls 56.7%, $P = 0.04$; TDT, 59.7% of transmission versus 50%, $P = 0.06$) (Table 2). The GRR test showed a significant increase of the C/C genotype and an excess of C-allele-containing genotypes in patients (Table 3).

The linkage disequilibrium test showed a weak linkage disequilibrium between SNP1 and SNP2 ($D' = 0.33$), and were thus considered independent. The results of the haplotypic TDT analysis showed a significant undertransmission of the SNP1/SNP2 *GA* haplotype (21 versus 37, $P = 0.03$), and a trend for an overtransmission of the two haplotypes containing the C allele of SNP2 (data not shown).

Table 2

Affected family-based control and transmission disequilibrium test analyses for single nucleotide polymorphism (SNP)1 and SNP2 in sample 1 of rheumatoid arthritis trio families

Allele	Affected family-based controls			Transmission disequilibrium test		
	Rheumatoid arthritis cases	Controls	*P*	Transmission (%)	*n*	*P*
SNP1						
A	0.360	0.320	0.39	54.4	90	0.39
G	0.640	0.680				
SNP2						
C	0.665	0.567	0.04	59.7	92	0.06
A	0.335	0.433				

n, number of heterozygote parents.

Table 3

Genotype relative risk analysis for single nucleotide polymorphism (SNP)1 and SNP2 in sample 1 of rheumatoid arthritis trio families

Genotype	Rheumatoid arthritis cases	Controls	*P*
SNP1			
A/A	16	7	0.1 (global)
A/G	40	50	
G/G	44	43	
SNP2			
C/C	45	32	0.03 (C/C versus C/A + A/A)
C/A	38	46	0.36 (C/C + C/A versus A/A)
A/A	13	18	

When stratifying the sample for the families with the index presenting at least one *PTPN22-620W* allele or the *HLA-DRB1* allele shared epitope status, no correlation with the *ITGAV* genotypes could be observed (data not shown).

Test for association and linkage in sample 2

The significant association observed for SNP2 in sample 1 led to a replication test in a second set of 100 French Caucasian Trio families (sample 2) on the hypothesis of an association of the C allele.

In this sample, we observed a trend for association and linkage of the C allele with RA (AFBAC, RA index cases 63.1% versus controls 59.6%, $P = 0.4$; TDT, 52.6% of transmission, $P = 0.6$) (Table 4). The GRR test showed a trend for an excess of the C-allele-containing genotype in RA index cases compared with controls (90 RA index cases versus 79 controls, $P = 0.09$) but not for the C/C genotype (Table 5).

The combination of the two samples, authorized by the absence of any significant clinical difference between them, showed a marginally significant association of the C allele (AFBAC, RA index cases 64.8% versus controls 58.2%, $P = 0.05$; TDT, 56.1% of transmission, $P = 0.09$) and a significant excess of the C-allele-containing genotype in RA index cases compared with controls (173 RA index cases versus 157 controls, $P = 0.02$).

Test for association and linkage in sample 3

The trend for association of the C allele observed in sample 2 was in the same direction as the significant association observed in sample 1, without reaching statistical significance – notably due to a lack of power (the power to detect a significant association in sample 2, based on the allelic frequencies in sample 1, with $P < 0.05$, was only 51%). A larger replication test (265 families, sample 3) was therefore conducted on the hypothesis of an association of the C allele and of the C-allele-containing genotype.

Table 4

Affected family-based control and transmission disequilibrium test analyses for single nucleotide polymorphism 2 in sample 2 of rheumatoid arthritis trio families

Allele	Affected family-based controls			Transmission disequilibrium test		
	Rheumatoid arthritis cases	Controls	*P*	Transmission (%)	*n*	*P*
C	0.631	0.596	0.4	52.6	95	0.6

n, number of heterozygote parents.

Table 5

Genotype relative risk analysis for single nucleotide polymorphism 2 in sample 2 of rheumatoid arthritis trio families

Genotype	Rheumatoid arthritis cases	Controls	*P*
C/C	33	39	0.4 (C/C versus C/A + A/A)
C/A	57	40	0.09 (C/C + C/A versus A/A)
A/A	8	19	

We observed a significant RA association and linkage for the C allele (AFBAC, RA index cases 64.4% versus controls 57.8%, $P = 0.03$; TDT, 57% of transmission versus 50%, $P = 0.04$) (Table 6). This increase was supported by a significant increase of the C-allele-containing genotype in patients (199 RA index cases versus 182 controls, $P = 0.02$) (Table 7).

Test for association and linkage in the combined samples 1 + 2 + 3

The combination of the three samples, authorized by the absence of a significant clinical difference between them, confirmed association and linkage for the C allele (AFBAC, 64.6% versus 58.1%, $P = 0.005$; TDT, 56.5% of transmission, $P = 0.009$) (Table 8). The GRR test showed an excess of the C-allele-containing genotype in patients (372 RA index cases versus 339 controls, $P = 0.002$, odds ratio = 1.94, 95% confidence interval = 1.3–2.9) (Table 9).

Discussion

We studied the *ITGAV* gene, a good RA candidate gene for its function implicated in angiogenesis, and its chromosomal location (in one of the 19 suggested non-*HLA* loci of our dense genome scan) [5]. We observed a significant RA association for the C allele of rs3738919 in a first sample of French Caucasian families, the same trend in replication sample 2, and again a significant association in replication sample 3 (European Caucasian families). Finally, significant RA association and linkage were observed when all sets were combined.

The association and linkage evidences provided by the present study remain nevertheless statistically modest, suggesting at most a minor RA susceptibility marker. Further studies in independent samples will be needed to definitively establish association and linkage of the *ITGAV rs3738919-C* allele to RA. For the observed allelic frequencies of 64.6% in patients versus 58.1% in controls, a sample size of 350 families would be required to obtain, with 80% power ($P < 0.05$), an independent replication of the association evidence reported here.

Once this association had been replicated, resequencing would be necessary to identify exonic and promoter SNPs to refine the associated haplotype.

In the same way, the chromosome 2 linkage suggestion observed in the genome scan of our laboratory could not be totally explained by the findings of the *ITGAV* linkage; hence, with the overtransmission observed in the TDT, the allele sharing expected for the affected sib-pair siblings would be about 53% and would necessitate thousands of sibling pairs to be revealed. Other RA genes in this chromosomal location and/or epistatic effects could be expected to be stronger RA factors that remain to be discovered.

Since the association evidence is modest, no genetic testing would be clinically indicated. Instead, the clinical relevance of the finding is likely to come through better understanding of the RA pathophysiology and may lead to new therapeutic targets.

Contrary to the result of the GRR test in sample 1, which suggested a recessive effect of the *ITGAV rs3738919-C* allele, the result of the larger combined sample is more in favour of a dominant effect of this marker. This difference could be explained by the relatively small size of the first sample.

Table 6

Affected family-based control and transmission disequilibrium test analyses for single nucleotide polymorphism 2 in sample 3 of rheumatoid arthritis trio families

Allele	Affected family-based controls			Transmission disequilibrium test		
	Rheumatoid arthritis cases	Controls	*P*	Transmission (%)	*n*	*P*
C	0.644	0.578	0.03	57	200	0.04

n, number of heterozygote parents.

Table 7

Genotype relative risk analysis for single nucleotide polymorphism 2 in sample 3 of rheumatoid arthritis trio families

Genotype	Rheumatoid arthritis cases	Controls	*P*
C/C	88	76	0.2 (C/C versus C/A + A/A)
C/A	111	106	0.02 (C/C + C/A versus A/A)
A/A	22	39	

Table 8

Affected family-based control and transmission disequilibrium test analyses for single nucleotide polymorphism 2 in the combined samples 1 + 2 + 3

Allele	Affected family-based controls			Transmission disequilibrium test		
	Rheumatoid arthritis cases	Controls	*P*	Transmission (%)	*n*	*P*
C	0.646	0.581	0.005	56.5	387	0.009

n, number of heterozygote parents.

Table 9

Genotype relative risk analysis for single nucleotide polymorphism 2 in the combined samples 1 + 2 + 3

Genotype	Rheumatoid arthritis cases	Controls	*P*
C/C	166	148	0.1 (C/C versus C/A + A/A)
C/A	206	191	0.002 (C/C + C/A versus A/A)
A/A	43	76	

Finally, regarding the key function of angiogenesis in others diseases, and in particular in cancers, it would be interesting to test the *ITGAV rs3738919-C* allele in these phenotypes.

Conclusion

The present study showed a significant association and linkage for the *rs3738919-C* allele of the *ITGAV* gene with RA in the European Caucasian population, suggesting *ITGAV* as a new minor RA susceptibility gene in this population.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LJ, CP, EG and SG carried out the molecular genetic studies. LJ, CP, SBa, SG, PD, LM, HM, VHT, BP, EP-T and FC performed acquisition and analysis of the data. LM, SL, IL, PQ, PH, PM, AB, RW, PB, HA, CV, MF, DP-S, SBo, JD, TRR, PVR, LvdP, AL-V, TB, and the European Consortium on Rheumatoid

Arthritis Families contributed to the recruitment of families and to the acquisition of clinical data. All authors read and approved the final manuscript.

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PTPN22 R620W polymorphism is not associated with pemphigus

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Sir, Pemphigus is a blistering autoimmune disease characterized by the production of autoantibodies against desmosomal cadherins: desmoglein (Dsg) 1 in pemphigus foliaceus (PF) and Dsg3 in pemphigus vulgaris (PV). These autoantibodies are pathogenic and cause keratinocyte detachment or acantholysis. It is widely known that pemphigus, like many other autoimmune diseases, has a genetic basis involving several different genes, mainly at the HLA locus.[1] PTPN22, a gene encoding a lymphoid tyrosine phosphatase, has recently been described as a candidate gene for autoimmune diseases. Indeed, a functional single nucleotide polymorphism (SNP) +1858C/T in PTPN22 (rs2476601), that leads to a change at codon 620 from arginine to tryptophan (R620W), has been reported to be associated with rheumatoid arthritis (RA) and type 1 diabetes (T1D).[2] Based on these data we analysed the association of this SNP with pemphigus in order to test the involvement of this gene's variability in susceptibility to pemphigus.

Blood samples were obtained from 154 unrelated patients with pemphigus (100 PF, 54 PV) with a median age of 46 years (range 20–80) and a female/male sex ratio of 4 : 1. One hundred and fifty healthy persons matched for age, sex and origin were studied as controls. Genomic DNA was purified from fresh peripheral blood leucocytes by standard methods. Genotyping of the PTPN22 rs2476601 polymorphism was carried out with a Taqman® 5′ allelic discrimination assay using an ABI 7500 (Applied Biosystems, Foster City, CA, U.S.A.) real-time polymerase chain reaction system (custom assay). Ten per cent of samples randomly chosen were regenotyped in order to evaluate genotyping accuracy; they were 100% identical. T allele and genotype frequencies were compared between cases and controls using a χ^2 test and a Fisher exact test.

The C/T genotype was detected in 3% and 1·8% of patients with PF and PV, respectively (Table 1). No patients, either with PF or with PV, had the homozygous state T/T. No significant association between PTPN22 polymorphism and the disease was observed ($P > 0{\cdot}05$). We also assessed our population for Hardy–Weinberg equilibrium and confirmed that there was no deviation in either patient or control groups.

To our knowledge, we are reporting the first case–control study describing PTPN22 R620W in pemphigus. Our results showed that the SNP 1858T is present in 1·3% of the Tunisian population. We found no association between the R620W phenotype and either form of the disease (PV or PF). These findings suggest that this polymorphism does not contribute to disease susceptibility.

The 1858T allele was first described in association with T1D in two different populations. This result was confirmed by others in family-based and case–control studies. PTPN22 R620W was found to be associated also with rheumatoid arthritis, Graves disease, systemic lupus erythematosus, Hashimoto thyroiditis and juvenile idiopathic arthritis.[2,3] Although confirmed in different populations, this association was not reported in either T1D or RA in a Colombian population.[2] Similar negative associations were reported with coeliac disease, Crohn disease, Sjögren syndrome and autoimmune thyroid disease in a large affected Tunisian family.[2,4] These

	Pemphigus foliaceus		Pemphigus vulgaris	
Genotype 1858	Patients (n = 100)	Controls (n = 100)	Patients (n = 54)	Controls (n = 50)
C/C	97 (97%)	98 (98%)	53 (98·2%)	48 (96%)
C/T and T/T	3 (3%)	2 (2%)	1 (1·8%)	2 (4%)
f (C)	98·5%	99%	99·1%	98%
f (T)	1·5%	1%	0·9%	2%

Table 1 Genotype and allele frequencies (f) of the PTPN22 1858C/T polymorphism in patients with pemphigus foliaceus (PF) and controls and in patients with pemphigus vulgaris (PV) and controls. No statistical difference was observed between patients (PF or PV) and controls for the PTPN22 1858C/T polymorphism

data revealed ethnic variations which could explain this discrepancy and suggested also that diseases such as systemic lupus erythematosus, T1D and RA share a common mechanism that would not play a crucial role in predisposing to pemphigus. Studies that reported a positive association sought an interaction between this polymorphism and HLA, the main and common locus that implicates autoimmunity. In the present report, the small number of pemphigus patients with the T allele precludes reporting a specific interaction between the HLA locus and PTPN22.

PTPN22 is a negative regulator of T-cell receptor signalling. The +1858T allele encodes an amino acid substitution (R620W) leading to a gain of function that may act in a central or a peripheral way, leading to autoimmunity. Our report that the +1858T allele has no effect on susceptibility to pemphigus may be a reflection of the small number of patients, particularly in the PV group. It also raises the question about the influence of other candidate genes that are involved in the autoimmune response. The rarity of multiplex families presenting pemphigus has made investigation of linkage analysis difficult.[1] As a consequence, the candidate gene strategy is best suited to identify the genes participating in pemphigus susceptibility.

Accordingly, case–control studies have assessed polymorphisms of Dsgs, immunoglobulins, CTLA4 and genes encoding cytokines such as tumour necrosis factor, interleukin (IL)-1, IL-4, IL-6 and IL-10.[5–7] These data revealed a weak association with Dsg1 polymorphism 809C/T in French and Tunisian patients with PF.[8] Moreover, two Dsg3 haplotypes, defined by five SNPs, were also associated with British and Indian patients with PV.[9] Among cytokine genes, only IL6 was suspected to contribute to pemphigus pathogenesis.

This discrepancy of the associated allele reflects that common autoimmune susceptibility alleles may not be shared among all autoimmune diseases but among groups. Although a minor effect of PTPN22 could not be ruled out, there is no evidence to suggest that it could be a more modest factor requiring a large dataset to be observed. Moreover, studying other polymorphisms within PTPN22 would clarify its 'real' influence in the autoimmune process. Accordingly, two other PTPN22 polymorphisms (the −1123 G→C promoter polymorphism and the +2740C→T polymorphism within the 3' untranslated region) were described in Asian T1D.[10] Finally, when looking for a possible association with polymorphisms of other candidate genes, their interaction with the environment will be helpful to elucidate pemphigus aetiology.

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SHORT COMMUNICATION

HSPD1 is not a major susceptibility gene for rheumatoid arthritis in the French Caucasian population

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Abstract The heat shock 60-kDa protein 1 (HSP60) is involved in immune and inflammatory reactions, which are hallmarks of rheumatoid arthritis (RA). HSP60 is encoded by the *HSPD1* gene located on 2q33, one of the suggested RA susceptibility loci in the French Caucasian population. Our aim was to test whether *HSPD1* is a major susceptibility gene by studing families from the French Caucasian population. Three single nucleotide polymorphisms (SNPs) were studied in 100 RA trio families, and 100 other families were used for replication. Genetic analyses were performed by comparing allelic frequencies, by applying the transmission disequilibrium test, and by assessing the genotype relative risk. We observed a significant RA association for the *C*/*C* genotype of rs2340690 in the first sample. However, this association was not confirmed when the second sample was added. The two other SNPs and the haplotype analysis did not give any significant results. We conclude that *HSPD1* is not a major RA susceptibility gene in the French Caucasian population.

Keywords *HSPD1* · Rheumatoid arthritis · Candidate gene · Heat shock protein · Chaperonin

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Introduction

Rheumatoid arthritis (RA) is a common human systemic autoimmune disease, for which previous studies have suggested the importance of genetic factors (Seldin et al. 1999). Two susceptibility genes have been established so far (and are confirmed by the results from the sample sets of the present study), *HLA-DRB1* and *PTPN22*, which account for 19 and 1% of the familial aggregation respectively (Tezenas du Montcel et al. 2005; Michou et al. 2007). A genome scan suggested 19 non-*HLA* susceptibility loci in a French Caucasian population (Osorio et al. 2004). *HSPD1* is located in one of these regions (2q33), and encodes for a member of the chaperonin family (heat shock 60-kDa protein 1, HSP60). Heat shock proteins constitute a family of proteins involved in cell homeostasis, immune and inflammatory reactions. They can regulate gene expression, cell proliferation, and death. HSP60 is a mitochondrial chaperonin, highly preserved during evolution, and responsible for protein folding (Cappello et al. 2004). Homozygous *Drosophila melanogaster HSPD1* homolog –/– mutants die early on during embryogenesis (Perezgasga et al. 1999). In human cells, knockdown of the *HSPD1* gene compromises the folding of the mitochondrial matrix enzymes, indicating that HSP60 plays an essential role (Corydon et al. 2005). Furthermore, HSP60 can downregulate adaptive immune responses and is involved

in apoptosis in patients with systemic autoimmune diseases (Zanin-Zhorov et al. 2006; Jamin et al. 2005). Interestingly, DNA vaccination with the *HSPD1* gene can inhibit adjuvant arthritis in Lewis rats (Quintana et al. 2003).

HSPD1 is thus a positional and functional RA candidate gene. The aim of this study was to test whether *HSPD1* is a major susceptibility gene by analyzing families from the French Caucasian population.

Material and methods

Rheumatoid arthritis families were recruited through a media campaign, and then individuals who fulfilled the 1987 American College of Rheumatology criteria were selected (Arnett et al. 1988). All subjects provided informed consent, and an ethics committee (Kremlin-Bicêtre, France) approved the study. The two samples included DNA from 100 unrelated French Caucasian trio families (a patient and both parents) along with four grandparents of French Caucasian origin (Table 1).

DNA was isolated and purified from whole blood according to standard protocols (Sambrook et al. 1989). The single nucleotide polymorphisms (SNPs) were selected with a required minor allele frequency of >18% among the European Caucasian population and $r^2 > 0.8$ using the QuickSNP web server (http://bioinformoodics.jhmi.edu/quickSNP.pl).

Genotyping

Genotyping of rs2340690 was performed by polymerase chain reaction followed by the restriction fragment length polymorphism method (Botstein et al. 1980). The designed primers are available on request. The resulting 434-bp fragment was digested with *Alu*I, generating three fragments for the *C* allele (68, 103, 263 bp), and two for the *T* allele (103, 331 bp, the 103 bp being an internal control for restriction). Allelic discrimination assays (assay C 8744787 10, C 16261693 10; Applied Biosystems, Foster City, CA, USA) were used to genotype rs788016 and rs2605039, respectively, following the manufacturer's protocol. Genotypes were assessed blindly by two investigators (LJ-CP), and 10% of the samples (chosen randomly) were regenotyped for quality control.

Table 1 Characteristics of the rheumatoid arthritis index cases

	Sample 1 (n = 100)	Sample 2 (n = 100)
Females (%)	87	90
Mean age at disease onset (years)	32 (±10)	31 (±6)
Mean disease duration (years)	18 (±7)	16 (±8)
Patients with bone erosion (%)	90	79
Patients seropositive for rheumatoid factor (%)	81	76
Patients carrying at least one *HLA-DRB1* shared epitope allele* (%)	78	80

**DRB1*0101*, *DRB1*0102*, *DRB1*0401*, *DRB1*0404*, *DRB1*0405*, *DRB1*0408*, *DRB1*1001*

n is the number of cases

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was checked for with virtual controls consisting of parental alleles untransmitted to RA index cases. Association and linkage were examined using three methods. Affected family-based controls (AFBAC) were used to compare transmitted and untransmitted allelic frequencies. The transmission disequilibrium test (TDT) was used to detect linkage through preferential transmission of one allele to the affected subjects. The genotype relative risk (GRR) was calculated to compare the genotypic distributions in patients and controls (Spielman et al. 1993; Thomson 1995; Lathrop 1983). Significance of the P value was assessed at the 5% level, which led to a replication test in 100 other families (sample 2) if at least one test was significant. In case of a positive replication, 265 European Caucasian families were available.

Power calculation

Using the European population minor allele frequencies of 20, 43 and 25% for rs2340690, rs788016 and rs2605039, respectively, with a sample size of 100 patients and 100 controls, as previously described (Garnier et al. 2006), we had 80% power to detect an association ($P < 0.05$) when the difference in allelic frequencies between patients and controls was at least 10.7, 12.4 and 11.4%, respectively.

Results

There are three SNPs that tag the three different LD blocks that exist in *HSPD1* (without containing the other genes, rs2340690, rs788016 and rs2605039). In a recent large-scale genome-wide association study in the British population, one of them (rs788016) and one SNP in LD with rs2605039 (rs8539) were tested without significant results being obtained (Wellcome Trust Case Control Consortium 2007). As we performed a different approach (family-based) in a different population (French Caucasian), we tested these three SNPs. The observed genotype

frequencies were in accordance with the HWE in controls. We observed a trend for overtransmission of the *C* allele with the *C/C* genotype significantly more frequent in cases than in controls for rs2340690 ($P = 0.04$, Table 2). This association led to a replication test in sample 2 with the hypothesis of a *C/C* genotype association with RA. This finding was not confirmed, and the allelic frequencies were nearly identical between cases and controls. The combined analysis of the two clinically identical samples (200 families) did not yield a significant result (Table 2).

Neither significant association nor linkage to RA was found for rs788016 and rs2605039 (Table 3), in agreement with the British study (Wellcome Trust Case Control Consortium 2007). The absence of linkage disequilibrium (LD) between the three SNPs was confirmed, and the results of the haplotype TDT analysis were not significant (data not shown).

When stratifying the sample for families with index presenting at least one *PTPN22-620W* allele or the *HLA-DRB1* allele shared epitope status, no correlation with the *HSPD1* genotypes was observed (data not shown).

Discussion

We studied *HSPD1*, a positional and functional RA candidate gene, since we hypothesized that it is a major susceptibility gene by analyzing families from the French Caucasian population. We observed a significant RA association of the *C/C* genotype for rs2340690 in the first sample, which was not replicated in the second sample.

These results exclude the *rs2340690-C* allele from being a major RA genetic factor, but they cannot totally exclude a minor association. However, it would require 550 trio families with 80% detection power ($P < 0.05$) to find this association, and several thousands to confirm a definitive association ($P < 10^{-6}$).

To test our hypothesis, we studied the three tag SNPs corresponding to our criteria. Thus, we cannot totally exclude the notion that a very minor RA susceptibility allele exists in *HSPD1*.

This 2q3 susceptibility locus contains 101 genes (68 known) including one recently suggested susceptibility gene (Remmers et al. 2007, http://www.ensembl.org/index.html). It seems very unlikely that *HSPD1* is involved in this susceptibility locus.

In conclusion, this study provides evidence that *HSPD1* is not a major RA susceptibility gene in the French Caucasian population.

Table 2 Affected family-based controls (AFBAC), transmission disequilibrium test (TDT), and genotype relative risk (GRR) analyses for rs2340690

Allele and sample	AFBAC			TDT		
	Cases	Controls	*P*	% of trans	*n*	*P*
C, sample 1	0.24	0.17	0.09	60	65	0.1
C, sample 2	0.21	0.20	0.7	52.3	63	0.7
C, sample 1 + 2	0.23	0.18	0.14	56.2	128	0.15

Genotype	Cases	Controls	*P*
GRR sample 1			
C/C	7	3	0.04 (*C/C* vs. others)
C/T	32	27	
T/T	55	64	
GRR sample 2			
C/C	3	2	0.9 (*C/C* vs. others)
C/T	34	33	
T/T	55	57	
GRR Sample 1 + 2			
C/C	10	5	0.2 (*C/C* vs. others)
C/T	66	60	
T/T	110	121	

n is the number of heterozygous parents

Table 3 AFBAC, TDT and GRR analyses for rs788016 and rs2605039

Allele and sample	AFBAC			TDT		
	Cases	Controls	*P*	% of trans	*n*	*P*
Rs788016-A, sample 1	0.49	0.53	0.4	45.9	98	0.4
Rs2605039-G, sample 1	0.26	0.28	0.7	48	77	0.7

Genotype	Cases	Controls	*P*
GRR			
Rs788016-A/A	27	28	0.49
Rs788016-A/G	43	49	
Rs788016-G/G	28	21	
Rs2605039-G/G	8	8	0.9
Rs2605039-G/T	37	40	
Rs2605039-T/T	55	52	

n is the number of heterozygous parents

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The *MMP2 rs243865-T* allele is not a major genetic factor for rheumatoid arthritis in the French Caucasian population

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Summary

The *MMP2 rs243865-T* allele was recently suggested to be associated with rheumatoid arthritis (RA) in a case–control study. *MMP2* is a positional RA candidate gene. Our aim was to test rs243865 in a French family based study. No significant result was shown. The *MMP2 rs243865-T* allele is not a major rheumatoid arthritis genetic factor in this population.

Introduction

Rheumatoid arthritis (RA) is a common human systemic autoimmune disease, for which previous studies have suggested the importance of genetic factors (Seldin *et al.*, 1999). Two genes have been established so far and confirmed with family-based studies, *HLA-DRB1* and *PTPN22* (Deighto *et al.*, 1989; Dieudé *et al.*, 2005). A genome scan performed in a French Caucasian population suggested 19 non-*HLA* regions (Osorio *et al.*, 2004). The *MMP2* gene is located in one of these regions (2q33), and was recently suggested as a susceptibility gene (*rs243865-T* allele), in a case–control Spanish study (Rodriguez-Lopez *et al.*, 2006). This gene is a good functional RA candidate because of its role in the degradation of extracellular matrix of the cartilage. Furthermore, the rs243865 C/T single nucleotide polymorphism (SNP) is located in the promoter sequence, and the *T* allele displays a lower promoter activity (Goldbach-Mansky *et al.*, 2000; Price *et al.*, 2001; Buisson-Legendre *et al.*, 2004). The case–control approach cannot avoid imperfect matching between cases and controls, and the association may consequently be under- or overestimated. The family-based studies are known to be more robust, avoiding this imperfect matching and testing directly Mendel's law by the transmission disequilibrium test.

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The aim of our study was to test this SNP in the hypothesis of a major, clinically relevant genetic factor supporting the RA susceptibility locus on 2q33, using a family-based analysis from the French Caucasian population.

Materials and methods

DNA samples were from 100 French Caucasian Trio families (one patient and both parents) with the four grandparents of French Caucasian origin (the characteristics of the sample population are presented in Table 1). RA families were recruited through a French media campaign followed by selection of individuals who fulfilled the 1987 American College of Rheumatology criteria (Arnett *et al.*, 1988). All subjects provided informed consent, and the ethics committee of Hôpital Bicêtre (Kremlin-Bicêtre, France), approved the study. DNA was isolated and purified from whole blood according to standard protocols (Sambrook *et al.*, 1989).

Genotyping was performed using a Taqman 5′ allelic discrimination assay (assay C_3225943-10, Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Genotypes of 30 individuals were checked again randomly for quality control.

Association and linkage were examined using three methods. Affected family-based controls (AFBAC) were used to compare transmitted and untransmitted allelic frequencies across all families. The transmission disequilibrium test (TDT) was used to detect linkage through preferential transmission of one allele to the affected subjects. The genotype relative risk (GRR) was calculated to compare the genotypic distribution in patients and

Table 1. Characteristics of rheumatoid arthritis index cases (*n*: number of cases)

	Sample (*n* = 100)
Females	87
Mean age at disease onset (years)	32 ± 10
Mean disease duration (years)	18 ± 7
Patients with erosions (ER+)	90
Patients seropositive	81
Patients carrying at least	78

One *HLA-DRB1*shared epitope allele.

Table 2. Affected family-based controls (AFBAC) and transmission disequilibrium test (TDT) analysis for rs243865 (*n*: number of heterozygote parents)

	AFBAC			TDT		
Allele	RA index cases	Controls	*P*	% of Trans	*n*	*P*
rs243865-T	0.242	0.212	0.4	54.8	62	0.4
ER + cases	0.253	0.242	0.8	52.5	59	0.5

RA, rheumatoid arthritis.

Table 3. Genotype relative risk (GRR) analysis for rs243865 (ER + cases)

Genotypes	RA index cases	Controls	*P*
C/C	58 (51)	60 (49)	0.7 (0.6)
C/T	33 (30)	34 (35)	
T/T	7 (7)	4 (4)	

RA, rheumatoid arthritis.

controls (Lathrop, 1983; Spielman *et al.*, 1993; Thomson, 1995). The significance of *P*-value was assessed at 5% and led to a replication test in another 100 families sample if at least one test had a significant result. A study of the erosive subgroup (ER+) of patients with RA was also done, according to the *MMP2* gene function.

Power calculation: with an allele frequency of the *rs243865-T* of 24.5% and 20.3%, in RA index cases and controls, respectively (Spanish study allelic frequencies), a sample size of 100 patients and 100 controls, and the arc sinus transformation method precedently described by Garnier *et al.* (2006), we had a 63.7% power to detect a significant association ($P < 0.05$).

Results and discussion

The observed rs243865 genotype frequencies were in accordance with the Hardy–Weinberg equilibrium in 'virtual controls', constituted by parental untransmitted alleles to RA index cases and controls. We observed neither significant linkage nor association between rs243865 and RA. We found, however, a trend for the *T* allele (TDT 54.8% of transmission vs. 50%, $P = 0.4$, AFBAC RA index cases 24.2% vs. controls 21.2%, $P = 0.4$) and for the *T/T* genotype (seven RA index cases vs. four controls, $P = 0.7$). We did not find any more evidence in the ER + subgroup (Tables 2 and 3).

The aim of this study was to detect a major RA susceptibility allele located at the 2q33 susceptibility locus and suggested in a case–control study. Our results allow to exclude the *rs243865-T* allele as a major genetic factor in the French Caucasian population, but a minor significant RA association cannot be totally excluded. However, a 80% detection power (based on the allelic frequencies of our study) would require 1200 Trio families ($P < 0.05$) and several thousand Trio families for a definitive association ($P < 10^{-6}$).

In conclusion, this study provides evidence that the *MMP2 rs243865-T* allele is not involved in the RA susceptibility locus on 2q33 and is not a major, clinically relevant RA susceptibility genetic factor in the French Caucasian population.

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Association of *IRF5* gene polymorphisms with rheumatoid arthritis in a Tunisian population

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Objective: A strong genetic association of rheumatoid arthritis (RA) with the interferon regulatory factor 5 (*IRF5*) gene has been described previously in a Swedish population, although this result was not confirmed in a French population. We undertook an association study between *IRF5* and the RA phenotype, as well as a study with serological markers of RA, in a Tunisian population.
Methods: A single-nucleotide polymorphism (SNP; rs2004640) was genotyped using a Taqman 5′ allelic discrimination assay on an ABI 7500 real-time polymerase chain reaction (PCR) instrument in 140 RA patients and 185 controls. Rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA) were determined by enzyme-linked immunosorbent assay (ELISA). Association was assessed based on the χ^2 test and odds ratios (ORs) with 95% confidence intervals (CIs).
Results: The frequency of the TT genotype of the *IRF5* SNP rs2004640 differed significantly between patients and controls ($p=0.01$). This difference was greater when a subgroup of patients with another 'autoimmune' disorder was considered ($p=0.007$). A weak but significant association was also found in a subgroup of patients who were positive for ACPA ($p=0.04$) or erosion ($p=0.01$).
Conclusions: Our results indicate that the TT genotype of the *IRF5* (rs2004640) dimorphism is associated with RA in a Tunisian population.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by progressive joint destruction and autoantibody formation. Antibodies elaborated include anti-citrullinated protein/peptide antibodies (ACPA) and anti-rheumatoid factor (RF). Both genetic and environmental factors, and their interaction, play a role in the development of RA (1–3). A common set of alleles at the HLA-DRB1 locus (the 'shared epitope' alleles) has been associated with RA in populations with white European and Asian ancestry (4, 5). This locus, however, does not account for the whole genetic component of RA, and multiple loci with smaller contributions to disease susceptibility are likely to exist. Numerous candidate gene studies have been performed in RA, but findings from one population have been difficult to replicate in other populations. The protein tyrosine phosphatase N22 (*PTPN22*) gene was discovered as a risk factor for RA by genome-wide association scanning of functional single-nucleotide polymorphisms (SNPs) (6) and has been replicated in many white RA cohorts. However, the *PTPN22* risk allele (R620W) is extremely rare in Asian populations and there is no evidence of association of this gene with RA in non-white populations (7, 8). By contrast, the peptidylarginine deiminase type 4 (*PADI4*) gene, which codes for one of the PADI enzyme isotypes, has genetic variants that confer susceptibility to RA in Asian but not in European populations. The solute carrier family 22 (*SLC22A4*) gene, an organic cation transporter, and the Fc receptor-like-3 (*FCRL3*) gene have been associated with RA in a Japanese population and replicated in other Asian groups (9–11), but have given weak or negative results in populations with European ancestry (12, 13). These divergent results suggest genetic heterogeneity of RA across the major ethnic groups (14).

It is relatively common that patients affected by an autoimmune disease suffer from another autoimmune disease, and that members of the same family suffer from different autoimmune diseases.

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For example, in families with systemic lupus erythematosus (SLE; OMIM 1527000), RA (OMIM 180300) occurs more frequently than in the general population (15). Such observations suggest shared genes or involvement of common cellular pathways in these diseases. This hypothesis is supported by reports on genes found to be associated with more than one autoimmune disease, such as *PTPN22* and *IRF5* in RA and SLE, respectively (16–18).

The type I interferon (IFN) system has been postulated to play a key role in autoimmune diseases (19). Increased expression of IFN-induced genes has been detected in autoimmune diseases such as SLE (20), RA (21), Sjögren's syndrome (22), and in a subgroup of patients with multiple sclerosis (MS) (23). The interferon regulatory factors (IRFs) are major regulators of genes activated by the type I IFNs (24) and a role in the regulation of the immune system is well established for the majority of the members of the *IRF* family of nine genes. The role of *IRF5* in the immune response is not as well established as for other *IRF*s, but *IRF5* has recently received attention in studies on autoimmunity. The *IRF5* gene displays a complex transcription pattern with three alternative non-coding 5′ exons and at least nine alternatively spliced mRNAs (25). *IRF5* is expressed in dendritic cells, monocytes, and B cells, but its expression can be induced in other cell types by the type I IFNs (26). *IRF5* regulates the Toll-like receptor (TLR)-dependent activation of inflammatory cytokines and functions downstream of the TLR–MyD88 pathway, where it is activated by MyD88 and tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (27). The recent finding of an association between the *IRF5* gene and SLE (17), which has been replicated in multiple populations, as well as the association between *IRF5* and RA (18) and inflammatory bowel diseases (IBD) (28), provides additional support for the important role of *IRF5* and the type I INF system in autoimmune diseases. Inspired by these findings, we investigated whether polymorphism in the *IRF5* gene would also be associated with RA in the Tunisian population. We considered a T/G SNP within the *IRF5* gene (rs2004640). The *IRF5* gene (rs2004640) is located 2 bp downstream of the intron–exon border of exon 1B, creating a consensus GT donor splice site (29). In this report, we demonstrate that the *IRF5* rs2004640-TT genotype confers susceptibility to RA in a Tunisian population.

Patients and methods

Samples

Our study was undertaken in 140 patients with RA (25 men and 115 women) and 185 healthy controls (60 men and 125 women). The average age of RA onset was 53 years. Erosions were present in 98 of the patients and rheumatoid nodules in 15 of the 98. Fifty-eight patients had another autoimmune disease (thyroid autoimmune disorders, type 1 diabetes, and Gougerot Sjögren syndrome), 94 were rheumatoid factor positive (RF+), and 92 were positive for ACPA. All patients were Tunisians living in the south of Tunisia. All cases satisfied the 1987 American College of Rheumatology (ACR) criteria (30). The 185 controls lived in the same area as the patients. The mean age at analysis was 43.5 years. There was no clinical evidence of family history of autoimmune disease and inflammatory joint disease.

Autoantibody analysis

Patient sera obtained at the time of diagnosis were examined for RF by nephelometry and for ACPA by enzyme-linked immunosorbent assay (ELISA) (second-generation test; Euro-Diagnostica, Arnhem, the Netherlands).

Molecular genotyping

Genomic DNA was purified from fresh peripheral blood leucocytes by standard methods. Genotyping of the *IRF5* rs2004640 was carried out with a Taqman 5′ allelic discrimination assay on an ABI 7500 real-time polymerase chain reaction (PCR) instrument (assay: C_9491614_10). CEPH (Centre d'Etude du polymorphisme Humain) controls (1347-02 and 884-15) were co-genotyped with all our samples for quality control.

Statistical analyses

The results from the control subjects and RA unrelated patients were compared using the χ^2 test (2×2 contingency tables) for statistical significance. The genotype relative risk (GRR) method (a single genotype vs. the others) was used to compare the genotype distribution in controls and patients. The GRR test adjusts the genotype frequencies in the controls to the expected Hardy–Weinberg proportions and yields more accurate risk estimates (31). p-values < 0.05 were considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with Woolf's method (32). Hardy–Weinberg equilibrium was checked in the control group using a standard χ^2 test.

Power calculation

We calculated the empirical power of the study by bootstrapping from the dataset of patients and controls (random sampling with replacement of the same number of patients and controls from the original

dataset). For each bootstrap a GRR test was calculated. 100 000 bootstraps were used and the empirical power was estimated as the number of times the GRR test exceeded the χ^2 threshold at a 5% significance level (3.84) divided by the total number of bootstraps. Calculations were performed using a program written in R language (www.r-project.org).

Results

In the control subjects, the genotype frequency of *IRF5* polymorphisms conformed to Hardy–Weinberg equilibrium. Table 1 shows the allelic and genotypic distribution of the *IRF5* rs2004640 polymorphism in patients and controls. As can be observed, the two groups were very similar for the *IRF5* rs2004640-T allele distribution (58.9%) in patients and controls (54.8%) (χ^2=1.07, p=0.30, OR=1.1, 95% CI 0.86–1.62). Moreover, no significant association was detected when patients were subgrouped by clinical data (erosive, nodules, other autoimmune disease) or immunological profile (the presence of RF and/or ACPA) (p>0.05).

At the genotypic level, the genotype TT was more frequent in patients with RA (42.1%) than in the controls (31.4%). The inverse was observed for the GG genotype, while the frequency of the heterozygote genotype is similar in the two groups (Table 1). There is a significant increase of TT genotype in patients compared to controls (χ^2=5.91, p=0.01, OR=1.59, 95% CI 1.01–2.51).

We next stratified our analysis with regard to RF and ACPA for patients. The frequency of the risk genotype (TT) was not higher in the subsample of RA patients testing positive for RF (40.4%) compared to controls (31.3%) (χ^2=3.43, p=0.06). Similar negative results were found in the subgroup of patients negative for ACPA when compared to controls (43.7% vs. 31.3%; χ^2=3.57, p=0.058), whereas a slight increase was observed in patients positive for ACPA compared to controls (41.3% vs. 31.3%; χ^2=3.94, p=0.046).

Compared to the controls, we observe a significant increase in the TT genotype in the RA subgroup positive for erosion and associated autoimmune diseases (p=0.013 and p=0.0078, respectively).

Discussion

The data presented here provide evidence of an association between the *IRF5* gene variant (rs2004640) and RA and show that the IFN pathway may be involved in this disease. Of note, we detected the most significant association with the *IRF5* gene in the subgroup of patients who carried another autoimmune disorder, supporting the hypothesis that *IRF5* is a common susceptibility gene to different autoimmune disorders (17, 18, 28).

To assess the informativeness of our case–control sample, we computed the empirical power, which is the probability of detecting an association. We found a power of 20% for the allelic test and 51% for the genotypic test. This explains the negative allelic association and the significant genotypic association.

In contrast to these positive results, a previous study reported no RA linkage in 100 French Caucasian trio families; there was no over-transmission of the *IRF5* rs2004640-T allele from heterozygotic parents to affected patients (p=0.76) (33). Moreover, the rs2004640-T allele frequency was slightly lower in patients with RA than in the virtual controls derived from untransmitted parental chromosomes (27% vs. 30.5%, p>0.05).

In three case–control cohorts from Spain, Sweden, and Argentina (34), no statistically significant differences in allele or genotype frequencies of the rs2004640 and rs2280714 *IRF5* polymorphisms were observed between RA patients and controls. This discrepancy between different populations may reflect genetic heterogeneity in the genetic susceptibility basis of susceptibility to RA.

More recently, an analysis of five SNPs (rs729302, rs3757385, rs2004640, rs752637 and rs3807306) in

Table 1. Allele and genotype frequencies of the *IRF5* T/G SNP (rs2004640) in Tunisian RA patients stratified by clinical and immunological data and in Tunisian controls.

	Allele association			Genotype association				
	G	T	p-value	GG	GT	TT	p-value	OR† (95% CI)
Patients (n=140)	115 (41.1)	165 (58.9)	0.3	34 (24.3)	47 (33.6)	59 (42.1)	0.014*	1.59 (1.01–2.51)
Anti-ACPA$^+$ (n=92)	76 (41.3)	108 (58.7)	0.39	22 (23.9)	32 (34.7)	38 (41.4)	0.046*	1.54 (0.92–2.59)
Anti-ACPA$^-$ (n=48)	39 (40.6)	57 (59.4)	0.42	12 (25)	15 (31.2)	21 (43.8)	0.058	–
RF$^+$ (n=94)	78 (41.5)	110 (58.5)	0.41	22 (23.4)	34 (36.2)	38 (40.4)	0.06	–
AID (n=58)	42 (36.2)	74 (63.8)	0.09	12 (20.0)	18 (31.1)	28 (48.9)	0.0078*	2.04 (1.12–3.72)
Erosive (n=97)	78 (39.8)	118 (60.20)	0.22	23 (23.4)	32 (32.7)	43 (43.9)	0.013*	1.71 (1.03–2.84)
Nodules (n=15)	17 (56)	13 (44)	0.22	5 (33.3)	7 (46.7)	3 (20)	0.46	–
Controls (n=185)	167 (45.1)	203 (54.9)	–	40 (21.6)	87 (47.0)	58 (31.4)	–	–

ACPA, anti-citrullinated protein/peptide antibodies; RF, reumatoid factor; AID, autoimmune diseases. Frequencies given as n (%). *Significant p-value (p<0.05). †Odds ratios (ORs), and 95% confidence intervals (CIs) for the TT genotype compared to others.

patients with RA and controls from a Swedish population showed that four of the five SNPs were associated with RA ($p < 0.05$), and the SNP rs3807306 exhibited the strongest association ($p = 0.00063$). This SNP is in high linkage disequilibrium with SNP rs2004640 (18). However, the SNP rs752637 did not show any association with RA, supporting the hypothesis that the mechanisms by which susceptibility genes confer susceptibility to autoimmune diseases may, in part, be disease specific (18).

Because *IRF5* is an important mediator of signals from TLR7/TLR9 (35, 36), we speculate that certain expression patterns of the *IRF5* gene can modify IRF5-dependent induction of type I IFN, pro-inflammatory cytokines (IL-6, TNFα, IL-12, and IL-1β), and several chemokines (37, 38). Furthermore, the level of TLR7 expression is increased in RA synovium and can contribute to synergistic cytokine production by dendritic cells (39). These cytokines can obviously influence the development and expression of inflammatory diseases, both at the level of the underlying autoimmune process and by promoting the inflammatory process. However, IRF5 can also increase the expression levels of several genes encoding proteins that mediate cell growth arrest and apoptosis (37). Consequently, polymorphisms within the *IRF5* gene may affect several cellular functions of importance for RA susceptibility, besides the type I IFNs. Finally, understanding the possible functions of the allele variants of the *IRF5* gene on the molecular level requires further experimental studies.

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Research article

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Association of *MICA* with rheumatoid arthritis independent of known *HLA-DRB1* risk alleles in a family-based and a case control study

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Abstract

Introduction The gene *MICA* encodes the protein major histocompatibility complex class I polypeptide-related sequence A. It is expressed in synovium of patients with rheumatoid arthritis (RA) and its implication in autoimmunity is discussed. We analyzed the association of genetic variants of *MICA* with susceptibility to RA.

Methods Initially, 300 French Caucasian individuals belonging to 100 RA trio families were studied. An additional 100 independent RA trio families and a German Caucasian case-control cohort (90/182 individuals) were available for replication. As *MICA* is situated in proximity to known risk alleles of the *HLA-DRB1* locus, our analysis accounted for linkage disequilibrium either by analyzing the subgroup consisting of parents not carrying *HLA-DRB1* risk alleles with transmission disequilibrium test (TDT) or by implementing a regression model including all available data. Analysis included a microsatellite polymorphism (GCT)n and single-nucleotide polymorphisms (SNPs) rs3763288 and rs1051794.

Results In contrast to the other investigated polymorphisms, the non-synonymously coding SNP *MICA*-250 (rs1051794, Lys196Glu) was strongly associated in the first family cohort (TDT: $P = 0.014$; regression model: odds ratio [OR] 0.46, 95% confidence interval [CI] 0.25 to 0.82, $P = 0.007$). Although the replication family sample showed only a trend, combined family data remained consistent with the hypothesis of *MICA*-250 association independent from shared epitope (SE) alleles (TDT: $P = 0.027$; regression model: OR 0.56, 95% CI 0.38 to 0.83, $P = 0.003$). We also replicated the protective association of *MICA*-250A within a German Caucasian cohort (OR 0.31, 95% CI 0.1 to 0.7, $P = 0.005$; regression model: OR 0.6, 95% CI 0.37 to 0.96, $P = 0.032$). We showed complete linkage disequilibrium of *MICA*-250 ($D' = 1$, $r^2 = 1$) with the functional *MICA* variant rs1051792 ($D' = 1$, $r^2 = 1$). As rs1051792 confers differential allelic affinity of MICA to the receptor NKG2D, this provides a possible functional explanation for the observed association.

CCP+: positive for anti-cyclic citrullinated peptide antibodies; CI: confidence interval; LD: linkage disequilibrium; LRT: likelihood ratio test; OR: odds ratio; PCR: polymerase chain reaction; RA: rheumatoid arthritis; SD: standard deviation; SE: shared epitope; SNP: single-nucleotide polymorphism; TDT: transmission disequilibrium test.

Conclusions We present evidence for linkage and association of *MICA*-250 (rs1051794) with RA independent of known *HLA-DRB1* risk alleles, suggesting *MICA* as an RA susceptibility gene. However, more studies within other populations are necessary to prove the general relevance of this polymorphism for RA.

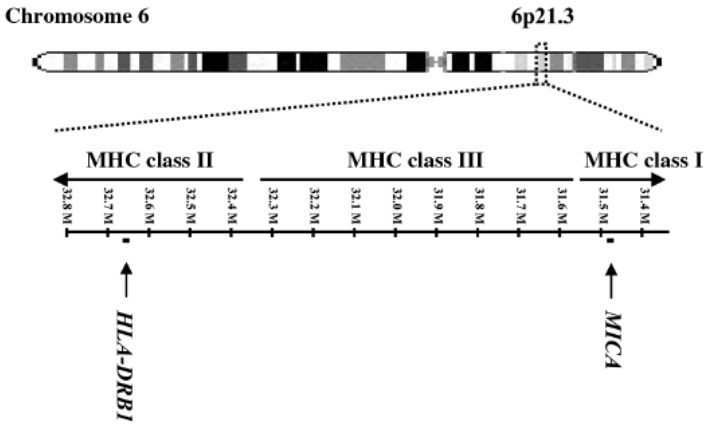
Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammatory changes of joints and inner organs. It is estimated that at least 50% of the risk to develop RA is determined by genetic factors [1]. Considerable efforts have been made to elucidate these genetic factors to better understand the disease. However, even after the advent of genome-wide association studies, only somewhat more than half of the estimated genetic risk for RA has been assigned to specific genetic determinants [2]. There is strong evidence [3-5]. that additional genetic risk factors reside within a genomic region containing the strongest known genetic determinants of RA susceptibility, alleles of the *HLA-DRB1* gene. Identification of additional risk factors within the *HLA-DRB1* gene region is complicated by the extraordinarily high local linkage disequilibrium (LD): Standard association analyses of genetic variants in candidate gene and genome-wide association studies are prone to confounding due to LD with *HLA-DRB1* alleles. Successful identification of additional genetic risk factors in this region needs to account for risk conferred by different *HLA-DRB1* alleles. Within the shared epitope (SE) hypothesis, *HLA-DRB1* alleles *0101, *0102, *0401, *0404, *0405, *0408, and *1001 are most commonly reported to be associated with risk for RA in European Caucasians [6]. Recently, a new classification of *HLA-DRB1* alleles was proposed by du Montcel and colleagues [7], taking into account risk-modifying effects of neighboring amino acids. This classification emerged as especially reproducible and reliable [8].

MICA is located within the same genomic region as *HLA-DRB1* (Figure 1). It encodes the protein major histocompatibility complex class I polypeptide-related sequence A. This protein interacts with the C-type lectin activatory receptor NKG2D (also known as KLRK1) found on natural killer cells, γδ T cells, and certain subgroups of αβ T cells. MICA-NKG2D interaction is believed to be important for eliminating infected or tumorous cells [9]. This interaction is also described to increase inflammatory cytokine production and proliferation of a certain subset of T cells. In consequence, implications in autoimmunity have been discussed [9-12]. MICA is expressed in RA synovium but not in osteoarthritis synovium [12]. Local NKG2D expression is induced by tumor necrosis factor and interleukin-15 [12]. These findings make *MICA* an interesting candidate gene for association studies in RA.

The highly polymorphic gene *MICA* (122 frequency-validated single-nucleotide polymorphisms [SNPs] in SNP database [dbSNP] build 129) was investigated in various RA association studies in different populations. For several SNPs and for a microsatellite marker, associations with protection or risk were shown [4,13-17]. Results for different *MICA* variants

Figure 1



Location of *MICA* relative to the *HLA-DRB1* locus. Despite a distance of more than one megabase from the rheumatoid arthritis risk factor *HLA-DRB1* in the major histocompatibility complex (MHC) class II region, there is considerable linkage disequilibrium between markers in both genes. Therefore, *HLA-DRB1* status must be considered for interpretation of genetic association data.

were not conclusive but point toward association with RA. Heterogeneity between results of these studies may be due at least partially to confounding of results by LD with *HLA-DRB1* alleles.

Some studies reported association analyses without controlling for LD of *MICA* with *HLA-DRB1* alleles at all [14,17]. This makes a conclusion about an independent association of *MICA* intricate. If association analysis is done under the condition of no significant LD between *MICA* and *HLA-DRB1* alleles [16], the problem prevails: Even weak, non-significant LD may bias *MICA* association analysis since effect sizes of known *HLA-DRB1* risk alleles are considerably large. Other authors restricted analysis to the patient subgroup without *HLA-DRB1* risk alleles, ignoring large parts of the data [13]. Alternatively, stratification of data in SE and non-SE subgroups ignores variance of the individual risk of SE alleles within the SE subgroup [15]. In a recent study, case-control pairs were matched 1:1 by *HLA-DRB1* genotype to control confounding [4]. However, as a disadvantage of this method, large proportions of typical RA patient and control collections are excluded from analysis since certain *HLA-DRB1* genotypes are common in patients but rare in controls and vice versa.

Our aim was to investigate the role of DNA polymorphisms of *MICA* in French Caucasian RA family trios and in a German Caucasian case-control cohort. Confounding by *HLA-DRB1* risk alleles was controlled by analysis of the subgroup negative for known *HLA-DRB1* risk alleles and by logistic regression including all data.

Materials and methods

Patients

We analyzed 600 French Caucasian individuals belonging to 200 families grouped in two cohorts of 100 family trios. Characteristics (gender, age at onset, disease duration, erosions, seropositivity, and SE) as well as details on DNA preparation were described previously [18]. Seventy-six percent of French RA index patients were positive for anti-cyclic citrullinated peptide antibodies (CCP^+). For case-control analysis, 272 German Caucasians were analyzed. Controls were 182 healthy blood donors (mean age ± standard deviation [SD] was 50 ± 7 years, and 80% were female) from the Institute of Transfusion Medicine, University Hospital Leipzig, Germany, and cases were 90 RA patients from the Medical Clinic IV, University Hospital Leipzig, Germany, with the following characteristics: mean age (± SD) at disease onset was 47.1 ± 15.7 years, mean (± SD) disease duration was 26.7 ± 20.5 years, 92% were RA patients seropositive for rheumatoid factor, and 78% were female. All individuals provided informed consent, and the ethics committees of Hôpital Bicêtre (Kremlin-Bicêtre, AP-HP, France) and of the University of Leipzig (Leipzig, Germany) approved the study.

Genotyping

We investigated three polymorphisms spanning *MICA* for association with RA. For SNP selection, we required frequency validation, a map weight of 1, and a minor allele frequency exceeding 5% in Caucasians. Among 775 SNPs available within the MICA region in Ensemble version 24, 7 were frequency-validated and had a map weight of 1. Within the promoter region, defined as within 5 kb upstream of the start of the gene, we selected *MICA*-300 (rs3763288). According to TESS (Transcription Element Search System) [19], *MICA*-300 co-localizes with a binding site for the transcription factor ETV4. Within the coding region, we selected the non-synonymously coding SNP *MICA*-250 (rs1051794, Lys196Glu) as validation information for this variant was previously published [20,21]. In addition, variant *MICA*-210 (a trinucleotide repeat (GCT)n microsatellite polymorphism within the transmembrane domain) was selected as various associations of this variant with RA were reported previously [15-17,22].

Genotyping was done by applying single-base extension followed by mass spectrometry ('GenoSNIP') as described [23] but with the following modifications: polymerase chain reaction (PCR) and genotyping primers for *MICA*-210: CCTTTTTTTCAGGGAAAGTGC, CCTTACCATCTCCAGAAACTGC [22], and bioCCATGTTTCTGCTG(L)TGCTGCT; *MICA*-300: GGAAGGCTGTGCAGTAATCTAGG, TCCCTTTTCCAGCCTGCC, and bioCTGTGCAGT(L)ATCTAGGCTGAAGG; and *MICA*-250: AAGGTGATGGGTTCGGGAA, TCTAGCAGAATTGGAGGGAG [21], and bioCTCAGGAC(L)ACGCCGGATT. For the *MICA*-250 assay, a genotyping primer bioCTCCAGAG [L]TCAGACCTTGGC, differentiating between a paralogue sequence variant of *MICA* and *MICB*, was genotyped in 558 (63.8%) samples. This assay always indicated amplification of *MICA* and never of *MICB*. PCR products were checked by agarose gel electrophoresis for correct size and sufficient yield. Within the studied population, no Mendel error occurred. No significant departure ($P \leq 0.05$) from Hardy-Weinberg equilibrium was observed in controls (French samples: $P = 0.240$ for non-transmitted chromosomes; German controls: $P = 0.233$; chi-square test with one degree of freedom).

HLA-DRB1 was genotyped previously using sequence-specific PCR primers and hybridization of PCR products with probes specific for *HLA-DRB1* alleles, as described for the French family sample [18] and the German case-control sample [24]. Distribution of *HLA-DRB1* alleles can be found in the online supplement (Additional data file 1).

Statistical analysis

For association analysis, we chose a multistep approach. In a first cohort of 100 family trios, selected polymorphisms were tested for association with RA. Those showing nominal association at a significance level of 0.05 or below were tested in

a second cohort of 100 French family trios. A decrease in *P* value in the combined French cohorts was taken as strong evidence in favor of association. These polymorphisms were further analyzed in a German Caucasian case-control cohort.

Haplotypes were estimated using the software HAPLORE (HAPLOtype REconstruction) [25]. For these estimations, data of SNPs located between *MICA* and *HLA-DRB1* were included (rs1800629, rs909253, rs3093553, and rs3093562 for the second French cohort and additionally rs1043618, rs2075800, rs1799964, rs1800630, rs3093662, and rs3093664 for the first French cohort; data available online [26]). We successfully assigned haplotypes for 95% of all families (minimum posterior probability was 90% and mean posterior probability was greater than 99.9%).

Transmission disequilibrium test (TDT) for association and linkage with RA was calculated as described by Spielman and colleagues [27]. For subgroup analyses, the subgroup without *HLA-DRB1* risk alleles was defined by the absence of SE alleles. This is identical with allele L according to the classification by du Montcel and colleagues [7]. Derived haplotype information allowed identification of transmitted and non-transmitted chromosomes.

For conditional logistic regression analysis of families, LogXact (Cytel Inc., Cambridge, MA, USA) was used. Within this analysis, *HLA-DRB1* allele classification was according to du Montcel and colleagues [7]. The S3P allele consisted of alleles *0101, *0102, *0404, *0405, *0408, and *1001, and the S2 allele consisted of *0401. We applied the convention that allele L denotes alleles S1, S3D, and X as the associated risk for RA of the latter three alleles was found to be of similar magnitude [7,8]. Of the index patients of all 200 French families, 53% and 45% contributed to allele groups S3P and S2, respectively. Twenty-one percent were homozygous for allele L. In regression analysis, we modeled the transmission probability of a haplotype toward affected children given the competitive haplotype of a parent. This method is known as conditional logistic regression. To include *HLA-DRB1* alleles in the model, allele L was used as the reference group. To ensure independence of *MICA* association from *HLA-DRB1* risk alleles, a likelihood ratio test (LRT) was done. Here, the likelihood of the model including *HLA-DRB1* alleles and *MICA* was compared with a model including *HLA-DRB1* alleles only. A significant increase of the model's likelihood that includes polymorphism *MICA* (that is, an LRT *P* value of less than 0.05) indicates an association of the *MICA* polymorphism independent of the known association of *HLA-DRB1* alleles. Analogously, we checked for interactions between *MICA* and *HLA-DRB1*. Additional methodological remarks to this method are given in the online supplement (Additional data file 2).

Within the case-control cohort, haplotyping was not resolvable with the same accuracy as for the family cohorts. Hence, the logistic regression model was based on unphased data of *MICA*-250 and *HLA-DRB1*. It included all case-control individuals, accounting for *HLA-DRB1* risk alleles. *HLA-DRB1* classification according to du Montcel and colleagues [7] as described above was applied. Cases of the case-control cohort contributed to allele groups S3P (42%) and S2 (36%). Twenty-one percent were homozygous for allele L. Within the model, genotypes were coded (0, 1, and 2), with 2 coding for the homozygous minor allele. Thus, an additive model was implemented. LRTs were done similarly to the conditional logistic regression model described above. Multimarker LD analysis was done using the software MIDAS (Multiallelic Interallelic Disequilibrium Analysis Software) [28]. For the exact Mantel-Haenszel test, the software StatsDirect was used [29]. If not indicated otherwise, *P* values were not corrected for multiple testing.

Results

Association of *MICA* with rheumatoid arthritis within the first French family cohort

We analyzed three polymorphisms within the gene *MICA*: *MICA*-300 (rs3763288) within the 5' region of the gene (promoter region), *MICA*-210 (trinucleotide repeat (GCT)n microsatellite polymorphism within the transmembrane domain), and *MICA*-250 (non-synonymously coding SNP, rs1051794, Lys196Glu).

In standard analysis (TDT without accounting for linkage with *HLA-DRB1*), we found significant undertransmission of *MICA*-250A in the first French family cohort (Table 1a). Our first strategy to account for potential LD with *HLA-DRB1* was to restrict analysis to parents negative for *HLA-DRB1* risk alleles. Here, we also found protective association of *MICA*-250A and RA (Table 1b). In our second strategy, we controlled for LD with *HLA-DRB1* risk alleles by conditional logistic regression. *MICA*-250A again emerged as a protective factor as haplotypes including *MICA*-250A were significantly undertransmitted to affected children. The LRT was significant, demonstrating that *MICA*-250 is associated with RA independent of known *HLA-DRB1* risk alleles (Table 1c).

Association of *MICA*-250 with RA was stronger compared with association of other analyzed single markers (Table 1) and with three-marker haplotypes consisting of *MICA*-300, *MICA*-250, and *MICA*-210 (data not shown). Therefore, only *MICA*-250 was included in further validation studies within a second independent French Caucasian family cohort and a case-control cohort of German Caucasian origin.

Association analysis within the second and combined first and second French family cohorts

Within the second French family cohort, we found the same trend for protective association of *MICA*-250A with RA in standard analysis and in both the *HLA-DRB1* risk allele-negative subgroup analysis and conditional logistic regression

Table 1

Association of *MICA* polymorphisms within the first French Caucasian family cohort

	MICA-210					*MICA*-250	*MICA*-300
(a) French population 1 – all individuals without controlling for LD with *HLA-DRB1*							
Minor allele	4	5	5.1	6	9	A	A
Frequency in cases/controls[a]	7%/12%	12%/7%	42%/36%	26%/25%	13%/20%	23%/34%	8%/4%
Minor allele transmitted/untransmitted	13/21	21/13	44/36	35/33	17/29	26/48	15/7
Transmission rate	38%	62%	55%	51%	37%	35%	68%
TDT *P* value	0.172	0.172	0.376	0.815	0.080	0.011	0.091
(b) French population 1, subgroup without *HLA-DRB1* risk alleles							
Minor allele transmitted/untransmitted	5/10	5/4	18/15	18/12	5/10	6/18	3/1
Transmission rate	33%	56%	55%	60%	33%	25%	75%
TDT *P* value	0.200	0.740	0.600	0.270	0.200	0.014	0.317
(c) French population 1, all individuals, controlling for LD with *HLA-DRB1* by conditional logistic regression							
OR (95% CI)[b]	0.59 (0.25–1.34)	1.48 (0.64–3.54)	1.28 (0.77–2.16)	1.23 (0.71–2.17)	0.51 (0.24–1.05)	0.46 (0.25–0.82)	1.2 (0.37–4.15)
P value	0.235	0.428	0.379	0.518	0.072	0.007[d]	0.944
LRT[c] *P* value	0.165	0.319	0.314	0.433	0.048	0.005[d]	0.728

[a]Controls are non-transmitted alleles; [b]odds ratio of transmission of minor allele versus transmission of major allele as determined in logistic regression; [c]likelihood ratio test evaluating model including *HLA-DRB1* alleles S2 and S3P and *MICA*-250 versus S2 and S3P only. For the *HLA-DRB1* locus, allele L was used as reference. [d]*P* value corrected for multiple testing less than 0.05. CI, confidence interval; LD, linkage disequilibrium; TDT, transmission disequilibrium test.

(Table 2). In combined analysis of both French family cohorts, association of *MICA*-250 was comparable with the association in the first French family cohort in standard analysis and in analysis of the subgroup negative for *HLA-DRB1* risk alleles. In conditional logistic regression analysis, association in the combined cohorts was even more significant than in the first cohort alone (Tables 1c and 2c). Additionally, conditional logistic regression was done with a model in which S3P alleles were differentiated into three groups as described [8], accounting for potential differences in risk of these three groups for RA. Within this analysis, 79, 56, and 15 individuals contributed to the S3P*01, S3P*04, and S3P*10 alleles, respectively. This analysis gave similar results (data not shown). Interactions between *MICA*-250 and *HLA-DRB1* alleles were not significant (data not shown). Full details of the regression model are shown in the online supplement (Additional data file 3). When the analysis of the combined first and second French cohorts was restricted to CCP^+ RA, the protective association with *MICA*-250 A was also found (odds ratio [OR] 0.53, 95% confidence interval [CI] 0.33 to 0.83, $P = 0.005$; LRT P value = 0.003).

Association analysis within the case-control cohort

After demonstrating association of *MICA* with RA in French Caucasian family trios and its independence from HLA risk alleles, we analyzed the effect of *MICA* within a German Caucasian case-control cohort. Frequencies of *MICA*-250A were similar within the German and French populations (33% in controls). Again, we found protective association of *MICA*-250A with RA in standard analysis and within the subgroup of the case-control cohort not carrying SE alleles (Tables 3a and 3b). Logistic regression including all individuals demonstrated a significant protective effect as well. Significance in the LRT showed that this association was independent of *HLA-DRB1* risk alleles (Table 3c). Details of the regression model are given in the online supplement (Additional data file 4). Additionally, conditional logistic regression was done with a model in which S3P alleles were differentiated into three groups (S3P*01, S3P*04, and S3P*10) as described [8], accounting for potential differences of these three groups in risk for RA. This analysis resulted in similar results (data not shown).

Table 2

Association of *MICA* polymorphism within the second and combined first and second French Caucasian family cohort

	2nd French family cohort	1st + 2nd French family cohort
(a) All individuals without controlling for LD with *HLA-DRB1*		
Minor allele	A	A
Frequency in cases/controls[a]	27%/32%	25%/33%
Minor allele transmitted/untransmitted	37/46	63/94
Transmission rate	45%	40%
TDT *P* value	0.328	0.015
(b) Subgroup without *HLA-DRB1* risk alleles		
Minor allele transmitted/untransmitted	12/16	18/34
Transmission rate	43%	35%
TDT *P* value	0.450	0.027
(c) All individuals, controlling for LD with *HLA-DRB1* by conditional logistic regression		
OR (95% CI)[b]	0.68 (0.4–1.15)	0.56 (0.38–0.83)
P value	0.158	0.003
LRT[c] *P* value	0.122	0.002

[a]Controls are non-transmitted alleles; [b]odds ratio of transmission of minor allele versus transmission of major allele as determined in logistic regression; [c]likelihood ratio test evaluating model including *HLA-DRB1* alleles S2 and S3P and *MICA*-250 versus S2 and S3P only. For the *HLA-DRB1* locus, allele L was used as reference. Effects of S2 and S3P alleles are presented in the online supplement (Additional data file 3). CI, confidence interval; LD, linkage disequilibrium; TDT, transmission disequilibrium test.

Analysis of linkage disequilibrium

LD was analyzed within parents of the family cohorts and in the case-control cohort. As the German cohort was smaller, power to detect LD was decreased compared with power to detect LD within the French cohorts. Significant LD was found between *HLA-DRB1*-S3P and *MICA*-250A within parents of the French family cohorts ($D' = +0.21$, $P < 0.001$). Interestingly, this LD was positive between *HLA-DRB1* risk alleles of subgroup S3P and the protective allele *MICA*-250A. In-depth analysis of the S3P group revealed that this resulted mainly from LD between *HLA-DRB1**01 and *MICA*-250A, which was significant within parents of the family cohorts and cases from the case-control cohort ($D' = +0.38$ and $+0.25$ with *P* values of 2×10^{-7} and 0.047, respectively). Significant negative LD was found between *HLA-DRB1*-S2 and *MICA*-250A ($D' = -0.51$, $P < 0.01$) in French parents. No significant LD was found between *HLA-DRB1*-L within the family cohorts and individuals of the case-control cohort. In consequence, there was no significant correlation of carriage of *MICA*-250A with carriage of positive or negative SE status. LD was also analyzed between *MICA*-250 and rs1051792, another coding SNP with functional implications [30]. Within a representative sample of 182 French Caucasian and 181 German Caucasian cases and controls, both polymorphisms were in perfect LD ($r^2 = 1$, $D' = 1$).

Representation of association analysis in all informative families controlling for linkage disequilibrium with HLA-DRB1

An advantage of the conditional logistic regression approach is the integration of all data from all informative parents with respect to *HLA-DRB1* and *MICA*. A single statistic reveals independent association of *MICA*-250. However, it is of interest to compare subgroup analysis of parents negative for *HLA-DRB1* risk alleles with results of the regression model analyzing all data in detail (Tables 2b and 2c). A major difference is that the regression model additionally includes information of parents that are informative (that is, heterozygous) for *MICA* and that are also heterozygous for *HLA-DRB1* risk alleles. How can the effect of *MICA*-250 on transmission be represented within these parents, devoid of the effect of *HLA-DRB1* risk alleles? We propose to stratify *HLA-DRB1* heterozygous parents according to their genotype. The transmission ratio under the null hypothesis of no association within these parents will differ from a 50/50 ratio reflecting the different risk levels of both *HLA-DRB1* alleles. However, under the null hypothesis of no association of *MICA*-250, a two-marker haplotype consisting of *MICA*-250A and a certain *HLA-DRB1* allele should have the same transmission rate as a two-marker haplotype consisting of *MICA*-250G and the same *HLA-DRB1* allele. A deviation from this transmission rate repre-

sents an independent effect of *MICA*-250A quantifiable as an OR of *MICA*-250A transmission. As we applied the classification of du Montcel and colleagues [7] of *HLA-DRB1* alleles, three different independent strata of *HLA-DRB1* heterozygote parents exist: S3P/S2, S2/L, and S3P/L. Within all of these strata, we always found a decreased transmission of haplotypes carrying *MICA*-250A compared with the respective haplotype carrying *MICA*-250G (OR 0.33, 95% CI 0.02 to 5.11; OR 0.45, 95% CI 0.04 to 6.76; and OR 0.44, 95% CI 0.04 to 2.73, respectively, data of all families) (Additional data file 5). These observations are consistent with the significant protective association of *MICA*-250A revealed by conditional logistic regression (Table 2).

When we additionally include data from parents homozygous for *HLA-DRB1*, we can analyze the OR of *MICA*-250A on transmission within these parents when we compare the observed transmission ratio of *MICA*-250A versus the expected transmission ratio (Additional data file 5). The expected transmission ratio is 50/50 (transmitted/non-transmitted) within these parents under the null hypothesis of no effect of *MICA*-250A. We now can combine information from all parents informative for *MICA*-250 by combining all four ORs of all four independent strata with exact Mantel-Haenszel methodology. This analysis confirmed a significant undertransmission of *MICA*-250A within all data of all families (OR 0.48, 95% CI 0.25 to 0.91, $P = 0.02$, Fisher exact test).

Discussion

The aim of this study was to analyze the association of polymorphisms of *MICA* with risk for RA while controlling for the effects of *HLA-DRB1* risk alleles. We successfully identified *MICA*-250A as a new independent marker associated with protection from RA susceptibility. We analyzed the association of three genetic variants of the gene *MICA* with susceptibility to RA in a French Caucasian family cohort. In validation studies (including an additional independent French Caucasian family cohort and a German Caucasian case-control cohort), we focused on the non-synonymously coding SNP *MICA*-250 (rs1051794, Lys196Glu). In our first French family cohort, this SNP presented with the strongest evidence for association in terms of *P* values and transmission rate (Table 1). Association of three-marker haplotypes of *MICA* with RA was not statistically significant. Therefore, we did not investigate haplotype association further. However, it cannot be excluded that association of *MICA*-250 with RA may be related to an unknown allelic variant in linkage with these haplotypes as haplotypes were inferred and have error margins.

Within all combined French families, we found a significant undertransmission of *MICA*-250A in the TDT (Table 2). Therefore, we hereby provide evidence for linkage and association of *MICA*-250A with RA. This transmission analysis within trio families would not be affected by hidden population stratification. The association was also evident in conditional logistic regression analyses including all parents informative for *MICA*-250A and controlling for LD with *HLA-DRB1* risk alleles (Table 2c). We did not find any indication that the observed protective effect of *MICA*-250A is especially present on the background of certain *HLA-DRB1* alleles as interaction analyses of *MICA*-250 and *HLA-DRB1* alleles in the regression model did not result in a significantly increased likelihood (data not shown). Additionally, detailed transmission analysis of *MICA*-250 within parents heterozygous or homozygous for *HLA-DRB1* always resulted in a protective effect of *MICA*-250A of comparable magnitude irrespective of present *HLA-DRB1* alleles (Additional data file 5). Analysis of the CCP^+ subset showed that *MICA*-250 also associates with CCP^+ RA. We confirmed the protective effect in a German Caucasian RA case-control cohort (Table 3), which indicates that the protective effect may not be restricted to the French Caucasian population alone.

True association of *MICA*-250 with RA may be either feigned or masked by LD with known risk alleles. Therefore, we controlled for the separate contributions of *MICA*-250 and *HLA-DRB1* alleles (S3P, S2, and L) to the observed effect by logistic regression. This allowed us to make use of data from all patients. However, it could be argued that this logistic regression might be affected by stratification of the individual *HLA-DRB1* risk alleles in the groups used in the model. Hence, we also analyzed the subgroup of patients not carrying *HLA-DRB1* risk alleles. Naturally, this subgroup does not contain data from all patients, but results are completely independent from the excluded *HLA-DRB1* risk alleles. Both methods showed association of *MICA*-250A with RA.

In this context, it is of interest that, within all genome-wide association studies of RA published thus far, *MICA*-250 was found to be nominally associated: *MICA*-250A had a protective effect (OR 0.82, 95% CI 0.73 to 0.92, $P = 0.0008$, not corrected for genome-wide testing) within CCP^+ RA in North American samples [31]. Similar findings result from a genome-wide study in a British RA cohort, in which data for an SNP in perfect LD with *MICA*-250 are available (rs1051792: OR 0.85, 95% CI 0.77 to 0.93, $P = 0.0008$, not corrected for genome-wide testing) [32]. These findings corroborate our observation of a protective effect of *MICA*-250A in CCP^+ RA. *MICA*-250 was also associated with RA in a genome-wide study in a Spanish Caucasian cohort ($P = 0.02$, not corrected for genome-wide testing) [33]. In these genome-wide studies, association analysis was reported without controlling for LD of *MICA* alleles with *HLA-DRB1* alleles. If LD structure in Caucasians in these genome-wide studies was similar to that in our study (that is, if positive LD between *HLA-DRB1**0101 and *MICA*-250A was present), LD-corrected protective association of *MICA*-250A would be even stronger than reported.

The microsatellite polymorphism *MICA*-210 was studied in different populations. In Spanish [15] and Canadian [17] Cau-

Table 3

Case-control analysis in German Caucasians

(a) All individuals without controlling for LD with *HLA-DRB1*	
Minor allele	250A
Allele frequency in cases/controls	22%/33%
Total alleles of RA cases/controls	178/368
OR (95% CI)	0.60 (0.4–0.9)
OR *P* value	0.016
(b) Subgroup without *HLA-DRB1* risk alleles	
Frequency in cases/controls	14%/34%
Total alleles of RA cases/controls	50/216
OR (95% CI)	0.31 (0.1–0.7)
OR *P* value	0.005
(c) All individuals, controlling for LD with *HLA-DRB1* by logistic regression	
OR (95% CI)	0.6 (0.37–0.96)
P value	0.032
LRT[a] *P* value	0.022

[a]Likelihood ratio test evaluating model including *HLA-DRB1* alleles S2 and S3P and *MICA*-250 versus S2 and S3P only. For the *HLA-DRB1* locus, allele L was used as reference. CI, confidence interval; LD, linkage disequilibrium; OR, odds ratio; RA, rheumatoid arthritis.

casians, a protective effect was seen for allele *MICA*-210 6.0, whereas in Korean Asians [16], a protective effect was seen for *MICA*-210 9.0. No association of *MICA*-210 was seen in another Spanish Caucasian RA study [14]. None of these studies additionally analyzed *MICA*-250. However, in our study, strong LD between *MICA*-210 9.0 and *MICA*-250A was found ($D' = 0.98$, $P < 10^{-15}$). Therefore, previous findings in Koreans are in accordance with our results. It is of interest that in this study only a single *HLA-DRB1* RA susceptibility allele (*0405) predominates and no LD was found with *0405 and *MICA*-210 9.0, so that association analysis was hardly influenced by linkage with known *HLA-DRB1* risk alleles. This is different from the Caucasian studies of the (GCT)n polymorphism: In our data, we found considerable LD between various *MICA*-210 alleles and *HLA-DRB1* risk alleles (data not shown). We might speculate that complex LD structure between *MICA*-210 alleles and *HLA-DRB1* alleles may at least partially explain differing results in Caucasian association studies of *MICA*-210 and RA. This is especially relevant as these studies either did not account at all or only partially accounted for LD with *HLA-DRB1* alleles.

In recently published work, *HLA-DRB1*-matched cases and controls were analyzed mainly in American Caucasians in order to identify genetic factors associated with CCP^+ RA in addition to known *HLA-DRB1* risk alleles [4]. Within the *MICA* genomic region, significant evidence for independent association with RA was found with a maximum association within HLA-C. This association was attributed to the risk of the A1-B8-*DRB1**03 haplotype. Additionally, haplotypes carrying *HLA-DRB1**0404 were described to be *HLA-DRB1*-independent risk factors. An analysis of *MICA*-250 was not reported in this study. There is evidence that association of *MICA*-250A in our data represents an additional disease-modifying factor, independent of described risk factors in the American Caucasian study. This evidence results from the observation that a protective association of *MICA*-250A is still observed when all parents carrying either *HLA-DRB1**03 or *HLA-DRB1**0404 were excluded (OR 0.56, 95% CI 0.35 to 0.89, $P = 0.013$; LRT P value = 0.009).

Generally, an observed association of a polymorphism with a phenotype need not arise from a direct functional effect of this polymorphism. It may simply originate from LD with a functional polymorphism. Therefore, it is of interest that the amino acid change due to *MICA*-250A (Lys196Glu) is predicted to influence Hsp70 binding [34]. Possibly even more relevant, SNP rs1051792, in perfect LD with *MICA*-250 in Caucasian HapMap data and in our data, was experimentally shown to influence binding of the NKG2D receptor [30]. Variant rs1051792A, corresponding to *MICA*-250A, was shown to strongly bind NKG2D. All other alleles lead to weaker binding. Several studies show that NKG2D expression is modulated by MICA expression level with consequences for immune reactions. Wiemann and colleagues [35] showed that persistent expression of MICA in transgenic mice resulted in downregulation of the amount of surface NKG2D. As a consequence, impaired immune reaction against bacteria and MICA-expressing tumors was observed. In a different context, Mincheva-Nilsson and colleagues [36] observed elevated levels of soluble

MICA/MICB and a decreased level of NKG2D within maternal blood of healthy pregnant women. The authors showed that soluble MICA/MICB downregulates NKG2D levels and immune reactions [36]. Therefore, we speculate that an increased affinity of MICA to NKG2D, as must be present in carriers of *MICA*-250A, may have similar effects as increased expression of MICA, resulting in decreased NKG2D expression levels.

In this context, the observation of RA remission during pregnancy may be of interest [37]. Apparently, decrease of NKG2D plays a central role in decreased immune response. During pregnancy, this seems to be triggered by increased levels of MICA/MICB and appears to contribute to tolerance against the fetus and disease remission in women with RA. As pregnant women show both downregulation of NKG2D due to increased MICA expression and remission of RA, it can be speculated that there may be a functional link between these two observations. If *MICA*-250A reports on stronger binding of MICA and if this also results in downregulation of NKG2D levels, this would be consistent with the observed protective effect of *MICA*-250A in our data. As there are many links between the innate and adaptive immune systems and involvement of pathogens in the initiation of RA is discussed (reviewed by Falgarone and colleagues [38]), differences in NKG2D levels induced by functional variants of MICA are not unlikely to have consequences for RA etiology.

Conclusions

In summary, we present evidence for linkage and association of *MICA*-250 (rs1051794) with RA independently of known *HLA-DRB1* association in French Caucasians and evidence for association in a German Caucasian population, suggesting *MICA* as an RA susceptibility gene. The association might be explained by functional evidence of rs1051792, an SNP in perfect LD with *MICA*-250. However, more studies within other populations are necessary to prove the general relevance of this polymorphism with RA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HK helped to carry out the molecular genetic studies, performed acquisition of the data, helped to perform analysis and interpretation of the data, and drafted the manuscript. HH and JB helped to carry out the molecular genetic studies. MS, DHe, BP, CP, EP-T, and FC helped to perform analysis and interpretation of the data. DHa and PA helped to perform analysis and interpretation of the data and to draft the manuscript. VHT and UW (and the European Consortium on Rheumatoid Arthritis Families) contributed to the recruitment of families and to the acquisition of clinical data. FE and US helped to draft the manuscript. All authors read and approved the final manuscript.

Additional files

The following Additional files are available online:

Additional data file 1

A table listing the distribution of *HLA-DRB1* alleles in the analyzed RA cohorts.
See http://www.biomedcentral.com/content/supplementary/ar2683-S1.pdf

Additional data file 2

Background information for the conditional logistic regression method applied for family based analysis.
See http://www.biomedcentral.com/content/supplementary/ar2683-S2.pdf

Additional data file 3

A table providing detailed results of conditional logistic regression models of all French families.
See http://www.biomedcentral.com/content/supplementary/ar2683-S3.pdf

Additional data file 4

A table providing detailed results of logistic regression models of the German case control cohort.
See http://www.biomedcentral.com/content/supplementary/ar2683-S4.pdf

Additional data file 5

A table providing a representation of association analysis in all informative families controlling for LD with DRB1.
See http://www.biomedcentral.com/content/supplementary/ar2683-S5.pdf

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Association of the X-Chromosomal Genes *TIMP1* and *IL9R* with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is an inflammatory joint disease with features of an autoimmune disease with female predominance. Candidate genes located on the X-chromosome were selected for a family trio-based association study.

Methods. A total of 1452 individuals belonging to 3 different sample sets were genotyped for 16 single-nucleotide polymorphisms (SNP) in 7 genes. The first 2 sets consisted of 100 family trios, each of French Caucasian origin, and the third of 284 additional family trios of European Caucasian origin. Subgroups were analyzed according to sex of patient and presence of anti-cyclic citrullinated peptide (anti-CCP) autoantibodies.

Results. Four SNP were associated with RA in the first sample set and were genotyped in the second set. In combined analysis of sets 1 and 2, evidence remained for association of 3 SNP in the genes *UBA1*, *TIMP1*, and *IL9R*. These were again genotyped in the third sample set. Two SNP were associated with RA in the joint analysis of all samples: rs6520278 (*TIMP1*) was associated with RA in general ($p = 0.035$) and rs3093457 (*IL9R*) with anti-CCP-positive RA patients ($p = 0.037$) and male RA patients ($p = 0.010$). A comparison of the results with data from whole-genome association studies further supports an association of RA with *TIMP1*. The sex-specific association of rs3093457 (*IL9R*) was supported by the observation that men homozygous for rs3093457-CC are at a significantly higher risk to develop RA than women (risk ratio male/female = 2.98; $p = 0.048$).

Conclusion. We provide evidence for an association of at least 2 X-chromosomal genes with RA: *TIMP1* (rs6520278) and *IL9R* (rs3093457). (J Rheumatol First Release xxxxx; doi:10.3899/jrheum.090059)

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Rheumatoid arthritis (RA) is an inflammatory joint disease with features of an autoimmune disease and a prevalence of about 1% in the European Caucasian population[1]. There is evidence for genetic influences on RA and heritability is estimated to be 60%[2]. Female sex is a well known risk factor for RA. The female to male ratio ranges between 3 and 4[3]. There may be a link between heritability and sex, as the female genome differs crucially from the male genome. The Y chromosome supplies males with several genes absent in the female[4], while incomplete X-inactivation or varying inactivation patterns may lead to gene-dosage skewing in females[5]. X-chromosomal abnormalities were observed in immunological diseases, e.g., a significantly higher rate of acquired X-monosomy[6] and significantly skewed X-inactivation[7]. Patients with Turner's syndrome are known to manifest common autoimmune features[8]. Whole-genome linkage studies for RA suggest among others the presence of loci of interest on chromosome X[9,10]. Thus, X-chromosomal genes are highly relevant candidate genes to test for association with RA.

For this study we selected 7 genes, *CD40LG*, *CD99*, *EIF2S3*, *IL9R*, *TIMP1*, *UBA1*, and *XIAP* (Table 1). Most of these genes are involved in pathways thought to be crucial for RA etiology and evidence for their involvement in other immunological diseases exists as well.

CD99 and *IL9R* are situated within pseudoautosomal regions and have a functional homologue on the Y chromosome, whereas the other genes are restricted to the X chromosome. To our knowledge none of the genes we selected, with the exception of *TIMP1*, has been investigated for association with RA in candidate gene studies.

CD40LG is involved in the regulation of B cell functions and the production of autoantibodies[11]. CD99 is described to play a role in transport regulation of MHC class I molecules[12], lymphocyte adhesion[13], and induced T cell death[14]. EIF2S3 is the γ-subunit of the eukaryotic translation initiation factor (EIF2) and is only partially affected by X-inactivation[15]. EIF2 is involved in stress responses and apoptosis[16]. Insufficient apoptosis of inflammatory cells in synovialis as well as increased apoptosis, especially within the synovial lining, has been demonstrated in RA[17,18]. IL9R is a receptor for the cytokine interleukin 9 (IL-9) expressed on many hematopoietic cells including T cells[19], and it is also involved in early T cell development[20]. *TIMP1* protects extracellular matrix from degradation by inhibiting metalloproteinases (MMP)[21]. Secretion of MMP is required for the initial stage of angiogenesis[22], contributing to pannus formation in RA[23]. *TIMP1* (SNP rs5953060) was described to be associated with RA in a small Japanese cohort[24] and has also shown association with other immunity disorders like Crohn's disease[25] and systemic sclerosis[26]. UBA1 (also known as UBE1) catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation[27]. Involvement of UBA1 in cell-cycle regulation and apoptosis can be demonstrated and provides a functional link to RA[28]. XIAP is a potent inhibitor of apoptosis and is involved in regulation of lymphocyte homeostatis[29].

Our aim was to investigate genetic variants of selected X-chromosomal genes in a candidate gene association study based on a family-trio approach in a European Caucasian population.

Table 1. Selected genes in order of chromosomal location (short arm p to long arm q). Data were acquired using Entrez Gene and Entrez Protein databases, as well as the UCSC Genome Browser Build March 2006. rs numbers according to dbSNP Build 127.

Gene	Name	Locus	SNP Investigated	Position/Type of Variation	Pseudo-autosomal	Inactivation Status[59]	Published Disease Associations
CD99	CD99 molecule	Xp22.32	rs311071 rs312258	Intronic Intronic	Yes	Not inactivated	—
EIF2S3	Eukaryotic translation initiation factor 2 subunit 3	X922.2–p22.1	rs16997659 rs12556742 rs12847067	Coding, nonsynonymous Intronic 3' downstream	No	Partial inactivation	—
TIMP1	Tissue inhibitor of metalloproteinase 1	Xp11.23	rs4898 rs6520278 rs5953060	Coding, synonymous Intronic Intronic	No	Partial inactivation	Rheumatoid arthritis[24] Asthma[57] Crohn's disease[25] Systemic sclerosis[26]
UBA1	Ubiquitin-like modifier activating enzyme 1	Xp11.23	rs4239963 rs2070169 rs4529579	Intronic Coding, nonsynonymous Intronic	No	Partial inactivation	—
XIAP	X-linked inhibitor of apoptosis	Xq25	rs7878958 rs7053190 rs9856	5' upstream Intronic Coding, 3' UTR	No	Unknown	—
CD40LG	CD40 ligand	Xq26	rs3092936	Intronic	No	Not inactivated	Systemic lupus erythematosus[11]
IL9R	Interleukin 9 receptor	Xq28	rs3093457 rs1973881	Intronic Intronic	Yes	Not activated	Asthma[58]

MATERIALS AND METHODS

Three sets of family trios, RA patient (i.e., the affected individual) and both parents, were genotyped. Detailed characteristics of the first 2 and parts of the third set have been described[30]. Briefly, the first 2 sets consisted of 100 family trios of French Caucasian origin. The third set consisted of 284 additional European Caucasian families, from France, Germany, Italy, Portugal, Spain, The Netherlands, and Belgium. All affected individuals fulfilled the American College of Rheumatology 1987 revised criteria for RA[31]. In addition the status of anti-cyclic citrullinated peptide autoantibodies (anti-CCP, also known as ACPA) was available for French and German RA patients (CCP-positive, n = 226; CCP-negative, n = 73). In our multistage approach all SNP were genotyped in the first sample set ("exploration set"). Markers with a significant association with RA (uncorrected $p < 0.05$) were then genotyped in the second sample set ("replication set"). When evidence increased in favor of an association, i.e., the p value of the association decreased in the combined analysis of both sets, markers were genotyped again in the third sample set (the multinational European replication set).

Genomic DNA was purified from fresh peripheral blood leukocytes or from Epstein-Barr virus-transfected cell lines using standard methods.

SNP were chosen based on their position in the gene, depending on gene length and validation status. Information from public databases (PupaSNP, UCSC Genome Browser, Ensembl) was used to aid in SNP selection. Selected SNP are listed in Table 1.

Genotyping was carried out using the genoSNIP technique (Bruker Daltonics, Billerica, MA, USA)[32]. Polymerase chain reaction primers were designed using MuPlex Vs 2.2. SBE-primer design was carried out using PrimExtend, an in-house software tool based on CalcDalton[33]. Primer sequences are shown in Table 2.

Samples of the third set were genotyped by applying a TaqMan 5' allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocols.

For quality control purposes Mendelian laws of inheritance and Hardy-Weinberg equilibrium (HWE) in nontransmitted controls had to be fulfilled ($p > 0.01$). HWE analysis for nonpseudoautosomal genes was carried out in healthy female controls (mothers) only. All genotyping results fulfilled the quality control criteria. Genotype call-rate was more than 95%.

Statistical analysis. HWE was investigated using a chi-square test with 1 degree of freedom. Linkage and association analyses were performed using the transmission disequilibrium test (TDT)[34] and the genotype relative risk (GRR) test[35]. The TDT compares the transmission of SNP alleles from het-

Table 2. Polymerase chain reaction (PCR) primer pairs and genotyping primers used in this study.

Gene	SNP (rs number)	PCR Forward Primer	PCR Reverse Primer	SBE Primer
CD40LG	rs3092936	ACGTTGGATGCAGGCCTTATTA TCTCCATT	ACGTTGGATGCAGTTCCCAG GGTAGAATC	bioTCCTTGGCTTTGACAG ATATTG
CD99	rs311071	ACGTTGGATGATACAGAGACAG GAAGTGGG	ACGTTGGATGGTGCTTTGCA AATACTGTGT	bioACTGTGTGTTTTA(L) AAACGGAAGG
CD99	rs312258	ACGTTGGATGGCTGGGATTTGG AAGAAG	ACGTTGGATGTCATTAGCCA GTACATTGGT	bioTGTGTGTGG(L)GCCC TCGT
EIF2S3	rs16997659	ACGTTGGATGACACCTGACGAG TTTCCTAC	ACGTTGGATGCACCGTTTAA CACCTCGA	bioAGCAAAAGAGAG(L)T CACCTGACTAAT
EIF2S3	rs12556742	ACGTTGGATGGTGCTAGGATTA CAGGCG	ACGTTGGATGATTTCTACCT CATTGGAAATAC	bioTTACCTACAAATG(L) CAGTTGAATGAT
EIF2S3	rs12847067	ACGTTGGATGTGGATGGGATCA ATGTACA	ACGTTGGATGGCCTTTCTGG ATTAGATTTAAT	bioGGATGGGAT(L)AATG TACAGATGACC
IL9R	rs3093457	ACGTTGGATGCCACTGTGGCAT TTGAGA	ACGTTGGATGCCTACAGGAC TGGAAATTAGT	bioAAATTTTGCCATCTC(L)AAGAGATAC
IL9R	rs1973881	ACGTTGGATGCAACCGGCCTGT TACCA	ACGTTGGATGCTGGCTCCTG ACACCTCTC	bioCTGTGAA(L)TTCCCG AGGGC
UBA1	rs4239963	ACGTTGGATGGTGCTATGGTTT CTATGGTTA	ACGTTGGATGGTCCTGGTTA AAGGAAGAGT	bioAGGAAGAGTGAGTCTC (L)GAAACAG
UBA1	rs2070169	ACGTTGGATGAGTGCCTCCAGG TATGTG	ACGTTGGATGCAGGTCTGAG CCAAACAC	bioTCCTCCCTTT(L)CAT TCCTTAGC
UBA1	rs4529579	ACGTTGGATGGCTGGTTATTTA TTTGTCATG	ACGTTGGATGGCATCTAGTG GGCAGAAGT	bioTTGTC(L)TGGGAGAG GGGATG
TIMP1	rs6520277	ACGTTGGATGATTCCTCACAGC CAACAG	ACGTTGGATGAGCTCAGCCA ATCACAAG	bioTGCACATCAC(L)ACC TGCAGTTT
TIMP1	rs6520278	ACGTTGGATGGAAGTAGCAGGG GAAGGAT	ACGTTGGATGTGTTCTGGGC TCTGTGTC	bioCTACCCTGCAGGT(L) AGCCCTT
TIMP1	rs5953060	TTCAGTCTATCAGAAGGCC	CAAGAGTCCATCCTGCAGT	bioTGGCT(L)AGCTGCCA AGCTG
XIAP	rs7878958	ACGTTGGATGGTTCATATCTCC CAGTTGAC	ACGTTGGATGTCCTGCTAGA ATATAAGCTCT	bioCACAAGGATCCT(L)G TTTTGTTCA
XIAP	rs7053190	ACGTTGGATGTACGCAGTGAGT GGCATT	ACGTTGGATGTGTCCAGAAT AGGCAAGTC	bioAGACAGAAAGTAGA(L)TACTGGTTGCC
XIAP	rs9856	ACGTTGGATGCAAATTTAGTTG AGCTTTCTAAG	ACGTTGGATGGCTGAGGAAG AAATTCACA	bioCTGTATGAGTCAAACT GAAA(L)TGATTATT

bio: biotinylated group; (L): photo-cleavable base.

erozygous parents to affected offspring with a transmission ratio of 50% as expected by Mendel's law. The GRR test compares differences in genotype distribution between RA cases and "virtual controls" reconstructed from nontransmitted parental alleles. Haploview 4.1 software was used for gene-wide haplotype analysis[36]. Tests were also done in sample sets stratified for sex or anti-CCP status of RA patients. We used a 2-tailed test of interaction to assess significance of differences between subgroups[37].

For nonpseudoautosomal genes XTDT was applied as implemented in Haploview 4.1[38]. As proposed[39], males were treated like homozygous females for comparing allele frequencies by allele counting. Additionally, for GRR tests (Lathrop tests) only maternal reconstructed control genotypes and genotypes from corresponding affected female children were included.

RESULTS

In the first set, consisting of 100 French Caucasian family trios, 3 genes, *IL9R*, *TIMP1*, and *UBA1*, showed evidence for association. SNP with evidence for association were again genotyped in the second French Caucasian family trio set (100 additional trios). A combined analysis of set 1 and set 2 revealed a decreased p value for 3 markers. These SNP, rs4239963 (*UBA1*), rs6520278 (*TIMP1*), and rs3093457 (*IL9R*), were genotyped in the third European Caucasian sample set (284 additional trios). These data are summarized in Tables 3, 4, and 5. Details of family trio-based association analysis for all markers are shown in Tables 6, 7, and 8.

While the *UBA1* SNP rs4239963 showed significant association with RA in the first 2 sample sets, it was not found to be associated with RA in the combined analysis of all 3 sets, although the trend was the same as in sets 1 and 2, with the minor allele (C) being undertransmitted (Table 3).

Table 3. Results of family-trio TDT analysis for UBA1 (rs4239963, minor allele C): results in all samples in a given sample set; Minor allele transmissions no. of transmitted alleles: untransmitted alleles) are shown.

	Set 1	Set 1 & 2	Set 1 & 2 & 3
No. of informative families	31	69	192
Minor allele transmission	10:21	21:48	86:106
TDT p value	0.048	0.001	0.149

Table 4. Results of family-trio TDT analysis for *TIMP1* (rs6020277, minor allele C; rs6520278, minor allele T). Minor allele transmissions (no. of transmitted alleles: untransmitted alleles) are shown.

rs6520277	Set 1, male	Set 1 & 2, male	Set 1 & 2 & 3, male
No. of families	6	9	NI
Minor allele transmission	0:6	1:8	NI
TDT p value	0.014	0.020	NI
rs6520278	Set 1	Set 1 & 2	Set 1 & 2 & 3
No. of informative families	45	78	189
Minor allele transmission	15:30	29:49	80:109
TDT p value	0.025	0.024	0.035
rs6520278	Set 1, male	Set 1 & 2, male	Set 1 & 2 & 3, male
No. of informative families	7	9	21
Minor allele transmission	0:7	1:8	9:12
TDT p value	0.008	0.020	0.513

Male subgroup: family trios with male patients. NI: not investigated.

In contrast, SNP rs6520278 (*TIMP1*) was found to be significantly associated with RA in general, which is indicated by significant p values in the combined analysis of all 3 sets (p = 0.035; Table 4). The TDT showed the minor allele T was undertransmitted. Association of rs6520278 in families with male offspring could not be replicated in the European replication set. Additionally, the test of interaction revealed no significant difference between female and male subgroups for the SNP, as effect sizes (GRR minor vs major genotype) of the 2 subgroups did not differ significantly (p for interaction = 0.071). Another SNP, rs6520277 of *TIMP1*, also showed significant p value in families with male offspring in set 1, but this result could not be replicated in the second sample set.

SNP rs3093457 of *IL9R* was found to be significantly associated with RA in 2 subgroups in the combined analysis: families with anti-CCP-positive patients (p = 0.037) and families with male patients (p = 0.010), while an association of rs3093457 was only marginally significant in all family trios (p = 0.056; Table 5) and was not significant in families with female RA patients. In both subgroups the association was due to an increase of the homozygous minor genotype rs3093457-CC in RA cases. We also performed an interaction test to identify specific effects concerning anti-CCP status and/or sex. Comparing the GRR of rs3093457-CC for male and female subgroups revealed that the SNP affected males significantly more than females (p = 0.048). GRR in families with male offspring was about 3 times greater in the combined analysis of all sample sets (ratio of male/female GRR 2.98, 95% CI 1.01–8.79; Table 9). No significant difference between effect sizes was observed for anti-CCP-positive and negative subgroups.

DISCUSSION

We investigated SNP in 7 X-chromosomal genes for association with RA and were able to detect evidence for association for markers of 2 genes, *TIMP1* and *IL9R*. SNP rs6520278 of *TIMP1* showed a significant association in the combined analysis of all 3 sets (n = 484 family trios), with the minor T-allele being undertransmitted in RA patients (affected children), indicating a protective effect for this allele.

SNP rs6520278 was measured directly in at least 3 whole-genome association studies (WGAS) [the Spanish Upstream Regulatory Region study[40]; the British Wellcome Trust Case-Control Consortium (WTCCC) study[39]; the North American Rheumatoid Arthritis Consortium and Swedish Epidemiological Investigation of Rheumatoid Arthritis[41] studies], but p values were not significant. This might be due to disease heterogeneity or, if the analyzed variant is not a causative variant, to differences in the link-

Table 5. Results of family-based case-control analysis of *IL9R* (rs3093457, minor allele C).

	Set 1	Set 1 & 2	Set 1 & 2 & 3
No. of cases	89	180	437
Homozygous minor genotype vs others (Lathrop) p value	0.028	0.020	0.056
Minor allele GRR (95% CI)	2.34 (1.1–5)	1.96 (1.2–3.4)	1.46 (1–2.1)
	Set 1, male	Set 1 & 2, male	Set 1 & 2 & 3, male
No. of cases	12	20	55
Homozygous minor genotype vs others (Lathrop) p value	0.013	0.005	0.01
Minor allele GRR (95% CI)	15.49 (1.8–130.9)	11.23 (2.1–60)	3.75 (1.4–10.2)
	Set 1, a-CCP+	Set 1 & 2, a-CCP+	Set 1 & 2 & 3, a-CCP+
No. of cases	68	132	209
Homozygous minor genotype vs others (Lathrop) p value	0.019	0.008	0.037
Minor allele GRR (95% CI)	2.71 (1.2–6.2)	2.33 (1.2–4.3)	1.76 (1–3)

Male subgroup: family trios with male patients; a-CCP+: subgroups positive for anti-cyclic citrullinated peptide antibodies, i.e., family trios with anti-CCP-positive patients.

age disequilibrium of the various sample groups. On the other hand, we found several markers in the WGAS in proximity (± 200 kb, as proposed[40]) to *TIMP1* associated with RA (Table 10) at the single-marker level.

UBA1 and *TIMP1* are both situated on the same chromosomal band (Xp11.23) and about 370 kb apart. We could not confirm an association of the *UBA1* gene with RA in the analysis of all 3 sample sets. However, in WGAS several SNP near the gene showed significant p values as well (Table 10). Given the proximity of *UBA1* and *TIMP1*, these data might indicate the presence of causative variants in this chromosomal region.

Linkage disequilibrium (correlation of alleles of 2 polymorphisms in a given population) was examined between SNP associated with RA in our study and SNP in proximity that are also associated with RA in WGAS. Because SNP data for rs4239963 (*UBA1*) were not available from HapMap (release 23) and the *IL9R* region was not covered by the cited WGAS, only *TIMP1* could be investigated. The SNP rs760580 correlated with rs6520278 of *TIMP1* as shown by high D' (0.545) and r^2 (0.222). Moreover, SNP rs760580 was associated with RA in the WTCCC study at the single-marker level (p = 0.044) and showed a protective effect of the minor allele, as did rs6520278.

TIMP1 SNP rs5953060 was described to be associated with RA in a small Japanese cohort (p = 0.02)[42]. While we could not replicate this association (p = 0.228; Table 6), we found linkage disequilibrium between rs5953060 and rs65020278 (D' = 1, r^2 = 0.607). Therefore it appears possible that rs5953060 in the Japanese study reflects association of the same unknown causative locus in the *TIMP1* region as did rs6520278 in our study due to different linkage disequilibrium among populations.

We did not find a significant sex-specific effect of rs6520278, although another *TIMP1* SNP investigated in our study, rs6520277, did hint at sex-specific effects of the gene. This SNP was significantly associated with RA in families with male children in the first set and in the combined analysis of the first and second sets. However, the small number of informative families of male RA patients did not allow for final conclusions. Further investigations are required to clarify possible sex-specific effects of *TIMP1*.

TIMP1 could influence the etiology of RA in several ways. It inhibits MMP[43,44] and subsequently prevents the degradation of cartilage[22]. The inhibition of MMP also may inhibit angiogenesis required for pannus formation[23,45]. A genetic association of *TIMP1* with RA therefore supports the hypothesis that modified angiogenesis might play an important role in the etiology of RA due to altered regulation of MMP via their interactions with TIMP1.

Synovial endothelial cells of patients with RA secrete decreased levels of TIMP1[46]. Levels of TIMP1 expression are affected by X-chromosomal inactivation[47,48], but *TIMP1* partially escapes X-chromosomal gene silencing[49]. *TIMP1* variants may also lead to differences in the level of expression, e.g., SNP might be involved in incomplete gene silencing or in other regulatory mechanisms. It remains to be seen whether allele-specific effects contribute to differences in TIMP1 expression.

Another SNP associated with RA in our study was rs3093457 in the *IL9R* gene. SNP near *IL9R* were not investigated in any of the WGAS, thus our findings are the only data available for this gene and this region. The homozygous minor genotype CC was marginally increased in all cases (p = 0.056) and was significantly increased in the anti-CCP-positive subgroup (p = 0.037) and in male RA patients (p = 0.01). The interaction test result further supports the sex-specificity of the association with males, who are 3 times more affected by this genotype than females. Sex-specific effects for *IL9R* have been described as well for bipolar disorder as well as childhood wheezing, an asthma characteristic, with associations limited to males[50,51]. The observed association of the X-chromosomal *IL9R* with RA would therefore provide further evidence for sex-specific disease mechanisms in RA.

There are several possibilities for *IL9R* involvement in the etiology of RA. Different *IL9R* splice variants affect the

Table 6. Results of TDT analysis for SNP in family trios of set 1.

SNP	Gene	TDT p value	Allele	Transmission Ratio
All family trios				
rs3092936	CD40LG	0.595	C	9:6
rs311071	CD99	0.569	T	41:36
rs312258	CD99	0.092	G	47:32
rs16997659	EIF2S3	0.819	C	10:9
rs12556742	EIF2S3	0.731	G	18:16
rs12847067	EIF2S3	0.739	G	19:17
rs3093457	IL9R	0.087	C	46:31
rs1973881	IL9R	0.887	G	25:24
rs4239963	UBA1	0.048	G	21:10
rs11558783	UBA1	0.513	G	12:9
rs4529579	UBA1	0.435	T	23:18
rs6520277	TIMP1	0.307	T	27:20
rs6520278	TIMP1	0.025	C	30:15
rs5953060	TIMP1	0.228	C	26:18
rs7878958	XIAP	0.758	C	22:20
rs7053190	XIAP	0.578	T	16:13
rs9856	XIAP	0.773	G	25:23
Family trios with male offspring				
rs3092936	CD40LG	1	—	0:0
rs311071	CD99	0.166	T	9:4
rs312258	CD99	0.206	G	7:3
rs16997659	EIF2S3	1	—	1:1
rs12556742	EIF2S3	0.564	G	2:1
rs12847067	EIF2S3	0.564	G	2:1
rs3093457	IL9R	0.011	C	9:1
rs1973881	IL9R	0.248	A	4:2
rs4239963	UBA1	0.564	G	2:1
rs11558783	UBA1	0.564	G	2:1
rs4529579	UBA1	0.655	C	3:2
rs6520277	TIMP1	0.014	T	6:0
rs6520278	TIMP1	0.008	C	7:0
rs5953060	TIMP1	0.103	C	5:1
rs7878958	XIAP	0.655	C	3:2
rs7053190	XIAP	0.655	T	3:2
rs9856	XIAP	1	—	2:2
Family trios with female offspring				
rs3092936	CD40LG	0.595	C	9:6
rs311071	CD99	1 > 0.1	—	32:32
rs312258	CD99	0.232	G	40:30
rs16997659	EIF2S3	0.808	C	9:8
rs12556742	EIF2S3	0.857	G	16:15
rs12847067	EIF2S3	0.862	G	17:16
rs3093457	IL9R	0.232	C	40:30
rs1973881	IL9R	0.647	G	23:20
rs4239963	UBA1	0.059	G	19:9
rs11558783	UBA1	0.637	G	10:8
rs4529579	UBA1	0.250	T	22:15
rs6520277	TIMP1	0.758	T	22:20
rs6520278	TIMP1	0.194	C	23:15
rs5953060	TIMP1	0.631	C	21:17
rs7878958	XIAP	0.746	C	20:18
rs7053190	XIAP	0.683	T	13:11
rs9856	XIAP	0.763	G	23:21

influence of IL-9, because they differ in IL-9-binding abilities[52]. Expression of IL-9 was shown to be correlated with inflammation events and infiltration of lymphocytes in allergic diseases[53]. The STAT pathway is the main signaling pathway of IL-9/IL-9R[54], and its role in RA is discussed[55]. *IL9R* is also involved in early T cell development[20], which is relevant for RA, as the balance between autoreactive T cells and regulatory T cells is essential for immune tolerance.

Table 7. Results of TDT analysis for selected SNP in family trios sets 1 and 2 combined.

SNP	Gene	TDT p value	Allele	Transmission Ratio
All family trios				
rs3093457	IL9R	0.191	C	83:67
rs4239963	UBA1	0.001	G	48:21
rs6520277	TIMP1	0.131	T	50:36
rs6520278	TIMP1	0.024	C	49:29
Family trios with male offspring				
rs3093457	IL9R	0.071	C	11:4
rs4239963	UBA1	0.157	G	6:2
rs6520277	TIMP1	0.020	T	8:1
rs6520278	TIMP1	0.020	C	8:1
Family trios with female offspring				
rs3093457	IL9R	0.391	C	73:63
rs4239963	UBA1	0.003	G	42:19
rs6520277	TIMP1	0.365	T	43:35
rs6520278	TIMP1	0.118	C	41:28

Table 8. Results of TDT analysis for selected SNP in family trios of sets 1, 2, and 3 combined.

SNP	Gene	TDT p value	Allele	Transmission Ratio
All family trios				
rs4239963	UBA1	0.149	G	106:86
rs6520278	TIMP1	0.035	C	109:80
Family trios with male offspring				
rs4239963	UBA1	0.853	C	15:14
rs6520278	TIMP1	0.513	T	12:9
Family trios with female offspring				
rs4239963	UBA1	0.056	G	91:67
rs6520278	TIMP1	0.023	C	96:67

Table 9. Results of interaction test for IL9R (rs3093457) in subgroups of the combined set 1 & 2 & 3.

	Male		Female
No. of cases	55		382
Minor allele GRR (95% CI)	3.75 (1.4–10.2)		1.26 (0.8–1.9)
Ratio of GRR (95% CI)		2.98 (1.01–8.79)	
p		0.048	
	a-CCP+		a-CCP–
No. of cases	209		72
Minor allele GRR (95% CI)	1.76 (1.0–3)		1.05 (0.3–3.1)
Ratio of GRR (95% CI)		0.6 (0.16–2.17)	
p		0.216	

GRR: genetic relative risk. Male and female subgroups: family trios with male or female patients; a-CCP+ and a-CCP– subgroups: anti-CCP-positive and negative subgroups, i.e., family trios with anti-CCP-positive or negative patients.

We provide evidence suggesting association of 2 X-chromosomal genes, *TIMP1* and *IL9R*, with RA. As in other studies of RA[39], the effects of the observed associations were modest. This might be a reason that only nominal significance was achieved. However, our multistage approach analyzing and combining multiple study cohorts allowed testing for such modest genetic effects[56]. It is necessary to verify the associations we observed in additional larger cohorts.

While our findings might not explain the female predominance in RA, they point out that different disease mechanisms might exist in females and males. To elucidate the genetic background of complex diseases such as RA it might be beneficial to consider sex-specific effects, e.g., using sex-stratified sample subsets for association studies.

Table 10. Comparison of SNP analyzed in our study with results of genome-wide studies. Minimum regional p value is the lowest significant p value of markers in a region ± 200 kb near a gene investigated in our study. Positions on chromosome X were according to dbSNP built 127. The following genome-wide data input was used: URR global data, NARAC/EIRA all available data, WTCCC RA cases versus CTL (58C, NBS = normal controls) for chromosomes 23 (= X) and 24 (pseudoautosomal X genes).

Gene	URR Minimum Regional	NARAC/EIRA Minimum	WTCCC Minimum Regional
CD40LG	0.095	0.035 [2]	NI
CD99	0.667	0.184	0.0004 [10]
EIF2S3	0.312	0.090	0.024 [2]
IL9R	NI	NI	NI
UBA1	0.044(1)*	0.021 (2)*	0.111
TIMP1	0.0065(3)*	0.058	0.0057(8)*
XIAP	0.021(3)*	0.160	0.0060(*)

* Number of regional SNP with significant p value; NI: no regional SNP investigated in whole-genome association studies; WTCCC: Wellcome Trust Case-Control COnsortium; NARAC: North American Rheumatoid Arthritis Consortium; EIRA: Epidemiological Investigation of Rheumatoid Arthritis; URR: Upstream regulatory region.

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Chapter 9

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