SAMI-BARNA KANAAN

X-LINKED GENETIC FACTORS BEHIND GENDER BIAS IN RHEUMATOID ARTHRITIS

DISSERTATION

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SAMI-BARNA KANAAN

FACTEURS DE RISQUE LIES AU CHROMOSOME X A L'ORIGINE DE LA PREDOMINANCE DES FEMMES DANS LA POLYARTHRITE RHUMATOÏDE

THESE SOUMISE A

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DEDICATION

I would like to dedicate this thesis to my future self.

Hopefully, one day, I will develop my career as a scientist in immunology (or perhaps genetics of immunology), a field for which I have grown a passion over the last few years. This is due in big part to the excellent academic atmosphere in the scientific park of Luminy, at the Aix-Marseille University (France), and to the awesomeness of all the people that have had outstanding influence on me during this journey (see acknowledgements).

This day, I will be sipping my coffee at my senior professor desk, and I will be thinking, as I remember this dissertation: “thank you past self, high five!”

“Let us suppose you are walking along and you see lightning strike the ground, and just at the place and moment where it hits, a basketball appears out of nowhere. That would be pretty amazing, and you would probably call it a magical event. However, let us suppose that you make the same trip every day, and that every time lightning strikes a certain place, a basketball appears – that you could logically call science. Let me explain what I mean. Sometimes it would appear that the only difference between science and magic is that science repeats.”

–Louis J. DeFelice (Biologist)

ABSTRACT (ENGLISH)

Women are at higher risk compared to men in most autoimmune diseases. Several hypotheses have been proposed to explain this gender bias. The aim of my PhD is to understand female predominance in rheumatismal autoimmune disease, with special focus on Rheumatoid Arthritis (RA) and on the X chromosome. This chromosome is of particular interest in this context as it harbors a significant number of genes with immune functions. For that purpose, we have analyzed i) the genetic and ii) epigenetic influence of the X chromosome in RA.

i) Genetic influence of X-linked genes

Toll-like receptor 7 (*Tlr7*) gene, an X-linked gene important in the innate immune system, has been reported to be essential in accelerating autoimmunity in males in an autoimmune murine model (Yaa model). We have investigated the possibility of a gene copy increase of *TLR7* and its paralog *TLR8* in men with RA. We present evidence that copy numbers of *TLR7* and *TLR8* genes significantly increase with age in peripheral blood cells of men, whether they are healthy or with RA. Importantly, this age mediated variation is not a 2-fold increase in all cells (*TLR7/8* duplication) as observed in the Yaa mouse model, but rather of small amplitude with an increase of only 20%. Moreover, such phenomenon is not observed in women.

We propose several hypotheses to explain the significant, but small, age- and sex-mediated variation in copy numbers. Among them are somatically acquired duplications that can affect some cells over time and result in an increase with age in men. In parallel, X chromosome monosomy, previously described in aging women, may explain the opposite phenomenon of TLR7/8 copy number decrease in those. Another explanation for men with X-linked gene copy increase can be due to the presence of female microchimerism, arising from foeto-maternal or twin sister exchange during in utero life. Such cells would carry 2 X chromosomes and contribute to the increased pool of X-linked genes among XY host cells. We have recently demonstrated that this can occur with the persistence of female cells from a female vanished twin in a 40 year male host. Nevertheless further studies are needed to explore whether

microchimerism increases with age and/or hormonal changes and whether increased copy number of *TLR7/8* genes observed in elder men may have a real influence on TLR7/8 mRNA expression levels.

ii) X chromosome epigenetic influence

On the other hand, we have studied the patterns of X chromosome inactivation (XCI) among women with RA. XCI is an epigenetic dosage compensation mechanism used by mammals to ensure that XX females and XY males equalize X chromosome gene expression. Generally, there is a random inactivation of either the paternally-derived or the maternally-derived X chromosome in female tissues. A non-random XCI has been associated with many autoimmune diseases. In RA, we observe that, not only XCI is skewed among women with the disease, but it also correlates with the presence of predisposing HLA alleles, bearing the so-called “shared epitope”. The presence of the shared epitope often indicates severity of RA resulting in chronic inflammation and provoking a skewed XCI pattern by clonal selection over time. An interesting perspective would be to investigate XCI, with respect to the shared epitope, in a cohort of women before they have RA symptoms.

Our findings highlight the importance of the X chromosome, on a genetic and epigenetic point of view, in the development of autoimmunity. The X chromosome, often neglected in studies on autoimmune diseases, is a fascinating chromosome for which we do not know all facets. A better understanding will not only allow explaining female predominance in autoimmune disorders, but also give clues on female advantages in immunity and life expectancy.

RESUME (FRANÇAIS)

Les femmes sont plus à risque que les hommes dans la plupart des maladies auto-immunes (MAI). Plusieurs hypothèses ont été soulevées pour expliquer ce biais sexuel. L'objectif de ma thèse est de comprendre la prédominance féminine dans les MAI rhumatismales, avec un accent particulier sur la polyarthrite rhumatoïde (PR) et sur le chromosome X. Ce chromosome est d'un grand intérêt dans ce contexte, puisqu'il contient un nombre important de gènes avec des fonctions immunitaires. Pour cela, avons analysé l'influence i) génétique et ii) épigénétique du chromosome X dans la PR.

i) Influence génétique de gènes liés au chromosome X

Le gène Toll-like receptor 7 (*Tlr7*), important dans le système immunitaire inné, est décrit comme essentiel pour l'accélération de l'auto-immunité chez des souris mâles dans un modèle murin d'auto-immunité (modèle Yaa). Nous avons étudié la possibilité d'une augmentation du nombre de copies du gène *TLR7*, et de son paralogue *TLR8*, chez les hommes atteints de PR. Le nombre de copies de *TLR7* et *TLR8* augmentent significativement avec l'âge dans les cellules du sang périphérique des hommes, en bonne santé ou atteints de PR. De plus, cette variation liée à l'âge n'est pas due à un dédoublement dans toutes les cellules comme chez la souris Yaa, puisque l'augmentation n'est que de 20%. En outre, un tel phénomène n'est pas observé chez les femmes.

Plusieurs hypothèses peuvent expliquer ces observations. L'une d'elles est la duplication somatique affectant certaines cellules au cours du temps et se traduisant par une augmentation avec l'âge chez les hommes. En parallèle, la monosomie du chromosome X, précédemment décrite chez les femmes vieillissantes, expliquerait le phénomène opposé chez celles-ci. Une autre possibilité chez les hommes est la présence de microchimérisme féminin, résultant de l'échange cellulaire fœto-maternel ou entre frère et sœur jumeaux au cours de la vie *in utero*. Ces cellules XX pourraient contribuer à l'augmentation du nombre de copies de gènes du X parmi les cellules hôtes XY. Nous avons récemment démontré que cela est possible, avec la persistance de cellules d'une jumelle évanescente chez un homme de 40

ans. Cependant d'autres études sont nécessaires pour déterminer si le microchimérisme peut augmenter avec l'âge et/ou les changements hormonaux ; et si l'augmentation du nombre de copies de gènes a un réel impact sur les quantités d'expression d'ARNm de TLR7/8.

ii) Influence épigénétique du chromosome X

D'autre part, nous avons étudié l'inactivation du chromosome X (ICX) chez les femmes atteintes de PR. L'ICX est un mécanisme épigénétique de compensation de dosage utilisé par les mammifères pour s'assurer que les femelles XX et les mâles XY expriment de façon égale les gènes du X. Généralement l'inactivation se fait au hasard touchant soit le chromosome X d'origine paternelle soit le chromosome X d'origine maternelle dans tous les tissus féminins. L'ICX est biaisée dans de nombreuses maladies auto-immunes. Dans la PR, nous observons une ICX biaisée chez les femmes atteintes et corrélée avec la présence d'allèles HLA prédisposant à la maladie, portant l'épitope partagé (EP). La présence de l'EP est souvent un indicateur de sévérité de la PR et entraîne une inflammation chronique qui peut provoquer un biais d'ICX par sélection clonale au fil du temps. Une des perspectives intéressantes est d'étudier l'ICX, dans le contexte de l'EP, dans une cohorte de femmes avant qu'elles ne déclenchent la maladie.

Nos résultats soulignent l'importance du chromosome X, d'un point de vue génétique et épigénétique, dans le développement de l'auto-immunité. Ce chromosome, souvent négligé dans les études sur les maladies auto-immunes, est fascinant et nous n'en connaissons pas encore toutes les facettes. Une meilleure compréhension permettra non seulement de mieux expliquer la prédominance féminine dans les MAI, mais donnera également certains indices sur l'avantage des femmes dans l'immunité et l'espérance de vie.

TABLE OF CONTENTS

LIST OF TABLES

Number *Page*

LIST OF FIGURES

Number *Page*

FREQUENTLY USED ABBREVIATIONS

ACA	Anti-centromere antibodies
ACPA	Anti-citrullinated protein antibodies
APC	Antigen presenting cell
ATA	Anti-topoisomerase antibodies
CD(4, 8, 20,…)	Cluster of differentiation (cell surface) molecule
CCP	Cyclic citrullinated peptide
CNVs	Copy number variations
dcSSc	Diffuse cutaneous scleroderma
DNA	Deoxyribonucleic acid
Fc	Fragment crystallizable region
FISH	Fluorescence in situ hybridization
FLEDR	Phenylalanine-Leucine-Glutamic acid-Aspartic acid-Arginine
FOXP3	Forkhead box P3
HLA	Human leukocyte antigen
IFN	Interferon
IgE/G/M	Immunoglobuline E/G/M
IL	Interleukine
lcSSc	Limited cutaneous scleroderma
Mc	Microchimerism
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	Natural killer
PAR	Pseudo-autosomal region
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
Q-PCR	Quantitative PCR
QKRAA	Glutamine-Lysine-Arginine-Alanine-Alanine
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
SE	Shared epitope
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
TGF-β	Transforming growth factor beta
Th	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAELDT	Threonine-Arginine-Alanine-Glutamic acid-Leucine-Aspartic acid-Threonine
Treg	Regulatory T cell
TSIX	XIST antisense transcript
X^2	Chi square
XCI	X chromosome inactivation
XIST	X-inactive specific transcript
Yaa	Y-linked autoimmune accelerator

PREFACE

Four years ago, right after I came from Lebanon, I started as an intern for my Master's degree in Jean Roudier's laboratory. Later it turned into a project that Nathalie proposed on a theme she has been working on for many years now. The goal is to understand why there is a remarkable female predominance in autoimmune diseases, and the project consisted in a genetic and epigenetic study focused on the X chromosome.

The present work summarizes the efforts done by our team to try to answer this question. First this thesis will introduce the reader to the concepts of autoimmune diseases, with two examples in rheumatoid arthritis and scleroderma. Up to now, little is known about the etiology of this kind of diseases. However, they get significant genetic susceptibility with a particularly strong influence from the Human Leucocyte Antigen locus on chromosome 6. Risk factors behind gender bias will also be explained. These factors can be physiological (hormones, microchimerism) as well as genetic (linked to the X chromosome).

We then present how we contributed to the better understanding of X-linked risk factors behind female predominance. The results include insights about genes on the X chromosome that vary in copy numbers, and epigenetic whole chromosome silencing that turns out to take different patterns in affected women compared to healthy women.

While harvesting those results, I have learned more things than I ever expected to learn. Achieving this knowledge has accelerated in three phases. The first is technical; it is thanks to all the experiments, set up under amazing and fun conditions. The second is intellectual, through all the discussions and meetings we have had between colleagues, all experts and highly competent, always ready to assist and push me forward; also through the many international meetings we have had that were nothing but brain-storming and rich. The third is the strong enjoyment I had while writing the present thesis, during the past three months. It was quite a challenging exercise and I always came away with new thoughts in each page I wrote and in every reference that I utilized to find inspiration.

So here you go, dear reader, the fruit of our efforts. I hope you too could gain a new perspective, after finishing reading.

PART 1 – *INTRODUCTION*

CHAPTER 1.1 – AUTOIMMUNE DISEASES

1.1.1 DEFINITION

Autoimmune diseases arise when the immune system turns its antimicrobial defenses upon normal components of the body such as insulin-producing pancreatic cells in type 1 diabetes or chromatin in systemic lupus erythematosus. This autoimmune attack could be restricted to certain organs, e.g. in type 1 diabetes, or could take a systemic nature and involve tissues and organs in different places, e.g. in systemic sclerosis which results in extensive fibrosis of the skin and complications in many internal organs.

According to *Witebsky's postulate* (revisited by Rose and Bona) [1, 2], the establishment of the autoimmune nature of a disease requires the demonstration of autoantibodies and/or the existence of autoreactive cells (B or T lymphocytes), the induction of a similar autoimmune response, accompanied by a disease similar to the human one, in an experimental animal, and the support of circumstantial evidence from clinical clues.

Autoimmune diseases often have common systemic and inflammatory features. A hypothesis has emerged stating that a particular autoimmune disease arises from a common undifferentiated condition depending on the genetic background of the patient. This is supported by the fact that mixed connective tissue disease can differentiate to rheumatoid arthritis, systemic sclerosis, or systemic lupus erythematosus depending on the human leukocyte antigen (HLA) genotype of patients [3].

1.1.2 STRIKING FEMALE PREDOMINANCE IN AUTOIMMUNE DISEASES

Almost 5% of the world population develops an autoimmune disease. Female predominance in these diseases is remarkable as of this 5% approximately 80% are women [4, 5]. For example, women represent 95% of patients with thyroiditis, 92% of patients with systemic sclerosis, 88% of patients with systemic lupus erythematosus, 75% of patients with rheumatoid arthritis, and 64% of patients with multiple sclerosis [5] (**Figure 1**). Therefore, gender is a good predictor of many autoimmune diseases. Nevertheless, the reasons why women are at greater risk remain speculative and there is still no convincing evidence supporting a single hypothesis. Autoimmunity is the result of both environmental and genetic components and, to date, gene

polymorphisms associated to a particular autoimmune disease, e.g. in the HLA haplotypes, account to only a partial contribution to the disease. Breakdown of self-tolerance can be caused by hormones, immunological challenge during pregnancy, microchimerism [6, 7], skewing of X chromosome inactivation [8] or X chromosome-linked abnormalities, going from gene copy number variations [9] to X-linked aneuploidies [10, 11].

We will further develop some of these risk factors and their association to two rheumatic autoimmune disorders mainly studied in our laboratory: Rheumatoid Arthritis (RA) and Systemic Sclerosis (SSc).

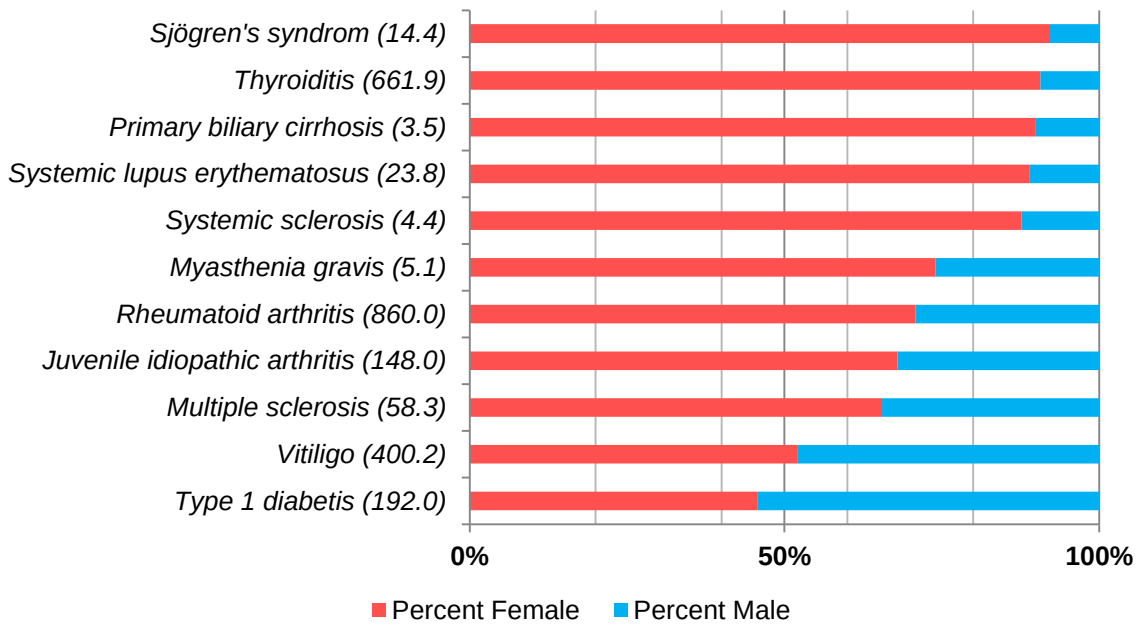


Figure 1. Sex distribution of some of the most important autoimmune diseases illustrates female predominance in autoimmunity.
Numbers between brackets represent the disease's prevalence (persons per 100 000).
Percentages are the means of those reported in *Cooper GS et al., 2003, Autoimmun Rev* and *Selmi C, 2008, Best Pract Res Clin Rheumatol*.

1.1.3 RHEUMATOID ARTHRITIS (RA)

1.1.3.1 Epidemiology

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis that cannot be cured and that has substantial personal, social, and economic costs. This autoimmune disease occurs worldwide, and 75% of people affected are women. Most prevalence studies are carried out on Northern American and European populations, giving a prevalence between 0.5 and 1.1% [12]. This varies, however, across ethnic groups, reflecting the influence of predisposing genes. Indeed, the prevalence tends to decrease in the south. Southern European countries show a prevalence of 0.3%–0.7% and it is lower in developing countries (0.1%–0.5%). The highest prevalence is recorded among Yakima Native Americans (5.3%–6%) and the lowest in some areas of rural Africa (0%–0.3%) [12, 13]. Men have generally a later onset of disease compared to women [14]. Furthermore, a British study indicates that female to male prevalence ratios change with age [15]. Ratios vary from 6.45 to 1 in the age group of 16–44 years to nearly 1.37 to 1 in the age group of 75+ years, thus indicating a tendency towards equalizing female-male proportions among older people (**Figure 2**).

1.1.3.2 Autoantibodies

RA is often characterized by the presence of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA; tested as anti-cyclic citrullinated peptide; anti-CCP). ACPAs can precede the clinical manifestation of RA by many years [16, 17]. The RF is present in 60–80% of cases. It is an immunoglobulin-M (IgM) isotype antibody reacting with the Fc fragment of human IgG antibodies. ACPAs are more specific to the disease as they are detected in 70–90% of cases with high disease specificity (90–95%), thus rarely found in other diseases or in healthy individuals. They recognize citrullinated forms of filaggrin, fibrin, fibrinogen, vimentin, types I and II collagens, and α-enolase [18].

Principal pathological features include chronic inflammation of the synovial membrane of peripheral joints, leading to gradual joint destruction and progressive bone erosion. These are the dominant cause of disability in RA. Loss of function and pain in joints with the typical deformations at later stages are the main symptoms of disease (**Figure 3**). Other systemic features include cardiovascular, pulmonary, psychological, and skeletal disorders [19]. Clinical

status of patients with RA is defined by the Disease Activity Score (DAS). The most commonly used version of this clinical test is the DAS28 in which the swollen status of 28 joints is examined, along with erythrocyte sedimentation rate and general health evaluation [20].

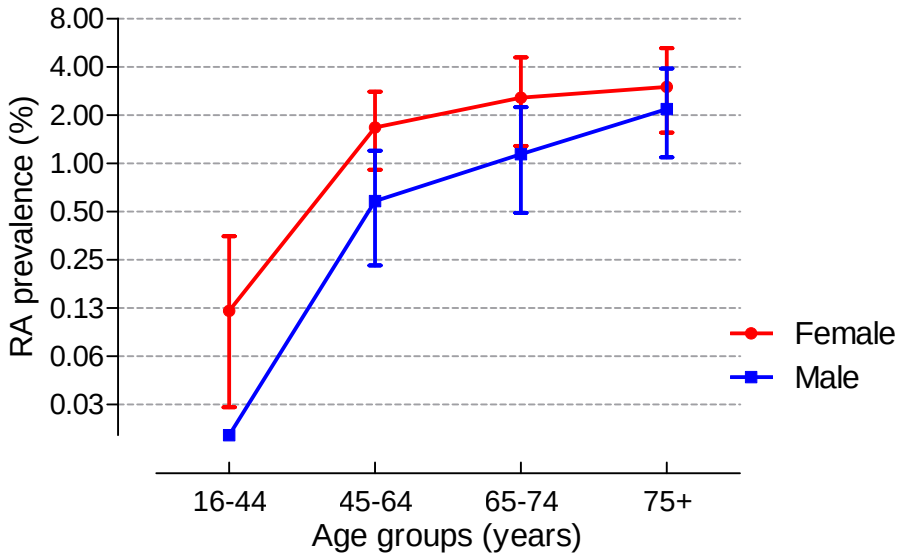


Figure 2. Age- and sex-specific prevalences of rheumatoid arthritis (RA).
Prevalence of RA is 6.45 folds lower in men compared to women in the youngest group of age and 1.37 folds lower in the oldest group of age. Prevalence in percentage is log scale. Error bars represent 95%-confidence intervals. Data are obtained from *Symmons D et al., 2002, Br J Rheumato*l.

1.1.3.3 Immuno-pathophysiology

The cause of RA is unknown. It is hypothesized [21] that, in genetically predisposed persons, an infective agent or another endogenous stimulus binds to toll-like receptors (TLRs) on dendritic cells and macrophages.

The first phase is a trigger by the innate immune system. A rapid response is then engaged involving a variety of innate effector cells, such as macrophages, dendritic cells, mast cells, neutrophils, and natural killer (NK) cells in the synovial tissues.

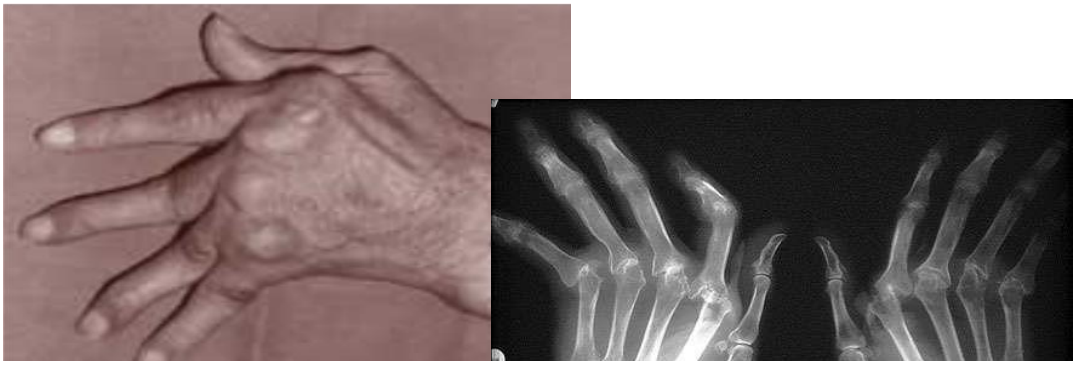
The second phase corresponds to the adaptive immune system, where antigen presenting cells (APC) migrate to lymph nodes to activate T cells. Indeed, the genetics of RA (with clear

Human Leukocyte Antigen [HLA] class II allele susceptibility) and the presence of autoantibodies place adaptive immunity at the center of its pathogenesis. T-cell activation requires two signals: first signal is generated by antigen, i.e. the peptide bound to HLA class II molecule on the APC stimulates the T-cell receptor. The second signal is generated by costimulation of the T cell surface molecule CD28, with CD80 or CD86 on the APC. Having two signals, activated T cells can proliferate and migrate into the joint, where they stimulate a multi molecular immune/inflammatory cascade. Abatacept (a fusion protein containing cytotoxic T-lymphocyte associated antigen 4 and the Fc fragment of IgG1) is successfully used in RA to disrupt antigen presentation by blocking CD28 costimulation [22]. The major T subset conventionally considered as a mediator of RA is type 1 helper T cell (Th1)[A]. However, attention has increasingly focused on the role of type 17 helper T cells (Th17), a subset that produces interleukin (IL)-17A, 17F, 21, and 22 and tumor necrosis factor α (TNF-α) [23]. Regulatory Foxp3+ T cells (Tregs) are also found in tissues from patients with RA but they appear to have inefficient regulating capabilities [24], an explanation could be that TNF-α impairs the activity of Tregs in the synovial environment [25].

The third phase is the establishment of a chronic inflammation. T cells produce interferon-γ and other pro-inflammatory cytokines which stimulate macrophages, fibroblasts, chondrocytes, and osteoclasts (via RANKL). Activated macrophages release a variety of pro-inflammatory cytokines (e.g. TNF-α, IL-1, 6, 12, 15, 18, and 23) [26]. Indeed, macrophages are central effectors of synovitis and their absence correlates with disease remission [27]. Neutrophils contribute to synovitis by synthesizing prostaglandins, proteases and reactive oxygen

[A] On the basis of cytokine patterns, CD4+ T cells are classified as Th1, Th2, Th17, Tfh, and Treg types. Th1 type promotes cell-mediated immunity (against viruses, intracellular bacteria and fungi), mainly produces interferon-γ and interleukin-2, and their target cells are macrophages and dendritic cells. Th2 type promotes humoral immunity mainly against multicellular parasites, produces interleukin-4, -5, and -13, and their target cells are eosinophils and basophils. Th17 type promotes an effect against extracellular bacteria and fungi, produces interleukin-17A, -17F, -21, and -22, and their target cells are neutrophils. Tfh type (follicular help) provides B-cell help in B follicles and their germinal centers, and produces interleukin-4, and-21. Treg type has regulatory functions on the other effector Th cells, and produces interleukin-10 (from *Miossec P et al., 2009, N Engl J Med; and Craft JE, 2012, Nat Rev Rheumatol*).

intermediates [28]. Mast cells also play a role. Interestingly, a fraction of ACPA belongs to the IgE class, which suggests an activation of mast-cell through Fc receptor ε [29]. Furthermore, the humoral adaptive immunity is essential to RA pathogenesis. Synovial B cells are localized in aggregates with T cells and their pathogenic role is confirmed by the efficacy of rituximab (anti-CD20 monoclonal antibody) against B cells [30]. Finally, endothelial cells perpetuate the immune response by enhancing cell recruitment to the joint, and joint damage occurs through the action of proteases, growth factors, and activated osteoclasts [31].



TNF-α is a central component in RA pathophysiology. It binds to type 1 TNF receptor (p55) and type 2 TNF receptor (p75), which are found on a variety of immune and endothelial cells [32]. Although there is no definite cure for the disease, treatment of RA with TNF inhibitors has shown great success. TNF inhibitors include a soluble p75 TNF-α receptor–IgG1 fusion protein (etanercept) and monoclonal antibodies against TNF-α (infliximab and adalimumab). A very effective strategy is to use TNF inhibitors in combination with the antimetabolite drug Methotrexate. Other drugs approved in treating RA include a monoclonal antibody against IL-6 receptor (tocilizumab) that proved very effective against the disease and an IL-1 receptor antagonist (anakinra) that has a rather low efficacy in RA despite good anti-inflammatory activity in other inflammasome-driven diseases [19]. Nonetheless, the efficacy of these drugs supports the pivotal role of those pro-inflammatory cytokines in the establishment of RA.

1.1.4 SYSTEMIC SCLEROSIS (SSC)

1.1.4.1 Epidemiology

Systemic sclerosis (SSc), or scleroderma, is a rare complex autoimmune connective tissue disorder, affecting between 44 and 300 per 1 million [5, 33] of the average population. Prevalence thus varies across ethnic groups. Interestingly, the highest prevalence is reported among Choctaw Native Americans reaching 660 per 1 million [34], reflecting therefor a genetic predisposition. SSc is a female predominant disease and approximately 92% of people affected are women [5]. Although SSc can occur at any age, it typically affects middle-aged persons for whom the average age of onset is 46 years [35].

1.1.4.2 Autoantibodies

Scleroderma is characterized by the presence of specific autoantibodies against various cellular antigens, in 90% of patients [36]. Some of the autoantibodies that can be found are: anticentromere antibodies (ACA), antitopoisomerase I antibodies (ATA), anti-RNA polymerase I and III (ARA), antipolymyositis, antifibrillarin (U3RNP), anti-endothelial cell (that induce apoptosis of endothelial cells), and antiplatelet-derived growth factor receptor (PDGFR) antibodies [37, 38]. ACA and ATA are the most characteristic of the disease, while the others are less specific. Importantly, patient with SSc typically produces only one of these two autoantibodies [39] and each one can be associated with a specific feature of the disease. For example, a strong association has been shown between: ATA and pulmonary fibrosis, ARA and renal involvement with a diffuse skin disease, and ACA and limited skin involvement with protection against pulmonary fibrosis [40]. Moreover, some SSc-specific autoantibodies can occur at different frequencies among different ethnic groups.

1.1.4.3 Classification

Scleroderma is one of the most complicated diseases to sub-classify. The main pathological features include vascular alterations and extensive fibrosis (**Figure 4**) which eventually leads to skin thickening, ischemia with ulcer formation on digital parts, and internal organ damage. Two major subclasses exist: a systemic and a localized scleroderma.

The localized subclass occurs in three forms: morphea, linear scleroderma, and general morphea. It is more frequent in children and carries a benign prognosis [41].

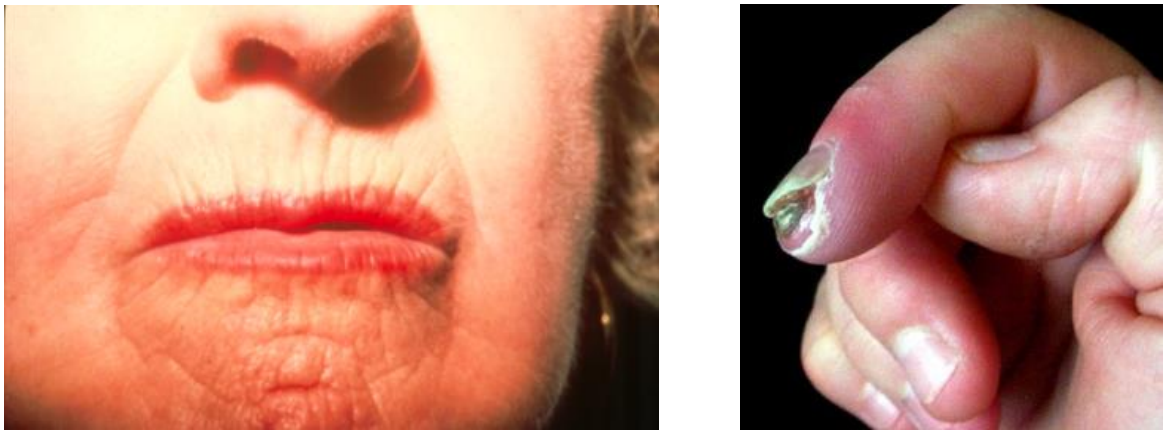


Figure 4. Examples of SSc features: fibrosis giving shape to lines around the mouth, and ulceration on finger.

The focus of this discussion will be on the more severe systemic subclass. A classification introduced by Leroy in 1988 [42, 43] indicates that, according to clinical manifestations and presence of biomarkers (autoantibodies), the systemic subclass is divided into two forms: limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc). LcSSc includes the CREST[B] syndrome and its involvement is characterized by symmetrical skin thickening, mainly restricted to the hands, arms, and face. ACA are hallmarks of this subgroup and occur in approximately 50–90% of lcSSc patients [44]. In dcSSc, the skin lesions are severe and extensive, affecting large areas of the body. The progression of the disorder is rapid and with multiple internal organ involvement (pulmonary, cardiovascular, gastrointestinal, and renal). ATA are hallmarks of this subgroup and occur in 40% of patients. ATA are associated with a severe prognosis.

[B] The CREST acronym stands for Calcinosis (calcium deposit in the skin), Raynaud's phenomenon (spasm of blood vessels in response to cold or stress), Esophageal dysfunction (acid reflux and decrease in motility of esophagus), Sclerodactyly (thickening and tightening of the skin on the fingers), and Telangiectasia (dilatation of capillaries causing red marks on the surface of the skin).

1.1.4.4 Immuno-pathophysiology

In SSc, vascular injury occurs as an early event. It precedes fibrosis and involves small vessels, particularly the arterioles. High levels of vascular endothelial growth factor (VEGF) are found in ischemic regions [45]. However, and for unknown reasons, there is a defect in vasculogenesis [46]. In later stages, fibrosis gradually replaces the vascular inflammation and ultimately causes damage to tissue architecture, giving rise to the main symptoms of disease [44].

Autoimmunity is best exemplified by the presence of non-overlapping autoantibodies which identify clinical subsets, e.g. ATA for dcSSc and ACA for lcSSc [38], thus indicating the participation of B cells. Nevertheless, their actual contribution to disease is unknown. A polymorphism in the promoter region of CD19 (a cell surface molecule crucial in B cell signal transduction) frequently detected in patients with SSc is associated with an increased expression of CD19 on B cells in SSc [47]. Furthermore, peripheral B cell homeostasis is altered. Naïve B cells are expanded while memory B cells (with higher antibody production potential) are diminished, but on the other hand are activated [48]. Even though the mechanistic role of specific autoantibodies on the pathophysiology is not known, treatment with rituximab against CD20+ B cells, commonly used in RA and systemic lupus erythematosus (SLE), is also effective in SSc [49].

Although B cell participation in SSc is significant, T cells are found to play an important role in SSc pathogenesis. In general, a significant number of studies suggests that peripheral blood and skin-infiltrating T cells show a predominant type 2 helper T cell (Th2) profile, elaborating the type 2 cytokines IL-4 and IL-13 [50, 51], which strongly stimulate production of transforming growth factor beta (TGF-β) (an important molecule in SSc [52]) and contribute to fibrosis [51]. CD4+CD25+Foxp3++ Treg cells may also contribute to SSc. Indeed, despite an increase in frequency in peripheral blood of patients with SSc, they have an attenuated suppressive and regulative activity [53].

While the adaptive immune system plays a pivotal role, as discussed above, recent studies highlight the importance of innate immunity in SSc pathogenesis. Type I interferons (e.g. IFN-α and -β) are early mediators of the innate immune response that influence the adaptive immune

response through direct and indirect actions on dendritic cells, lymphocytes, and natural killer cells. As a prominent IFN signature is well established in SLE [54, 55], recently, several studies observed the presence of a type I IFN gene-expression signature in SSc, similar to that of lupus [56, 57]. Levels of IFN-α mRNA is also found elevated in vascular and perivascular cells, suggesting local activation of leucocytes in the vasculature [58]. Moreover, sera levels of IFN-α correlate with the presence of ATA. In such a way, IFN-α correlates with a severe form of dcSSc and tissue injury [59]. Taken together, these data suggest that the development of certain pathogenic immune complexes may stimulate the IFN response in some SSc subsets, thereby producing more severe manifestations. However, they do not fully explain the mechanisms underlying the manifestations, nor do they explain why patients with SSc differ from patients with lupus, while sharing many overlapping IFN-responsive genes [56]. Accordingly, IFN signature in SSc merits further investigations.

CHAPTER 1.2 – GENETIC SUSCEPTIBILITY IN AUTOIMMUNITY

1.2.1 GENOME-WIDE ASSOCIATIONS

It is largely accepted that the pathogenesis of an autoimmune disease is multifactorial, with genetic and environmental or stochastic factors interplaying in disease onset and progression [60]. In this context, twin studies are the most useful tools to estimate heritability of each disease. Indeed, the finding of significantly higher monozygotic compared to dizygotic twin concordance is used as proof that genetic factors play a significant role in pathogenesis of numerous autoimmune diseases. For example, ankylosing spondylitis, celiac disease, and primary biliary cirrhosis showed the highest monozygotic twin concordance with pairwise concordance of 58%, 69%, and 77%, respectively. Almost all other autoimmune conditions registered concordances of less than 50%, presupposing a large contribution of stochastic events. Examples for such monozygotic twin pair concordance include: SLE 25%, RA 12.2%, and SSc 4.2%. Interestingly, monozygotic twin pair concordance for the presence of antinuclear antibodies is 90% in SSc [61].

To further identify disease-associated genes or loci responsible for autoimmune disease pathogenesis, a useful tool is genome-wide association studies (GWAS). Generally, GWAS involve the genotyping of several hundred thousands of single-nucleotide polymorphisms (SNP) throughout the genome in large case-control studies, and the application of a large number of statistical tests [62]. This is why stringent statistical thresholds are applied (*P* value < 5 × 10^−8) to establish evidence for association. A number of associated loci has been identified that include a wide range of immune-associated genes involved in lymphocyte activation (costimulation, receptor signaling pathways), microbial recognition, cytokines or cytokine receptors. Nonetheless, the most predominant locus involved in the majority of autoimmune diseases is the major histocompatibility complex (MHC) locus [63]. Based on data available [63-67], a non-exhaustive list of some important genes implicated in at least two autoimmune diseases is noted in **Table 1**. **Figure 5** represents genes implicated in RA and SSc [64, 65, 67], excluding the MHC locus which will be discussed below.

Table 1. Association of genomic loci with 2 or more autoimmune diseases

Genes	Function	Diseases*
MHC	Major histocompatibility complex	Most autoimmune disorders
PTPN22	T and B cell receptor signaling	RA, T1D, CeD, SSc, SLE
STAT4	Transcription factor, development of Th1 from naïve CD4+ T cells, IFN-γ production via IL-12	RA, T1D, SLE, SSc
6q23	Intergenic, between oligodendrocyte lineage transcription factor 3 (*OLIG3*) and *TNFAIP3*	RA, T1D, SLE
CTLA4	Transmits inhibitory signals to T cells	T1D, RA
CD2/CD58	Activation of T cells	RA, MS
IL23R	Unique component of the heterodimeric IL-23 receptor	IBD, PS, AS
IL10	Downregulates immune responses, including cytokines, MHC class II and costimulatory molecules	IBD, SLE, T1D
IL2/IL21	T cell trophic growth factors	CeD, IBD, RA, T1D
IL12B	P40 subunit common to IL-12 and IL-23	IBD, PS
IL2RA	IL-2 receptor alpha chain	MS, T1D, SSc, RA
TNFAIP3	Induced by TNF and pattern recognition receptor activation, inhibits NF-κB signaling	RA, SLE, SSc, PS
TNIP1	Interacts with TNFAIP3	SLE, SSc, PS
TIMP1	Tissue inhibitor of metalloproteinase (X-linked)	RA, SSc, CD
IRAK1	IL-1 receptor associated kinase 1 (X-linked)	SSc, SLE, RA
PRDM1	Transcriptional receptor of IFN-β, induces B cell maturation	RA, SLE
BLK	B lymphoid tyrosine kinase	RA, SLE, SSc
PTPN2	T cell protein tyrosine phosphatase	IBD, T1D

* AS (ankylosing spondylitis), CD (Crohn's disease), CeD (celiac disease), IBD (inflammatory bowel disease), MS (multiple sclerosis), PS (psoriasis), RA (rheumatoid arthritis), SLE (systemic lupus erythematosus), SSc (systemic sclerosis), T1D (type 1 diabetes mellitus)

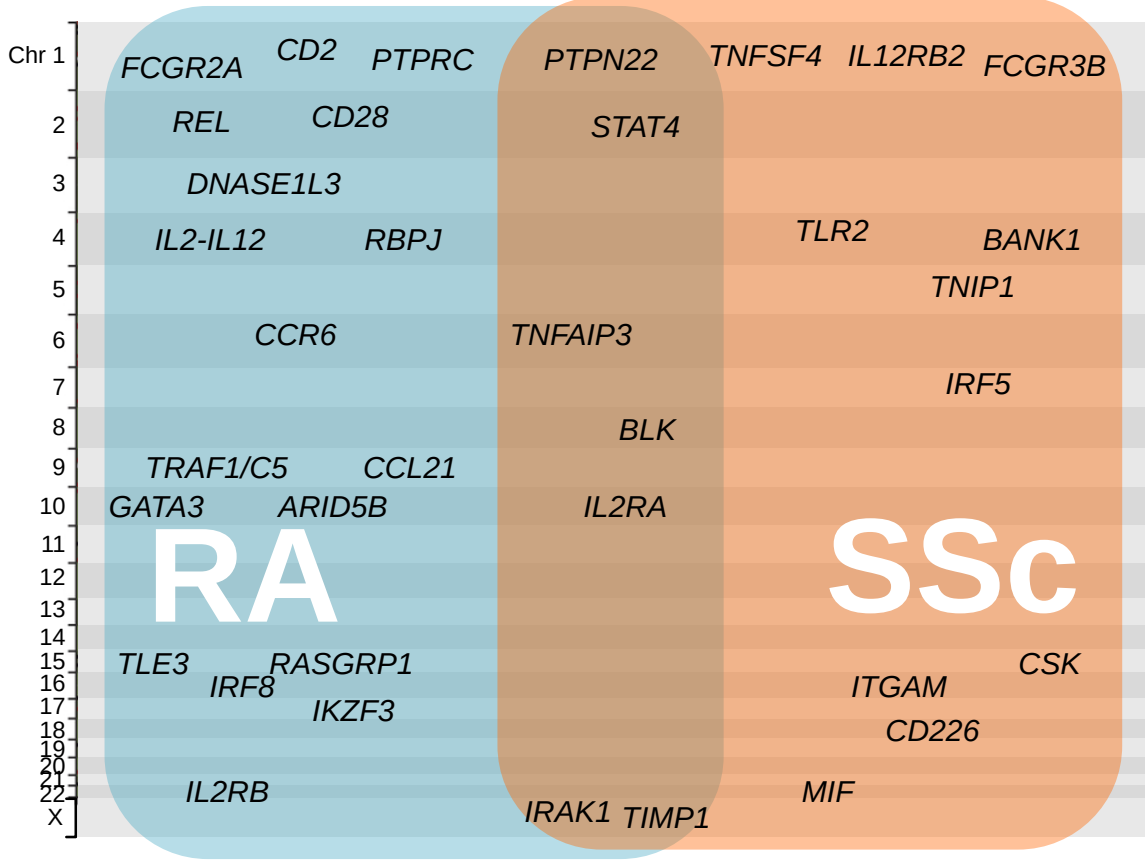


Figure 5. Non-HLA loci (affiliated to corresponding chromosome) conferring rheumatoid arthritis (RA) and/or scleroderma (SSc) risk (non-exhaustive).
Data from: *Eyre S et al., 2012, Nat Genet – Mayes M, 2012, Curr Opin Rheumatol – Amur S et al., 2012, J Autoimmun*.

1.2.2 The Major Histocompatibility Complex (MHC)

Major histocompatibility complex (MHC) genes are the most frequently reported genetic factors associated with autoimmune diseases. The strength of their contribution varies depending on the disease, as other genetic variants or environmental factors come along to play a part.

MHC molecules are encoded on the short arm of chromosome 6 and are essential for the normal function of the adaptive immune system, as they have a pivotal role in the presentation of antigens for recognition by T cells. The MHC is divided into 3 subregions: the telomeric MHC class I, class III, and the centromeric class II. Further functional classification divides MHC into: classical MHC molecules which participate in antigen presentation and non-classical MHC which have different functions, such as antigen processing (transporter associated protein [TAP]), complement cascade proteins, stress response (heat shock proteins), inflammation (tumor necrosis factor), and molecules like HLA-E, -F, and -G which are considered non-classical MHC class I [68]. Classical MHC class I genes include HLA-A, -B, and -C and classical MHC class II genes include HLA-DR, -DQ, -DP, -DO, and -DM (**Figure 6**).

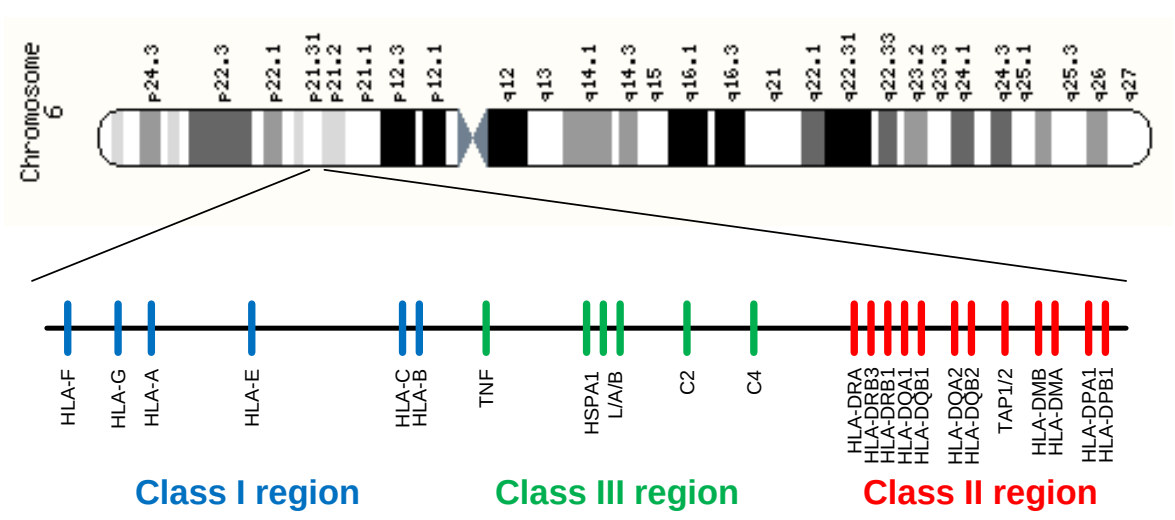


Figure 6. Organization of the HLA region on chromosome 6.
Positions of genes are approximates. Chromosome screenshot is from Ensembl.org and gene positions from *Horton R et al., 2004, Nat Rev Genet*.

HLA molecules from MHC class I are expressed on virtually all human cells and generally present endogenous peptides (processed from proteins in the proteasome) to CD8+ cytotoxic T cells. HLA class II molecules are expressed on cells of the immune system called antigen presenting cells (APC) and present exogenous peptides (processed from endocytosed proteins) to CD4+ helper T cells [69]. Peptides are bound on HLA molecule independently

from their amino-acid sequence, as long as the backbone position of their main chain fits with HLA binding groove shape. Conserved residues in peptide binding groove enable this sequence-independent binding by formation of hydrogen bonds and Van der Waals contacts. Amino-acid sequence and spatial conformation of HLA class I and class II molecules are then crucial for interaction (respectively) with T cell receptor (TCR) and CD8 on cytotoxic T cells, or TCR and CD4 on helper T cells [70].

1.2.2.1 Polymorphism and linkage disequilibrium in the MHC region

The allelic diversity of HLA molecules is higher than any other protein family, and this polymorphism is a major contributor to individual differences in immune response. Polymorphisms in HLA genes are concentrated in the areas encoding the peptide-binding groove, where an antigen to be presented is bound. From an evolutionary point of view, HLA alleles are under the influence of “balancing selection”, which is a natural selection process whereby no single allele is absolutely most fit, therefore conferring a high allelic diversity that responds to a high variety of pathogenic antigens.

A hallmark of the MHC region is strong linkage disequilibrium[C] between loci. This means that combinations of alleles occur in frequencies different than what would be expected from a random formation of haplotypes (based on observed frequencies of alleles in a population). This comes from the fact that alleles on the same chromosome tend to be inherited as whole blocks through generations. The term “ancestral haplotype” is then used to define conserved specific haplotypes.

[C] Due to chromosomal crossover, linkage disequilibrium decreases as distance between loci increases. In theory, it is a matter of time before linkage disequilibrium tends towards equilibrium; however, this process is as slow as the recombination rate (i.e. probability of a crossover) is small. Examples of linkage disequilibrium are well established between HLA loci. Nevertheless, factors other than the recombination rate influence disequilibrium strength, including molecular cooperative function of HLA heterodimers, DNA recombination occurring in preferential “hot spots”, positive selection for a specific allele with “hitch-hiking” effect on the accompanying allele, non-random mating, and genetic drift.

1.2.2.2 MHC associations in autoimmune diseases

Presence of autoantibodies and auto-reactive T cells in autoimmune disorders supports the hypothesis of a role played by MHC class II molecules. The first report of an association between an autoimmune disease and MHC genotype in humans was published in 1971 in SLE [71]. Few years later, more than 30 autoimmune diseases have been found to be associated with particular MHC genotypes [72].

Classical examples of HLA associations to autoimmune diseases include particular *HLA-DRB1*01* and *-DRB1*04* alleles with RA, the HLA-DR2 haplotype (-D*RB1*15 – DRB5*01:01 – DQB1*06:02*) with multiple sclerosis [73], and *HLA-DQA1*03:01 – DQB1*03:02* on the HLA-DR4 haplotype with type 1 diabetes [74]. Fewer correlations are found for MHC class I alleles, the most consistent being *HLA-B*27* with ankylosing spondylitis [75]. HLA alleles may be part of extended haplotypes in which different regions are associated with different diseases. An example is the ancestral haplotype 8.1-AH (*HLA-A*01 – C*07 – B*08 – C4AQ0 – C4B1 – DRB1*03 – DQB1*02*) which is carried by most Caucasians with *HLA-B*08*, and which predisposes to type 1 diabetes, SLE, myasthenia gravis, dermatitis herpetiformis, common variable immunodeficiency, IgA deficiency, and an acceleration towards acquired immunodeficiency syndrome (AIDS) [76]. Some MHC class II alleles are less frequent among patients suffering from autoimmunity, and called “protective”, e.g. *HLA-DRB1*01:03* and *-DRB1*04:02* for RA, *-DRB1*07* for autoimmune thyroiditis, RA, SSc, and Grave’s disease [65, 77].

1.2.2.3 RA: the Shared Epitope hypothesis

RA is an excellent example of the contribution of MHC in autoimmunity pathogenesis. Stastny first reported in 1976 that almost 70% of RA patients bear a predisposing HLA-Dw4 allele (later called *HLA-DRB1*04*) [78]. In 1987, Gregersen *et al.* proposed the so-called Shared Epitope (SE) hypothesis[D] which predicted that RA-associated DRβ1 molecules bind the same

[D] “Shared Epitope” appears to be somewhat confusing, because an epitope is, by definition, the part of an antigen recognized by the immune system (specifically by antibodies, B, or T cells). The “shared motif hypothesis” would be a better name in the context of a 5-amino-acid sequence shared by the

peptide(s) and thus facilitate the development of autoreactive T cells involved in the pathogenesis of RA [79]. Later, it has gradually become clear that RA was associated with several *HLA-DRB1* alleles (**01:01*, **01:02*, **04:01*, **04:04*, **04:05*, **04:08*, **10:01*, and **14:02*).

The molecular products of these RA-associated DRB1 alleles appears to carry a common 5-amino-acid sequence ^{70}Q/R–K/R–R–A–A^{74} (^{70}glutamine(or arginine)-lysine(or arginine)-arginine-alanine-alanine^{74}) in the third hypervariable region of the DRβ1 molecule (**Table 2**). This 5-amino-acid sequence is important as it is part of the so-called P4 peptide-binding pocket of the DRβ1 molecule. The K/R residue at position 71 is positively charged, which creates conditions to bind neutral or negatively charged peptides [80]. In this context, the Reviron model proposes that, while all RA-associated *HLA-DRB1* alleles are characterized by strong positive charge of P4 pocket, those with a weak positive charge do not confer risk of RA, and those with a neutral or negative charge are protective against RA [81].

The risk of disease follows a genotypic dosage effect among SE-positive *HLA-DRB1* alleles, in such a way that double dose genotypes have higher risk than single dose genotypes, while zero dose genotypes have neutral or even protective effect against RA development [82]. In addition, particular SE-positive heterozygous combinations can confer risk greater than SE-positive homozygous genotype or other genotypic combinations. This compound heterozygosity was suggested in the example of *HLA-DRB1*04:01/*04:04* that has 26 times more risk to develop RA than SE-negative individuals, compared to 8 times more risk in the case of another SE double dose genotype [83]. Recently, our laboratory has described compound heterozygosity, pointing out the influence of the combination of different alleles, with or without the SE, on disease risk. The genotypic combination conferring most risk is *DRB1*04:01/*10* with an odd ratio of 28.2, while the most protective genotype is *DRB1*03/*03* with an odd ratio of 0.2 [82]. It is interesting to note that men are more prone than women to having a double dose of SE [83, 84]. Therefore, it has been suggested that women acquire

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disease-associated HLA-DRB1 molecules. Nevertheless, the SE hypothesis became the most well-known hypothesis in the field of HLA and disease associations. However, until this day, a SE-binding peptide involved in RA pathogenesis has never been identified.

additional susceptibility to RA through the persistence of SE-positive microchimeric cells as consequence of pregnancy [85, 86] (microchimerism is discussed in more details in chapter 2).

Table 2. Amino-acid (AA) sequence (21 → 100) alignments of HLA-DRB1 alleles.
Based on the Reviron's model, the SE-amino-acid residues of RA-associated HLA-DRB1 alleles are highlighted in red. Non-highlighted residues belong to the no-risk-associated alleles. Residues highlighted in green are those of DRB1 alleles conferring protection against RA. (Amino-acid sequences were aligned from anthonynolan.org/hig with IMGT/HLA Database sequence alignment tool. (-) AA same as reference sequence, (*) unsequenced AA).

AA Pos.	30	40	50	60	70	80	90	100
DRB1*01:01	TERVRLLERC	IYNQEESVRF	DSDVGEYRAV	TELGRPDAEY	WNSQKDLLEQ	RRAAVDTYCR	HNYGVGESFT	VQRRVEPKVT
DRB1*01:02	----------	----------	----------	----------	----------	----------	----------	----------
DRB1*01:03	----------	----------	----------	----------	------I---	----------	----------	----------
DRB1*03:01	-----Y-D-Y	FH----N---	------F---	----------	----------	K-GR--N---	-----V----	-----H----
DRB1*04:01	-----F-D-Y	F-H---Y---	----------	----------	----------	----------	----------	-----Y-E--
DRB1*04:02	-----F-D-Y	F-H---Y---	----------	----------	------I---	----------	-----V----	-----Y-E--
DRB1*04:03	-----F-D-Y	F-H---Y---	----------	----------	----------	---E------	-----V----	-----Y-E--
DRB1*04:04	-----F-D-Y	F-H---Y---	----------	----------	----------	----------	-----V----	-----Y-E--
DRB1*04:05	-----F-D-Y	F-H---Y---	----------	------S---	----------	----------	----------	-----Y-E--
DRB1*04:06	-----F-D-Y	F-H-------	----------	----------	----------	---E------	-----V----	-----Y-E--
DRB1*04:07	-----F-D-Y	F-H---Y---	----------	----------	----------	---E------	----------	-----Y-E--
DRB1*04:08	-----F-D-Y	F-H---Y---	----------	----------	----------	----------	----------	-----Y-E--
DRB1*07:01	----QF---L	F-----F---	----------	------V--S	------I---	D --GQ---V--	----------	-----H-E--
DRB1*08:01	-----F-D-Y	F-----Y---	----------	------S---	------F---	D ---L------	----------	-----H----
DRB1*09:01	-----Y-H-G	------N---	----------	------V--S	------F---	R ---E---V--	----------	-----H-E--
DRB1*10:01	---------R	VH----YA-Y	----------	----------	----------	R ----------	----------	-----Q----
DRB1*11:01	-----F-D-Y	F-----Y---	------F---	-------E--	------F---	D ----------	----------	-----H----
DRB1*11:07	-----F-D-Y	F-----Y---	------F---	-------E--	----------	K-GR--N---	-----V----	----******
DRB1*12:01	---------H	FH----LL--	------F---	------V--S	------I---	D ----------	----AV----	-----H----
DRB1*13:01	-----F-D-Y	FH----N---	------F---	----------	------I---	D E---------	-----V----	-----H----
DRB1*14:02	-----F---Y	FH----N---	----------	----------	----------	----------	----------	-----H----
DRB1*15:01	-----F-D-Y	F---------	------F---	----------	------I---	A---------	-----V----	-----Q----
DRB1*16:01	-----F-D-Y	F---------	----------	----------	------F--D	----------	----------	-----Q----

1.2.2.4 MHC associations with SSc

MHC class II alleles have associations with SSc pathogenesis. However, they can differ across ethnic groups. Over the past two decades, SSc associations have been reported with *HLA-DRB1*11* and *-DRB1*03:01* in European and North American Caucasian subjects, with *HLA-DRB1*15:02* in Japanese and Koreans, and with *HLA-DRB1*08:04* in African-Amerricans [77, 87-90]. Other class II molecules are also associated, such as *DQA1*05:01*, *DQB1*03:01* (both belonging to the same haplotype: *HLA-DRB1*11:04–DQA1*05:01–DQB1*03:01*), *DQB1*06:01*, and *DPB1*13:01*[40, 77, 91]. On the other hand, class II alleles protective

against the occurrence of SSc are reported, such as the haplotype *HLA-DRB1*07:01–DQA1*02:01–DQB1*02:02* [77].

MHC class II associations differ across SSc clinical phenotypes and positivity for the various autoantibodies. Generally, presence of ATA related to the severe form of SSc is associated with *HLA-DRB1*11* and *-DPB1*13* [40, 77], and presence of ACA is associated with *HLA-DRB1*04*, and **08* [40, 77, 92].

A parallel to the Shared Epitope hypothesis in RA can be made in SSc. In patients with RA, the SE has a strong effect on the risk to develop ACPA positive RA (or at least RF positive ACPA negative RA) [93]. In SSc, most HLA-DR associated with disease have in common an amino-acid sequence ^{67}FLEDR^{71} on their β chain, and that sequence is associated with an ATA producing SSc profile [91] (*HLA-DRB1*11*, **08*, or in *HLA-DRB5*01:01* that is in linkage disequilibrium with the Asian susceptibility allele *DRB1*15:02* and coexpressed with it on the cell surface [91]). Similarly, most common *HLA-DQB1* susceptibility alleles code for a common ^{71}TRAELDT^{77} motif on their β chain, also associated with SSc positive for ATA (*HLA-DQB1*03*, **06*…) [94]. This observation supports the hypothesis that a particular HLA shared motif presents particular auto-antigenic peptides triggering a helper T cell response, which in turn conducts to a particular autoantibody production.

Linkage disequilibrium between specific *HLA-DRB1* and *-DQB1* alleles complicates the analysis of associations. For example, the susceptibility haplotype *HLA-DRB1*11:04–DQA1*05:01–DQB1*03:01* bears both FLEDR and TRAELDT motives and makes difficult assessing the primary effect of one or the other. Efforts are made to decipher relative contribution of alleles on the same chromosomic block, and a recent study conducted in our laboratory showed that the effect of FLEDR and TRAELDT are not necessarily inter-dependent [95].

CHAPTER 1.3 – PHYSIOLOGICAL RISK FACTORS FOR FEMALE PREDOMINANCE

1.3.1 HORMONAL FACTORS

The predominance of autoimmune disease among women suggests that sex hormones may modulate susceptibility. Most attention has been directed toward the sex steroids that are produced in the gonads. Testicles produce testosterones in males, and ovaries produce estrogens in females. The effect of sex hormones on the modulation of the immune system is well documented. Receptors for sex hormones are present on immune system cells, and numerous effects of estrogens and androgens have been reported on lymphoid precursors, maturation of B-cell, and regulation of macrophages and T cells [96-99]. In general, androgens are considered immunosuppressive, while estrogens are natural enhancers of immune response [100]. Th1 and Th2 balance seems to be influenced by hormones. High estrogen profile promotes Th2 cells, known to trigger antibody production, while low estrogen profile promotes Th1 cells, known to secrete proinflammatory cytokines and provoke cell-mediated immune responses [101]. Pregnancy, a high estrogen state, is characterized by Th2 dominance, and high Th1/Th2 ratios are associated with increased risk of “immunologically-induced” pregnancy loss [102, 103].

Sex hormones have been investigated in autoimmunity. In many autoimmune diseases, like RA, SLE, SSc, and multiple sclerosis, an estrogen/androgen imbalance towards elevated estrogen levels is often observed [104]. SLE may occur more frequently in women taking birth control pills and in post-menopausal women taking estrogen therapy [105, 106]. Nevertheless, these observations are less consistent with the fact that many autoimmune diseases do not have a peak of incidence during reproductive age but rather arise post-menopause, or with the fact that ameliorations are often observed in patients with RA and multiple sclerosis during pregnancy (characterized by high estrogen levels) [104, 107].

Although the sex hormone profile in women with autoimmune disease does not fully explain gender bias, this hormone/immunity inter-regulation supports the proposed view that evolution has endowed females with a “superior” immune system in order to face the immunological challenges of reproducing [108]. Therefore, in the context of a more “aggressive” immune system, the hormonal environment (e.g. concentration of estrogens, expression of estrogen

receptors, cell type involved) is thought to play a significant role in autoimmune and inflammatory diseases [109].

1.3.2 MICROCHIMERISM, LEGACY OF PREGNANCY

Presence of two or more populations of genetically distinct cells in one individual is called chimerism. *Micro*chimerism (Mc) corresponds to a small quantity of these cells (few per million of host cells). This chimerism can be acquired either naturally during pregnancy, or artificially by transplantation of organs or cells.

With the current methods of molecular biology, presence of fetal DNA in maternal plasma may be detected as early as the 5th week of pregnancy [110], while maternal DNA can be detected in fetal circulation at the 9th week. Throughout pregnancy, the exchange of cells between mother and fetus increases; at 5 weeks of pregnancy, fetal DNA is estimated to be on average 8–9 genome equivalent of fetal cells per millilitre (gEq/mL) of maternal plasma. This quantity increases throughout pregnancy up to more than 3000 gEq/mL of maternal plasma just before delivery. Amazingly, fetal DNA is rapidly cleared from maternal plasma right after delivery, with a mean half-life of 16.3 min [111].

Trafficking on the other way around also occurs and as it is known that maternal cells commonly enter the fetal circulation [112]. First demonstration of maternal passage was done in cord blood samples from male infants, using fluorescence *in situ* hybridization (FISH) with probes specific to the X and Y chromosomes. Female cells were then identified in more than 20% of the samples [113]. Subsequent studies utilizing classical PCR techniques identified maternal DNA with a frequency of 40% [114] to 75% [115] in cord blood samples.

Long term persistence of microchimeric cells is now well recognized (**Figure 11**). Based on methods using FISH with double labelling of X and Y chromosomes and PCR assays targeting the non-inherited HLA, maternal cells are shown to persist in immuno-competent adults [116, 117]. Mc of fetal origin is detected, using Y-chromosome-specific PCR methods, many years after women have been pregnant, both in the case of a live birth or a miscarriage or abortion [118-120]. Cells from a twin transferred to a fetus in utero is also possible, and that might happen even in case of singleton birth as the loss of an unrecognised "vanished" twin is not uncommon in healthy pregnancies [121]. Evidence for such a phenomenon have been

reported by our group in the case of a male patient with scleroderma-like syndrome [122]. Indeed, presence of female cells has been shown by FISH in this male patient. Further analyses by HLA-specific quantitative PCR have revealed the paternal origin of these cells, suggesting that cells from a vanished twin sister are the source of this Mc [122].

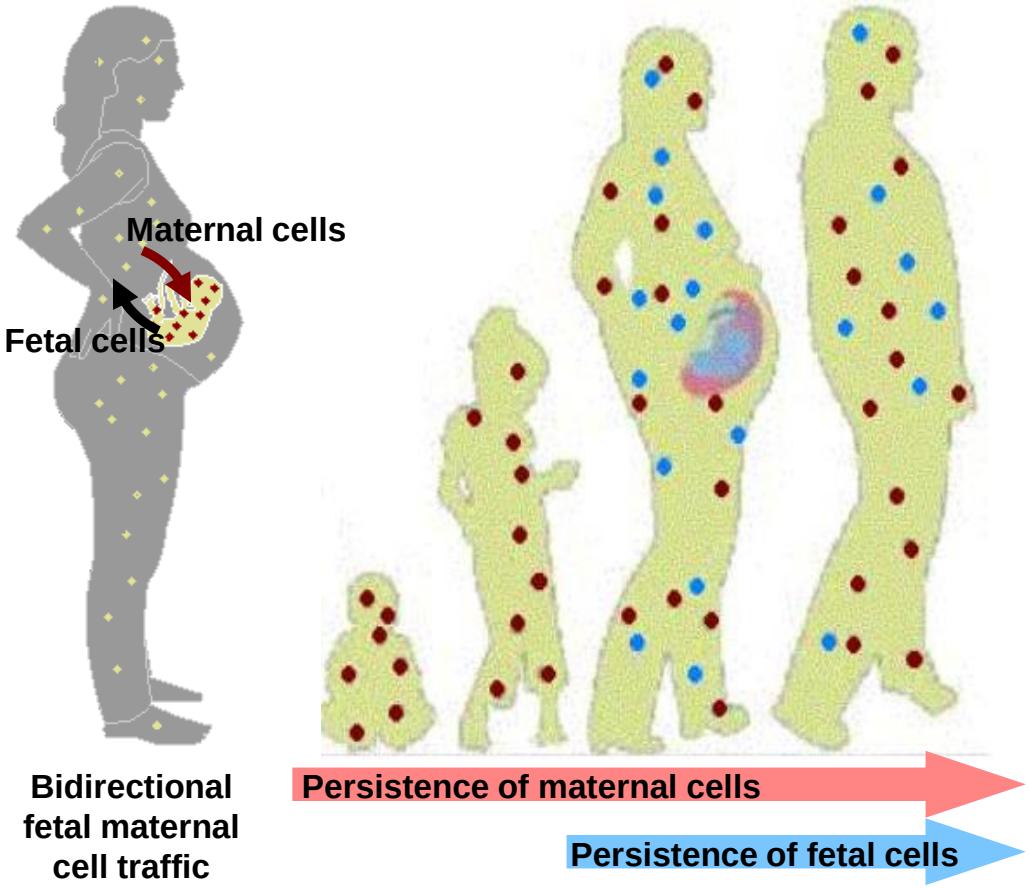


Figure 7. Natural microchimerism (Mc) occurs during pregnancy and persists for decades after delivery.
Fetal cells enter maternal circulation and, vice versa, maternal cells enter into the fetus. Several sources of Mc can coexist; parous women can have fetal Mc in addition to maternal and twin Mc common with men (adapted from: *Lambert N & Nelson JL, 2003, Autoimmun Rev*).

1.3.2.1 Phenotype of microchimeric cells

Mc has been described in a variety of human tissues, **Table 4** summarizes origin, location and presumed cell types. In fetuses, maternal Mc has been detected in thymus, lung, heart, pancreas, liver, spleen, kidney, adrenal gland, ovary, testis, and brain [123]. Infants had maternal Mc in the thymus, lung, pancreas, liver, spleen, thyroid, and skin [124, 125]. In addition, maternal Mc has been found in children and adults with or without autoimmune diseases in their heart, skeletal muscle, and islet β cells of pancreas [126, 127].

On the other hand, male Mc of presumed fetal origin has been described in cells with pluripotent characteristics, such as CD34+ and mesenchymal cells of healthy women [128, 129], but also in thyroid, lung, lymph node, skin, kidney, liver, heart, intestine, gallbladder, and cervix [130-133]. In the blood, Mc of maternal and fetal origin is detected in T and B cells, NK cells, monocyte/macrophages, and granulocyte populations [134-136].

Table 3. Reported cell types of naturally acquired Mc (Adapted from: *Nelson JL, 2012, Trends in immunology*).

Organ	Presumed cell type	Origin of Mc
Brain	Neurons (murine)	Fetal
Lymph node	Hematopoietic cells	Fetal
Thyroid	Epithelial cells, thyrocytes	Fetal
Blood	T and B cells, monocytes/macrophages, NK cells, granulocytes	Fetal and maternal
Blood	Lymphoid progenitor cells	Fetal
Heart	Cardiac monocytes	Fetal and maternal
Skin	Endothelial cells	Fetal
Skin	Keratinocytes	Maternal
Spleen	Hematopoietic cells	Fetal
Kidney	Renal tubular cells	Maternal
Pancreas	Islet beta cells	Maternal
Liver	Hepatocytes	Fetal and maternal
Gallbladder	Epithelial cells	Fetal
Intestine	Epithelial cells	Fetal
Cervix	Epithelial cells	Fetal

1.3.2.2 Potential role of microchimerism

The stem character of Mc cells as well as their possible “invisibility” to immune system might help their survival. The fact that these cells persist in healthy subjects indicates a possible tolerance of the immune system allowing them to survive. However, Mc is more frequent and quantitatively greater in women with certain autoimmune diseases [6, 7, 137]. Mechanisms by which Mc might affect autoimmune diseases are unknown but some possibilities can be considered.

The theory of a harmful role of Mc in autoimmunity has been supported by the findings that male microchimeric cells, presumed to be fetal in origin, is detected in disease-target organs such as thyroids of women with autoimmune thyroid diseases [138] as well as in blood and skin tissues of patients with scleroderma [117, 139-141]. Maternal T cells have been detected in myositis and the “immune response effector” role has been proposed for Mc [142]. Studies in RA have suggested that Mc can act as carrier of disease risk. Indeed, the majority of RA patients have the so-called “Shared Epitope” (SE) five-amino-acid motif in their HLA-DRβ1 molecules (as discussed in chapter 2). Some patients with RA, however, do not have the SE and two studies have presented evidence that microchimeric cells carrying the SE are detected with increased frequency and in higher amounts in these patients compared to controls, conferring some kind of “mini-dose” effect in RA risk, that is known to increase with the presence of the SE in an individual’s HLA genotype [85, 86].

On the other hand, maternal cells were found in pancreas of patients with type 1 diabetes (T1D). Importantly, these cells, of maternal origin, had differentiated into β-islet cells and were capable to produce insulin [127, 143]. These observations revolutionized opinions on Mc role, giving a regenerative function to microchimeric cells. Hypothetically, microchimeric cells, due to their stem-like character, have the capacity to differentiate and restore function of damaged organs. Little is known, however, about their migration and differentiation capacity, as well as their niche.

1.3.2.3 Gender bias in microchimerism

Natural Mc acquisition takes place during pregnancy as fetal cells enter maternal circulation and, vice versa, maternal cells are sources of Mc that can affect humans. A third source of Mc

is cells from a twin fetus. A fundamental difference between the sexes is the fact that men have fewer possibilities than gravid women to acquire natural Mc.

Mc is well described in the case of RA and SSc. Moreover, it is interesting to note that the course of disease tends to change after pregnancy. A study from Nelson's group highlights an overall reduction in disease risk for parous compared to nulliparous women, suggesting that pregnancy provides a "vaccine-like protection" against RA [144]. SSc has a peak incidence in women in thier postreproductive years [35] and shares clinical similarities with graft-versus-host-disease after hematopoietic cell transplantation. These observations suggest a particular and still unclear role for fetal Mc in the pathogenesis of both diseases. Men do not have the fetal source and are also less affected by autoimmune diseases [4, 5]. Therefore, Mc can be considered as an attractive explanation for female predominance in autoimmune diseases. Little is known, however, about the differences in frequencies and quantities of Mc between the sexes. It is expected that men with autoimmune diseases, having fewer sources of Mc, would acquire risk-conferring Mc at greater magnitude compared to women with same diseases. Investigating whether this hypothesis is true in the case of RA and SSc merits to be addressed.

CHAPTER 1.4 – X-CHROMOSOME-LINKED FACTORS BEHIND GENDER BIAS

1.4.1 GENES INVOLVED IN IMMUNITY ON THE X CHROMOSOME

In humans, the female karyotype includes two X chromosomes (one derived from each parent) while males carry one maternal X chromosome and one paternal Y chromosome. A single gene on the Y chromosome, i.e. *SRY*, acts as a signal to set the developmental pathway towards maleness [145], and in the absence of *SRY*, the default pathway leads to the development of female embryos.

The X chromosome contains about 1100 annotated genes, which is approximately 5% of the human genome [146, 147]. The Y chromosome, on the other hand, contains only about 100 genes [147] of which 78 are in the male specific region [148]. The X and Y chromosomes are believed to have originated from a common ancestor autosome, and still have homology in the pseudoautosomal regions (PAR). There are two PARs on the tips of each sex chromosome: PAR1 and PAR2 respectively at the termini of the short and long arm. Thanks to DNA homology in PAR1 and 2, sex chromosomes can exchange DNA by recombination during male meiosis. There are 54 functional genes homologous to both X and Y chromosome: 29 of them belong to the PARs and the remaining 25 are in homologous DNA blocks that became non-recombinant due to inversions, rearrangements and transpositions in the Y chromosome [146] (**Figure 8**). The genes in the PARs are present in two copies on both sexes and do not require inactivation. Nevertheless, genes in the female specific regions need to be silenced in one of the X chromosomes in females, in order to ensure that their expression equals that of males with only one X chromosome. The epigenetic phenomenon responsible for this silencing is the so called X chromosome inactivation.

Interestingly, the X chromosome carries numerous genes involved in immune functions, such as Toll-like receptor (*TLR*) *7* and *8*, CD40 ligand (*CD40L*), and forkhead box P3 (*FOXP3*) (**Table 4**). Further, it is suspected that additional genes and mutations relevant to the immune system are yet to be discovered [149]. In the context of female predominance in autoimmunity, associations of X-linked genes to autoimmune disease have been described. The X-linked genes identified include *TIMP1* (associated with SSc, RA, and Crohn's disease), *IRAK1* (SSc and SLE), *FOXP3* (Autoimmune thyroid disease), *CD40L* (SLE), and *IL9R* (RA) [65].

However, the relevance of the X chromosome in explaining risk of disease could account on the epigenetic level. Indeed, X chromosome inactivation, which silences one of the two X chromosomes in women, is naturally incomplete. Based on a human fibroblastic model, it is known that up to 15% of genes on the inactive X chromosome can escape permanent silencing. An additional 10% can have a heterogeneous on/off pattern of expression (depending on the cell line), leaving 75% of genes subject to inactivation [150]. The genes involved in immune functions are not an exception of this rule, as shown in **Table 4**.

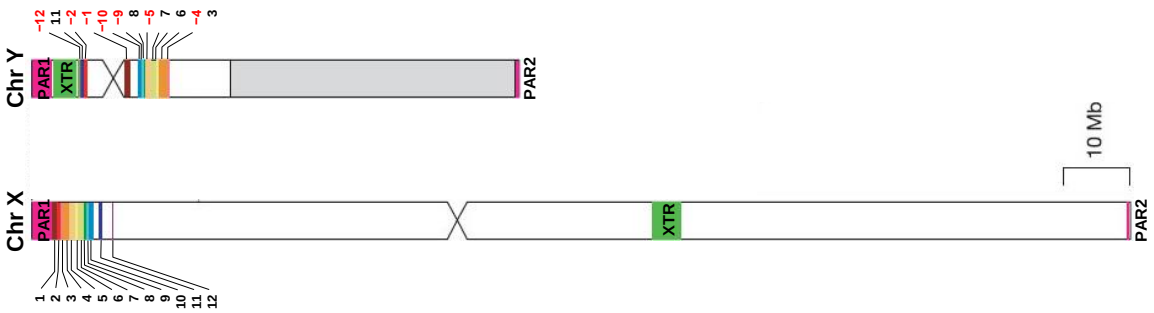


Figure 8. Shematic representation of major homologies between sex chromosomes.
X and Y chromosomes are shown on the same scale. Major heterochromatic region on the long arm of Y is in grey. Pseudoautosomal regions (PARs) are in purple. Outside PARs, homologies are in the non-recombining regions predominantly on the X chromosome short arm together with a large “X-transposed region” (XTR). These homologous sequences are colored and numbered (blocks 1 to 12). These blocks are rearranged on the Y chromosome and sometimes inverted relative to the X chromosome. In this case they are assigned red, negative numbers (Adapted from: *Ross MT et al., 2005, Nature*).

The significant number of genes (15%) that escape silencing could have implications in gender bias as these genes are expressed at characteristically higher levels in women compared to men. Moreover, 10% of X-linked genes have heterogeneous inactivation patterns, thus accounting for differences in expression among females themselves, predisposing some women more than others to autoimmune diseases.

Table 4. A list of genes located in succession on the X chromosome with a direct or indirect role in immune functions (*Libert C et al., 2010, Nat Rev Immunol*).
The depicted genes in the non-recombining region do not have any known Y homologues (except RAB9A → Y homology by BLAST). Data available on gene expression of human-fibroblastic inactivated

X chromosomes indicate genes that are subject to inactivation, escape inactivation, or are heterogeneously activated/inactivated (*Carrel L & Willard HF, 2005, Nature*).

Gene location	***Gene***	***Description***	***Function in immunity and/or disease***	***X chromosome inactivation***
Pseudo autosomal region 1 (PAR1)	*CRFL2*	Cytokine receptor like factor 2	Enhances dendritic cell (DC) maturation and T cell proliferation	n/a (not available)
	GMCSFR / CSF2RA	Granulocyte macrophage colony stimulating factor	Granulocyte and monocyte differenciation	n/a
	IL3RA	Interleukin (IL) 3 receptor alpha	Component of the IL-3 receptor	n/a
	CD99 / MIC2	CD99 antigen	T cell regulation	Escape (PAR)
Non-recombining region of the X chromosome (short arm)	*ARHGAP6*	Rho GTPase activating protein 6	Regulating the small GTP-binding protein of the RAS (Rat Sarcoma) superfamily	Subject to
	TLR7	Toll-like receptor 7	Viral recognition / SLE	n/a
	TLR8	Toll-like receptor 8	Viral recognition / susceptibility to tuberculosis	n/a
	RAB9A	Ras-related GTP-binding protein	Autophagy regulator during infection	Escape
	PIGA	Phosphatidylinositol glycan anchor biosynthesis class A	Paroxysmal nocturnal hemoglobinuria / involved in complement regulation	Subject to
	BMX	Bone marrow kinase X-linked	Growth and differentiation of hematopoietic cells	n/a
	RPS6KA3 / RSK2	Ribosomal protein S6 kinase alpha 3	Control of TLR signaling / required for endocytosis in DCs	Subject to
	CNKSR2	Connector enhancer of kinase suppressor of ras 2	Regulator of RAS signaling	n/a
	IL1RAPL1	IL1 receptor associated protein-like 1	Orphan receptor of the IL-1R superfamily	n/a
	CYBB / NOX2	Cytochrome b-245 beta polypeptide	X-chronic granulomatous disease (X-CGD), reactive oxygen species (ROS) production	n/a
	DUSP21	Dual specificity phosphatase 21	Phosphatase activity	Escape
	TIMP1	Tissue inhibitor of metalloproteinase	RA / Crohn's disease / wound repair, inhibition of metalloproteinase, erythroid potentiation activity	Heterogeneous
	PFC / CFP	Properdin P factor complement	Properdin deficiency / upregulating alternative complement pathway	Subject to
	WAS	Wiskott-Aldrich syndrome protein	WAS / cytoskeleton organization	Heterogeneous
	GATA1	GATA binding protein 1	Differentiation of erythrocytes and megakaryocytes	Subject to
	FOXP3	Forkhead box P3	Immune dysregulation-polyendocrinopathy-enteropathy X-linked (IPEX) syndrome / Autoimmune thyroid disease / differentiation of regulatory T cells	Subject to
Non-recombining region of the X chromosome (long arm)	*ARHGEF9*	CDC42 guanine nucleotide exchange factor (GEF) 9	Activation of CDC42	n/a
	VSIG4	V-set and immunoglobulin domain containing 4	Macrophage mediated phagocytosis / inhibition of T cell activation	n/a
	EDA2R / XEDAR	Ectodysplasin A2 receptor	Activation of NF-κB and JNK (c-Jun N-terminal Kinases) pathways	Heterogeneous
	EDA	Ectodysplasin A	X-linked hypohidrotic ectodermal dysplasia (XHED) / regulation of NF-κB and JNK pathways	Subject to
	IGBP1	Immunoglobulin (CD79A) binding protein 1	Inhibitor of apoptosis	Subject to
	IL2RG	Interleukin 2 receptor gamma	X-linked severe combined immunodeficiency (X-SCID) / common chain for IL-2, 4, 7, 9, 15, and 11	n/a
	CXCR3	Chemokine (C-X-C motif) receptor 3	Receptor for CXCR9, 10, and 11	Subject to
	FGF16	Fibroblast growth factor 16	Cell growth, morphogenesis, tissue repair, inflammation	n/a
	NOX1	Nicotinamide adenine dinucleotide phosphate (reduced) oxidase 1	ROS production	n/a
	BTK	Burton agammaglobulinaemia tyrosine kinase	X-linked Burton agammaglobulinaemia (XLA) / TLR4 and TLR2 signaling pathways	Subject to
	IL1RAPL2	IL1 receptor-associated protein-	Orphan receptor of the IL1R superfamily	n/a

		like 2		
	TSC22D3 / GILZ	Transforming-growth-factor-beta-stimulated clone-22 domain family member 3	Anti-inflammatory and immunosuppressive functions	n/a
	IL13RA2	IL-13 receptor alpha 2	IL-13 signaling (decoy receptor)	n/a
	IL13RA1	IL-13 receptor alpha 1	IL-13 signaling	Subject to
	NKRF	NF-kappaB repressing factor	Silencing of IFN-β through NF-κB inhibition	n/a
	NKAP	NF-kappaB activating protein	Regulation of NF-κB activation / T cell development	Subject to
	XIAP / BIRC4	Inhibitor of apoptosis X-linked	X-linked lymphoproliferative disease (XLP)2 / direct inhibition of caspases 3 and 7	Subject to
	SH2D1A / SAP	Signaling-lyphocyte-activation-molecule associated protein	XLP1 / Epstein-Barr virus infection	n/a
	AIFM1	Apoptosis-inducing factor	Inducer of apoptosis	n/a
	CD40L	CD40 ligand	X-linked hyper IgM syndrome (XHIM), lupus (SLE), malaria, human-immunodeficiency-virus (HIV)	n/a
	ARHGEF6	RAC/CDC42 guanine nucleotide exchange factor (GEF) 6	Activation of the RAS-like family of Rho proteins / cytoskeleton organization / apoptosis	Subject to
	BGN	Biglycan precursor	Endogenous ligand for TLR2 and TLR4 in macrophages / activation of p38 MAP kinase, ERK (extracellular regulated kinase), and NF-κB	Subject to
	DUSP9 / MKP4	Dual specificity phosphatase 9	Inactivation of MAP (mitogen activated protein) kinases	Subject to
	ARHGAP4	Rho GTPase activating protein 4	Regulating the small GTP-binding protein of the RAS superfamily	Escape
	IRAK1	IL-1 receptor associated kinase 1	TLR signaling / RA, SSc and SLE	Subject to
	G6PD	Glucose-6-phosphate dehydrogenase	G6PD-deficiency / required for oxidative burst by phagocytes and erythrocytes	Subject to
	IKBKG / NEMO	Inhibitor of kappaB kinase gamma	Incontinetia pigmenti / anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) / regulation of NF-κB signaling / susceptibility to mycobacterial infections	Escape
	GAB3	Growth factor receptor bound protein (GRB)2-associated binding protein	Macrophage differentiation	Escape
	MTCP1	Mature T cell proliferation 1	T cell proliferation	Subject to
Pseudo autosomal region 2 (PAR2)	*IL9R*	IL-9 receptor	RA / IL-9 receptor on several cells	Escape (PAR)

1.4.2 EPIGENETIC SUSCEPTIBILITY, THE X CHROMOSOME INACTIVATION

Epigenetics is the study of mechanisms responsible for functionally relevant changes in gene expression, with subsequent changes in cellular phenotypes, resulting from changes that do not implicate the DNA nucleotide sequence *per se* [151]. These epigenetic changes are stable and heritable; they are also reversible and are impacted by age and environmental factors, thus conferring potential risk to the development of autoimmune diseases [152].

The epigenetic control begins with the organization of DNA into chromatin, by forming tightly packaged nucleosomes (DNA warped around a histone protein octamer formed with H2A, H2B, H3, and H4). Then, epigenetic regulation occurs through four mechanisms. First, direct

chemical modification of DNA can occur by cytosine methylation, generally a marker of gene silencing. Second, core histones undergo post-transcriptional modifications, such as methylation, ubiquitylation, phosphorylation, and acetylation (this latter is often associated with the loosely condensed “euchromatin” which is more actively expressed than the tightly condensed “heterochromatin”). Third, the chromatin is packaged, via long-range interactions and DNA twisting mechanisms (influenced by topoisomerase, protein binding, etc.), into higher-order structures within the cell nucleus. Finally, non-coding RNAs, such as dosage compensation RNAs and small-interference RNAs, contribute to the epigenetic regulation. A clear example of RNA involvement is dosage compensation in mammals mediated by XIST RNA, resulting in a long-term silencing (i.e. inherited through cell division) of one of the two X chromosomes in female cells. This process is called X chromosome inactivation [151].

1.4.2.1 X chromosome inactivation, a dosage compensation mechanism

Dosage compensation is a regulatory process set on the X chromosome to ensure that X-linked genes have equivalent protein expression levels in female cells with two X chromosomes and in male cells with only one. Dosage compensation exists at different points of evolution and may be achieved in various ways. For example, in *Drosophila melanogaster*, X chromosome in males is two folds hyper-transcribed in order to match X-linked expression levels of female cells [153]. In contrast, the nematode *Caenorhabditis elegans* reduces in half the production of X-linked transcripts in XX hermaphrodites to attain the same level produced in XO males [154]. In the example of birds, in which ZW females and ZZ males differ in the number of Z chromosomes, it has been unclear whether Z-associated dosage compensation occurs. A recent report shows that, surprisingly, it is less effective than in other species [155].

In mammals (including humans), the equivalency between sexes is established and maintained by a complex and tightly regulated epigenetic process called X chromosome inactivation (XCI) [156], consisting in inactivating most of the genes on one of the X chromosomes in each female cell. It is to be noted that XCI is naturally incomplete and evidence suggest that from 5 [157] to 15% [150] of genes on this chromosome can escape permanent silencing.

Normally, the choice of which X chromosome to inactivate, the maternally-derived or the paternally-derived, is a random choice made in each cell. Random XCI starts at the blastocyst stage in the development of the embryo and is complete at the onset of gastrulation [158]. As a consequence, new-born females are a mosaic of areas with cells expressing either the maternal X-linked genes, or the paternal X-linked genes, with a ratio close to 50:50 due to the random nature of XCI (**Figure 9**). Throughout life, the daughter cells and all subsequent generations will keep the same parentally-derived inactive as was chosen by its original ancestor cell in the embryo [159].

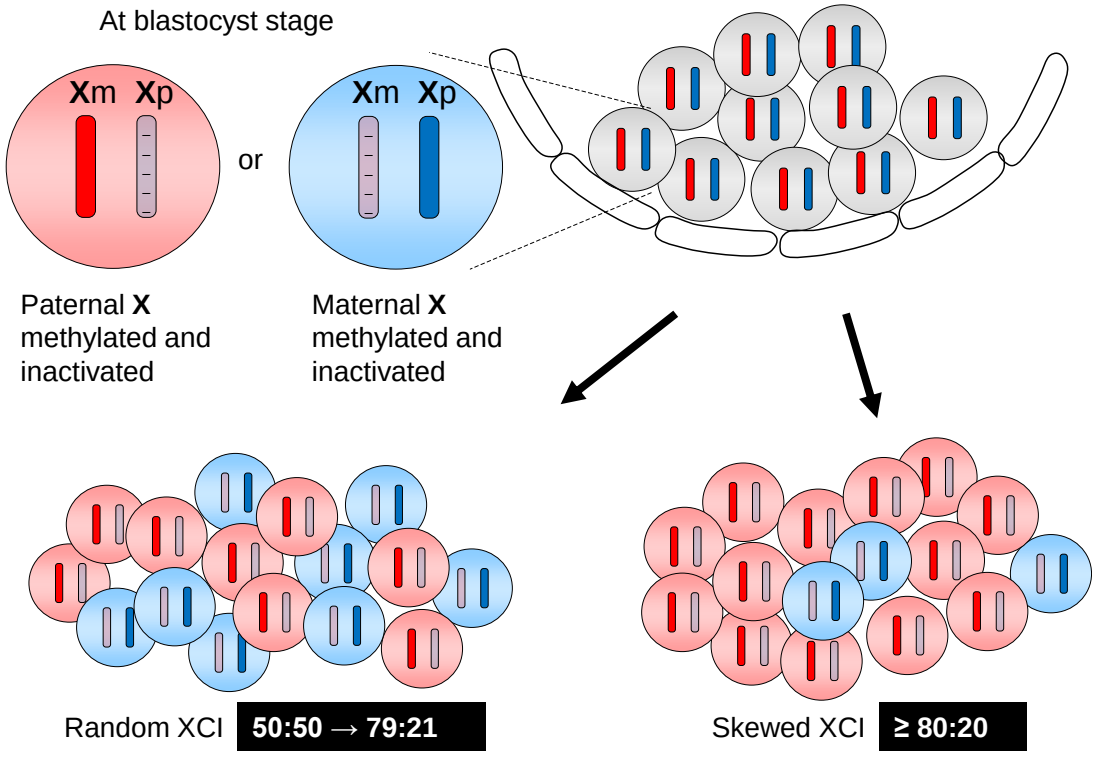


Figure 9. Schematic representation of X chromosome inactivation.
X chromosome inactivation (XCI) starts at the blastocyst stage and results in the formation of a mosaic of cells expressing either the maternally-derived (Xm) or paternally-derived (Xp) X chromosome, with a ratio close to 50:50 when XCI is random. A non-random (or skewed) XCI is considered when this ratio is ≥ 80:20.

The pattern of XCI in each embryonic cell originates in a region on the long arm of the X called "X inactivation center". This region contains a long non-translated (but functional) RNA: the X-

inactivation specific transcript (*XIST* in humans) [160], and its overlapping anti-sense counterpart *TSIX* [161]. At the early stages of embryogenesis *Xist* and *Tsix* are both expressed on both X chromosome. XCI is initiated by a break in symmetry of a transient phase of X to X pairing (**Figure 10**). It is believed that this event causes the redistribution of *Tsix* transcription factors thus designating the future active X [162]. On the future inactivated X chromosome, *Tsix* is downregulated. The loss of *Tsix* enables the long non-coding RNA: repeat A RNA (*RepA*) to target Polycomb repressive complex 2 (PRC2) and trimethylation of lysine 27 on histone H3 [163, 164]. This contributes to *Xist* upregulation. On the future activated X chromosome, *Tsix* expression persists and prevents Xist expression by silencing its promoter (via DNA methylation). Once *Xist* is stably silenced on the activated X chromosome, *Tsix* itself is also downregulated, while the expression of *Xist* persists on the inactive X chromosome [159]. *Xist* RNA transcript spread along the inactive X from the X inactivating center coating the chromosome and recruiting proteins and factors (PCR2 and trimethylation of H3) that further establish an inactive heterochromatin state [165].

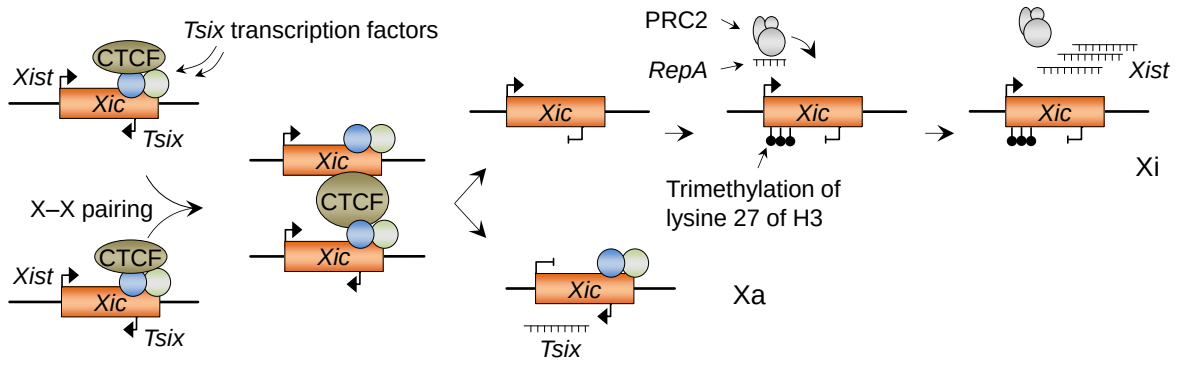


Figure 10. A transient X–X pairing enables break in symmetry and initiation of X chromosome inactivation.
X–X pairing at the X inactivation center's (*Xic*) location is initiated by CTCF transcription factor. A break in pairing would redistribute *Tsix* transcription factors on the future active X chromosome (Xa). On the future inactive X chromosome (Xi), *Tsix* RNA is downregulated, allowing *RepA* RNA to target PRC2 complex and trimethylation of lysine 27 on histone H3. *Xist* expression persists on the inactive X chromosome (Xi). Adapted from *Lee JT, 2011, Nat Rev Mol Cell Biol*.

1.4.2.2 Skewing of X chromosome inactivation

Many studies have shown that XCI patterns follow a normal distribution [166-168]. The largest one has included 1005 females having inactivation patterns with a mean ratio of 49:51 and a standard deviation of 17.2 [167]. The graphical aspect of the bell-shaped curve is represented in **Figure 11**. This shows that skewing, defined as a deviation in the 50:50 ratio of XCI and resulting in the overrepresentation of one of the parental X chromosomes in female tissues, can be observed in the general population. Furthermore, several studies have reported that skewed XCI significantly increases with age in blood cells of females from the neonatal period to late adulthood [169-171]. This suggests that, even though most of the choice of which X chromosome to inactivate happens in early embryonic life, the mosaic status is not stable and readjustments occur in adult life.

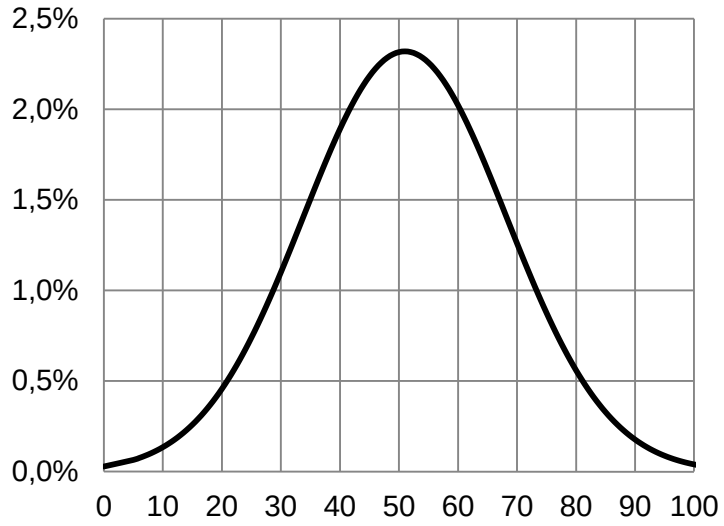


Figure 11. Bell-shaped curve describing the probabilities (in percentage) of XCI patterns with ratios going from 0:100 to 100:0.
The parameters of this normal distribution are 51:49 (mean) and 17.2% (standard deviation), according to data from 1005 normal females (*Amos-Landgraf JM et al., 2006, Am J Hum Genet*).

Although rarely observed in the general female population, a skewed XCI can occur in some women. Such skewing has been well documented in peripheral blood cells of women with autoimmune diseases, including autoimmune thyroid diseases, scleroderma, RA, and juvenile

idiopathic arthritis [172-178]. Interestingly, the study on XCI in scleroderma shows that purified T cells (CD3+), B cells (CD19+), monocytes (CD14+), plasmacytoid dendritic cells (BDCA+), and myeloid dendritic cells (CD1c+) have patterns of inactivation similar to those observed in PBMCs, suggesting that skewing takes place at the hematopoietic precursor level [178]. Another interesting inquiry is parental origin of inactivated X chromosome. Only the study of Uz *et al.* has analyzed this in blood samples from 10 patients with SSc and, although numbers were small, it reveals that the maternally-derived X chromosome is more often inactive than the paternally-derived one [177].

Reasons for biased XCI in autoimmunity are not yet fully understood. Nevertheless, it is becoming clear that this form of mosaicism is somehow associated with the occurrence of many autoimmune diseases, and therefore merits further investigations.

1.4.3 X CHROMOSOME SOMATIC MOSAICISM

The presence of genetically distinct cell populations in a single organism can occur. Many possible mechanisms can lead to such somatic mosaicism, including somatic mutations (e.g. single-base-pair mutations or CNVs), epigenetic changes in DNA (e.g. XCI mosaicism), and alteration in chromosomal structures and/or numbers (resulting from cell division errors).

A remarkable example of mosaicism in autoimmune diseases involves the loss of the X chromosome. Indeed, cases of women with SSc, primary biliary cirrhosis and autoimmune thyroid disease have been reported with higher X-chromosome monosomy in their blood cells compared to age-matched controls [179, 180]. It seems that such X-linked chromosomal abnormality is progressively acquired with age and parallels the incidence of autoimmune diseases. This supports the view that X-linked genetic dosage is critical to autoimmunity development.

1.4.4 GENOMIC STRUCTURAL VARIATIONS AND GENDER BIAS: THE YAA MODIFIER

1.4.4.1 Copy number variations

Polymorphism in the number of copies of chromosomal segments (both additions and deletions) among the human population is known as Copy Number Variation (CNV). A survey of 270 individuals from different ethnic groups from the human HapMap project has estimated

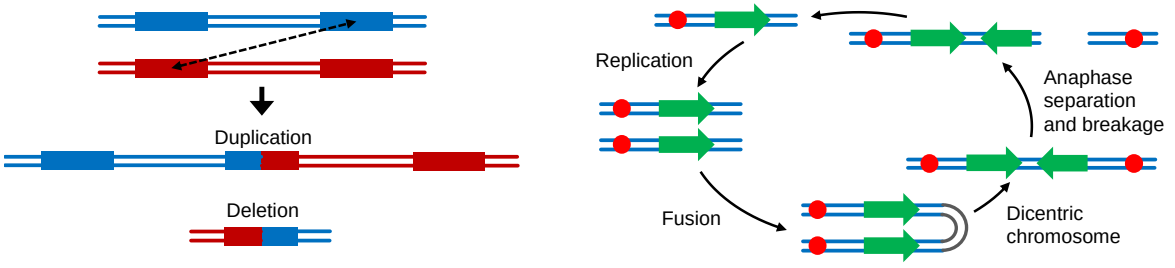
that up to 12% of the genome is covered by CNVs, and this may cause more overall sequence variation between humans than single nucleotide polymorphisms (SNPs) [181], a conclusion that has been reinforced by a study with an increased resolution of CNV discovery [182].

Copy number variants can arise both meiotically and somatically. They are enriched in genomic regions containing genes involved in immunity, olfaction and secreted proteins (genes relevant to the immediate environment) [183]. CNVs can be disadvantageous; they are involved in cancer formation and progression [184], they can lead to a variety of genomic disorders (Thalassemia, color blindness, X-linked ichthyosis…) [185], and are associated to autoimmune diseases, like RA, SLE, type 1 diabetes, psoriasis, and Crohn's disease [186].

CNVs seem to be more important as a driving force in the evolution period that shaped humans and great apes, compared to single-base-pair mutations [187, 188]. On the scale of populations, they contribute to the genotypic differences between individuals [181]. On the scale of the individual, they can affect some cells in the organism. This results progressively in somatic mosaicism, defined as the presence of genetically distinct cell populations in a single organism [189].

Change in copy number involves change in chromosome structure, such as two formerly separated DNA sequences can be joined. CNVs are results of these changes in the structure of chromosomes and they occur by two general mechanisms: homologous recombination and non-homologous recombination (for review [190]). Homologous recombination[E] requires extensive DNA sequence homology. It is likely that recurrent CNVs arise in regions with repeated sequences through a process called non-allelic homologous recombination (NAHR). By contrast, non-homologous recombination does not require sequence homology and can result in numerous structural changes of chromosomes such as translocations and CNVs [190] (examples in **Figure 12**).

[E] Homologous recombination is the basis of several mechanisms of accurate DNA repair that use another sequence that is practically identical to repair damaged sequence.



A- ***Non-Allelic Homologous Recombination*** **B-** ***Breakage-Fusion-Bridge cycle (non-homologous)***

Figure 12. Examples of change in copy number by homologous and non-homologous recombination.
Each line represents a single DNA strand, specific sequences are in bold, and centromeres are red circles. A- Non-allelic homologous recombination (NAHR) occurs by unequal alignment of homologous sequences that are present in repeats on the DNA molecule generating duplications as well as their reciprocal deletions. B- Breakage-fusion-bridge cycle is triggered when telomere is lost from a chromosome end. After replication, sister chromatids (both missing a telomere) fuse together, forming a dicentric chromosome. At the anaphase of mitosis, the 2 centromeres are pulled apart, causing the dicentric chromosome to break at a random position. This leads to the formation of a large inverted duplication. The new chromosome has once again an unprotected end and enters a new cycle. The cycle ceases when the chromosome acquires a telomere (Adapted from: *Hastings PJ et al., 2009, Nat Rev Genet*)

1.4.4.2 The Yaa translocation phenotype

Changes in copy number might modulate the expression levels of genes included in the affected regions and might lead to disease. One of the best examples in X-linked gene dysregulation leading to autoreactivity is described in a mouse model harboring an unbalanced translocation from the X to the Y chromosome. This translocation is called Y-linked autoimmune accelerator (Yaa) and consists of a 16-gene cluster including innate immunity genes *Tlr7* and *Tlr8*, coding respectively for Toll-like receptor 7 and 8 [191, 192]. This causes a 2-fold increase in copy numbers of those genes in the affected males. *TLR7* and *TLR8* are phylogenetically very close. Their natural ligand is single-stranded RNA from viral origin and stimulates human *TLR7* and *TLR8* and mouse *Tlr7*, but not mouse *Tlr8* [193] that seems to have only regulatory functions [194]. Moreover, it has been shown in the case of the Yaa translocation that only the duplication of *Tlr7* is required to accelerate autoimmunity in lupus susceptible male mice [195].

Yaa has been first described in the BXSB hybrid strain of mice which is a cross between a female from the common inbred C57BL/6 strain and a male from the immunity-altered SB/Le strain [196, 197]. BXSB spontaneously develops a lupus-like autoimmune syndrome that affects males much earlier than females. However, the reciprocal cross (SB/Le female and C57BL/6 male) does not show the same acceleration of disease in males. This is why the autoimmune-enhancing effect was attributed to the Y chromosome of the SB/Le strain [198]. The Yaa genetic abnormality by itself does not promote autoimmunity since non-autoimmune mouse strains, such as C57BL/6, are not affected by the Yaa mutation [199]. In contrast, combining Yaa with other mouse strains containing lupus susceptibility loci, such as NZB and NZW (New Zealand Black and White) induces the accelerated autoimmunity phenotype [199-202].

A genetic abnormality like the Yaa can occur in humans. Indeed, TLR7 and TLR8 are located at close proximity of each other in a syntenic region of the X chromosome in both humans and mice. They are closely related paralogs and are thought to be the outcome of a gene duplication in evolution. In humans, TLR7 and TLR8 both recognize the same natural ligand but show a difference in downstream inflammatory cytokine production [203]. Activation of TLR7 is oriented towards a type I interferon (IFN) production while activation of TLR8 is rather towards producing cytokines such as TNF-α, IL-1, IL-6.

1.4.5 X-LINKED TOLL-LIKE RECEPTORS

1.4.5.1 Toll-like receptors

TLR7 and TLR8 are part of a wider family of Toll-like receptors (TLRs). They are evolutionarily conserved innate immune proteins[F], critical in the first-line defense against foreign agents [204]. They mediate the recognition of conserved bacterial or viral ligands called pathogen-associated molecular patterns, and these ligands can activate dendritic cells, macrophages

[F] TLRs are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals. They are members of the “Toll” family, first described in *Drosophila* as a gene product essential for the development of embryonic dorsoventral polarity, and later shown to play a crucial role in the anti-fungal response of flies.

and other APCs and allow the effective presentation of microbial antigens to cells of the adaptive immune system. To date, there have been 10 human TLRs reported and they recognize different pathogen ligands. TLR1, 2, 4, 5, 6, and 10 are expressed on the cell surface and recognize microbial components including flagellin and lipopolysaccharide, whereas TLR3, 7, 8, and 9 are almost exclusively located in endosomal compartments and recognize nucleic acids [205].

TLRs activate the same signaling molecules that are used for interleukin 1-receptor (IL-1R) signaling [206]. The signaling pathways activated by different TLRs involve at least four protein adaptors that have a Toll/IL-1R homology (TIR) domain, such as myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing interferon-β (TRIF), TIR-associated protein (TIRAP) (also known as MyD88-adaptor-like [MAL]), and TRIF-related adaptor molecule (TRAM). MyD88 is critical for the signaling of all TLRs except TLR3 [204]. MyD88 and TRIF are responsible for the activation of distinct signaling pathways leading to the production of proinflammatory cytokines (IL-6, IL-12, TNF-α…) and type I IFN, respectively [204]. Molecules recruited and involved in these pathways include (in chronological order of involvement): kinases such as PKCε and Burton's tyrosine kinase (BTK), IL-1R-associated kinase-4 (IRAK4) and IRAK1, tumor-necrosis-factor-receptor (TNFR)-associated factor 6 (TRAF6), transforming-growth-factor-beta (TGF-β)-activated kinase 1 (TAK1), Mitogen-activated protein kinases (MAPK), I-kappa-B kinase epsilon (IKK-ε), IKK-γ/NFκB essential modulator (NEMO) complex. They activate transcription factors such as nuclear-factor-kappa-light-chain-enhancer of activated B cells (NFκB), interferon regulatory factor 5, 3, and 7 (IRF-5, -3, and -7), and activator protein 1 (AP-1) (**Figure 13**).

Cells prominently expressing TLRs include antigen-presenting cells (APCs) like dendritic cells (DCs), macrophages, and B cells. DCs are generally divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Expression of endosomal TLRs is different depending on the type of DC: cDCs express TLR3 but not pDCs, and pDCs are known to highly express TLR7 and TLR9, but not cDCs, leading to the production of IFN-α where IRF-7 plays a critical role [205]. Moreover, TLR-activated DCs and macrophages act as a source of immunomodulatory cytokines for directing the differentiation of T helper 1 (Th1) (via IL-12) and Th17 cells (via IL-6 and TGF-β, and later maintained by IL-1, -18, -23) [207].

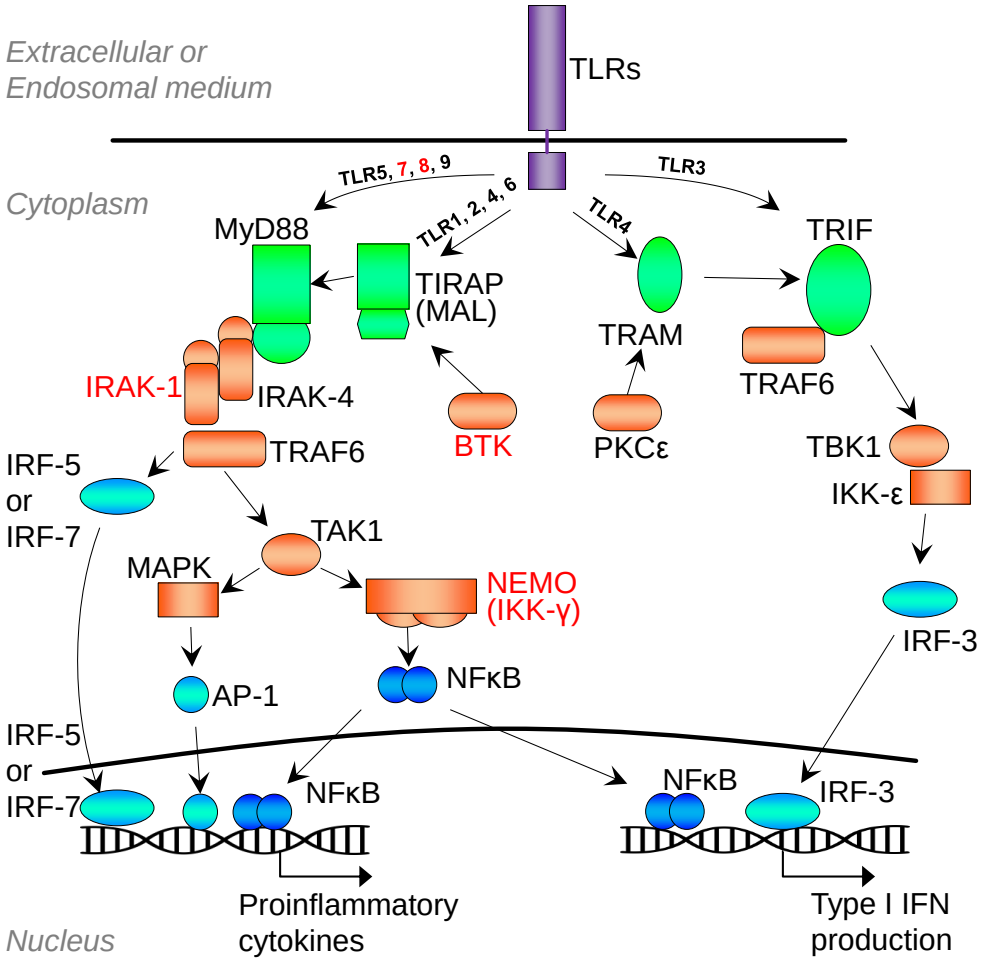


Figure 13. Simplified scheme of adaptor molecules in Toll-like receptor signaling in general. All TLRs, except TLR3 are dependent on MyD88 signaling pathway. TLR1, TLR2, TLR4 and TLR6 require TIRAP, for Myd88 recruitment, whereas TLR5, TLR7, TLR8 and TLR9 can bind MyD88 directly. In addition to the MyD88 pathway, TLR4 can signal through TRIF pathway, via TRAM molecule. TLR3 directly utilizes TRIF pathway. Molecule abbreviations are explained in the text. In green: adaptor molecules. In orange: intermediate signaling molecules. In blue: nuclear factors. TLR7, TLR8, IRAK-1, BTK, and NEMO (written in red) are coded by genes on the X chromosome (*Akira S et al., 2006, Cell*; *Baccala R et al., 2007, Nat Med*; *Goh FG & Midwood KS, 2012, Rheumatology (Oxford)* and *Doyle SL et al., 2007, J Biol Chem*).

1.4.5.2 Endogenous ligands of TLRs: role in autoimmunity

The long-standing viewpoint that the immune system functions by making a distinction between self and nonself does not present satisfying answers to many immunological

phenomena perceived by our body as foreign but harmless (e.g. fetus, gut microflora) or self but harmful (e.g. cancer, autoimmune diseases). Polly Matzinger proposed, over these unresolved issues, the *Danger Model*, in which antigen presenting cells are activated by danger/alarm signals from cells exposed to pathogens and toxins as well as injured cells (by cellular stress or mechanical damage, necrosis but not physiological apoptosis) [208, 209]. In such context, TLRs have been proposed to sense, in addition to pathogen-associated molecular patterns, endogenous molecules referred to as damage-associated molecular patterns.

There is accumulating evidence that endogenous TLR ligands have a role in triggering autoreactivity, thus contributing to autoimmune disease development. Indeed, activation of B cells is mediated by chromatin-IgG immune complexes and requires engagement of both B cell receptor and MyD88-dependent TLRs [210]. Moreover, B cells that are deficient in TLR7 or TLR9 have impaired response to such immune complexes [210, 211]. DNA-containing immune complexes within human SLE sera (but not protein-containing immune complexes from control sera) stimulate plasmacytoid dendritic cells via cooperative interaction between TLR9 and Fc-gamma receptor-IIa (CD32) [212]. Furthermore, direct autoantigen stimulation of innate immunity has been demonstrated by the findings that conserved RNA sequences within the small nuclear ribonucleoprotein particles (snRNP) from SLE-patients' sera, can stimulate TLR7 and TLR8, subsequently activating innate immune cells and the production of type I IFNs [213]. Similar findings are also reported in Sjögren's syndrome [214]. In RA, TLR3, 7 and 8 have increased expression in synovial tissues as compared to controls, and the activation of synovial fibroblasts is mediated by RNA released from necrotic synovial-fluid-cells in a TLR3-dependent manner [215, 216]. Moreover, a recent report demonstrates a pivotal role for TLR8 in systemic inflammatory diseases (in this case, juvenile idiopathic arthritis and Still's disease), as its transcripts levels positively correlate with levels of IL-1β, which was not the case with transcripts for TLR7 or TLR9 [217]. Interestingly, the anti-malarial drug chloroquine and related compounds which can block the activity of endosomal TLRs [218] have been used as anti-inflammatory drugs in various disorders, including SLE, psoriasis, and RA [219]. In addition, certain types of short oligodeoxyribonucleotides (ODNs) called immunoregulatory sequences, originally described in adenoviral vectors [220], inhibit endosomal TLRs, and are being

introduced as potential treatments for autoimmune diseases like RA [221]. Furthermore, these molecules have been used in mouse models of induced arthritis, and showed beneficial effects in reducing symptoms in mice [222, 223]. Other molecules that inhibit TNF-α production from human RA synovial tissues are mianserin (a tetracyclic serotonin receptor antagonist) and imiquimod (known as R-837, molecularly similar to the TLR7/8 agonist: R-848). Mianserin inhibits endosomal TLRs and imiquimod has an inhibitory effect on TLR8 [224].

Altogether, these findings suggest that endogenous DNA and RNA may contribute to the development of systemic autoimmune diseases via endosomal TLRs. However, whether other endogenous surface TLRs ligands effectively contribute to the onset or perpetuation of autoimmune disease is less clear-cut [225]. Other non-nucleic acid endogenous ligands include: breakdown products of the extracellular matrix (e.g. hyaluronate and heparan sulfate) [226-228], released molecules from necrotic or damaged cells (e.g. high-mobility group box 1 protein [HMGB1], fibronectin, heatshock proteins) [229-233], and fibrinogen [234]. They have been reported to stimulate TLR2 and/or TLR4. Many of these molecules accumulate in the joints of patients with RA [235], and are thought to contribute to the up-regulation of TLR2 and 4 in synovial fibroblasts or at sites of cartilage and bone erosion [236, 237], and to the increased osteoclastogenetic activity via stimulation of RANKL (receptor activator of nuclear factor kappa-B ligand) [238]. Nevertheless, the precise mechanism by which TLR2 and TLR4 recognize such a wide range of molecular structures, and the extent of redundancy between TLR2 and TLR4 in the detection of these danger-molecules, is not fully understood.

1.4.5.3 Examples in experimental autoimmune mouse models

Although mouse models of autoimmunity do not fully mimic human diseases, studies using mice have provided insights into the role of TLR activation in the initiation and exacerbation of autoimmunity. For example, mice deficient for the adaptor protein MyD88 are resistant to experimental autoimmune encephalomyelitis [239, 240] and experimental autoimmune uveitis [241]. In the collagen-induced arthritis model, symptoms arise after immunization with collagen and adjuvant containing killed bacteria, suggesting that disease is promoted by activation of TLRs (or other pattern recognition receptors) [242]. Interestingly, in two different reports, TLR2 has been found to either promote or inhibit inflammation in models of arthritis [243, 244].

However, the implication of TLR4 in murine arthritis is more consistent [244], and mice with bleomycin-induced scleroderma have attenuated dermal fibrosis when they have a mutated TLR4 [245]. Taken together, these studies suggest that TLR agonists may play a central role in triggering or maintaining autoimmunity.

Mouse models also helped to better understand the relation between innate and adaptive immunity when it comes to autoimmune manifestations. Indeed, there is evidence that pro-inflammatory cytokines, resulting from TLR-activation, from monocytes and dendritic cells promote induction of Th17 cells. Stimulation of TLR3, TLR4 or TLR9 induces MyD88 dependent production of cytokines, promoting the differentiation of IL-17-producing CD4+ T cells [246]. TLR2 activation also promotes the differentiation of Th17 cells and regulates Th17-mediated autoimmunity in experimental autoimmune encephalomyelitis [247]. Parallel to this in humans, monocytes stimulated with lipopolysaccharides, flagellin or other pathogen molecules promote IL-17 production by human CD4+ T cells [248]. In general, the induction of experimental autoimmune diseases in animals involves immunization with an antigen in the presence of an adjuvant. The killed M. tuberculosis in complete Freund's adjuvant promotes the expression of innate pro-inflammatory cytokines that direct the induction of Th1 and Th17 cells. It is then suggested that Th1 and Th17 cells (in other words IL-17+ INF-γ+ CD4+ T cells) may have pathogenic roles in autoimmune diseases, but their precise functions remain not fully resolved.

1.4.5.4 TLRs coded on the X chromosome

Among all TLRs, only TLR7 and TLR8 are coded by genes on the X chromosome. Therefore, not only they could play a role in the pathogenesis of autoimmune diseases, like RA, SSc and SLE (as discussed above), but they could also account for the gender bias observed in autoimmunity.

The Yaa phenotype in male mice, caused by the duplication of the *Tlr7* gene, provides the best example of TLR dysregulation leading to autoreactivity and inflammatory pathology. Such duplication could occur in humans, resulting in autoimmune disease in men, generally less predisposed compared to women.

The hypothesis of a *TLR7* gene copy number increase has been first tested by Kelley *et al.* who did not find any significant concordance between *TLR7* copy number and SLE neither in men or women [249]. However, a more recent and larger study found a correlation with susceptibility to childhood onset of SLE in a Mexican population [9]. Although results are contradictory, it is still interesting to see whether such phenomenon could occur in men with other autoimmune diseases like RA or SSc.

PART 2 – *OBJECTIVES*

The X chromosome contains many genes involved in immune functions, and such protein-coding genes are susceptible to X chromosome inactivation, cellular mosaicism, silencing escape and unusual patterns of evolution. Many genes on the X chromosome have been found implicated in autoimmune diseases in genome wide association studies (*TIMP1*, *IRAK1*, *FOXP3*, *CD40L*…). X-linked gene duplication has been described to potentiate autoimmunity in the Yaa mouse model. Perturbations in X chromosome inactivation are shown to be markers of several autoimmune diseases. Hypotheses about X chromosome's involvement in the remarkable female predominance in autoimmune diseases are currently well accepted. However, less is known about how this may lead to the breakdown of self-tolerance.

To better understand this gender bias, we propose, in the current thesis, to study risk factors associated to the X chromosome with special focus on rheumatoid arthritis. The study takes two aspects.

- The first one is genetic and involves the analysis of copy number variations of 2 X-linked genes in men with RA: Toll-like receptor 7 and 8, known to trigger autoimmunity in male mice of the BXSB strain, carrying the Yaa modifier.

- The second is epigenetic and consists in studying X chromosome inactivation patterns in women with RA, with regard to various disease parameters, including the presence of disease susceptibility *HLA-DRB1* genes. The latter study has been carried out in collaboration with Pr. Tayfun Ozcelik's group at Bilkent University, Ankara, Turkey.

The two studies give rise to two original articles where I am the first or co-first author with Onur E. Onat from Ozcelik's team.

Additionally, we present the case of a man in whom we demonstrated the presence of female microchimerism. We wonder if such phenomenon could contribute to the apparent copy number variation of X-linked *TLR7* and *TLR8*. The study of this case report has constituted part of my Master's project in our laboratory and is added to this manuscript. It represents a starting point to determine whether such phenomenon is common in men, and its role in autoimmunity.

Objectives ----------

We also present unpublished data on X chromosome inactivation studies in scleroderma, and compare them with our findings in RA.

Furthermore, as X chromosome inactivation mosaicism is susceptible to change with age, we test the hypothesis of a possible association of shortened telomere length with skewed X-chromosome inactivation in RA. Results on this subject give rise to an abstract published in the *Annals of the Rheumatic Diseases*.

PART 3 – *RESULTS*

CHAPTER 3.1 – TLR7 AND TLR8 COPY NUMBER VARIATIONS

In the context of female predominance in autoimmune diseases, we investigated the possibility of a gene copy increase of two X-linked genes important to the innate immune system, Toll-like receptor 7 (*TLR7*) and *TLR8* in men with rheumatoid arthritis. Such copy number increase has been already reported to potentiate autoimmunity in males from an autoimmune-background mouse strain.

For this purpose, we have developed a robust and sensitive real-time quantitative polymerase chain reaction (Q-PCR) assay to assess copy number variations of *TLR7* and *TLR8* genes in peripheral blood cells of patients with RA, compared to healthy controls. Contrary to expectations, instead of demonstrating a higher copy number in RA compared to controls, we show, for the first time, an influence of age and sex on copy numbers of those two X-linked genes. We discuss the possible consequences on autoimmunity and open this field to other topics such as X chromosome monosomy, and microchimerism…

3.1.1 ARTICLE 1 – [SUBMITTED]

COPY NUMBER VARIATION OF TLR7 AND TLR8 GENES IS AGE AND SEX BIASED IN PERIPHERAL BLOOD CELLS

Copy number variation of *TLR7* and *TLR8* genes is age and sex biased in peripheral blood cells

Running head: *TLR7* and *TLR8* gene copies increase with age in men

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ABSTRACT

Objectives. The X chromosome contains many immunity genes which may contribute to female predominance in autoimmune diseases. In the Yaa mouse model, a duplication of the innate immunity X-linked toll like receptor 7 (*Tlr7*) gene potentiates autoimmunity in males. We investigated whether such duplication is possible in humans and could contribute to the pathogenesis of rheumatoid arthritis (RA) in men.

Methods. A real-time quantitative PCR assay was developed to assess copy number variation (CNV) of *TLR7* gene and its paralog *TLR8* in DNA from PBMCs of 60 patients with RA (including 49 men) and 64 healthy controls (including 42 men). Among them, 31 men with RA and 18 healthy men were further screened for *TLR7/8* CNV in 4 subpopulations: B cells, T cells, granulocytes and the depleted fraction of the former 3.

Results. *TLR7/8* copy numbers significantly increased with age in PBMCs from all men ($p < 0.0001$), whether they had RA or not. This effect was observed in every cell subset. Such increase was not observed in women, healthy or with RA, but rather an opposite trend.

Conclusion. In conclusion, we show an age- and sex-mediated variation in *TLR7* and *TLR8* gene copy numbers and discuss its potential role in autoimmunity.

Introduction

Females have two X chromosomes, males have one. Women live longer than men, and their immune system is stronger against infections (1, 2). However, the prevalence of most autoimmune diseases is higher in women. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are examples of autoimmune disorders following this rule, with a women:men ratio ranging from 3:1 to 11:1 (3). The X chromosome is important in this context as it harbors many genes regulating hormonal and immunological features (4, 5). Unbalanced translocation of a 16-gene cluster from the X to the Y chromosome has been shown to be responsible for accelerating pathogenesis of autoimmunity only in males of the BXSB murine lupus strain (6). This translocation called Y-linked autoimmune accelerator (Yaa) includes innate immunity genes *Tlr7* and *Tlr8*, coding respectively for Toll-like receptor (TLR) 7 and 8. Only the duplication of *Tlr7* is required to accelerate autoimmunity in lupus susceptible male mice (7). Toll-like receptors (TLRs) are evolutionarily conserved innate immune proteins and are critical in first-line defense against foreign agents. *TLR7* and *TLR8* are located in endosomal compartments and both recognize single-stranded RNA of viral origin (8). *TLR7* and *TLR8* genes are phylogenetically related as they are paralogs. They are located at close proximity of each other in a region of the X chromosome syntenic in humans and mice.

Copy number variations (CNVs) are an important element of genomic polymorphism and population diversity (9). Furthermore, CNVs are more common in genomic regions containing immunity genes (10) and, in many cases, are associated with autoimmune diseases (11). The Yaa translocation reveals the importance of supplementary genetic material from the X chromosome in triggering autoimmunity. Copy number variation similar to that of the Yaa mouse model is possible in humans and could predispose men to autoimmunity. Indeed, importance of X-linked gene dosage has been described many times in autoimmunity. For example, men with the Klinefelter's syndrome (47, XXY) have 14 times higher risk of SLE than men with normal karyotype (12). Evidence for a strong association of Klinefelter's syndrome with other rheumatic autoimmune diseases including RA, juvenile idiopathic arthritis and systemic sclerosis has been implied by numerous case reports (for review (13)).

In this context, men with SLE would more often have 2 copies of *TLR7* when compared to healthy men. This hypothesis was first tested by Kelley et al. (14). They did not find a significant concordance between an increased *TLR7* copy number and SLE neither in men nor in women (14). However, a more recent and larger study found a correlation with susceptibility to childhood onset of SLE in a Mexican population (15). Such contradictory

results could be due to differences in methods, age of individuals, ethnicity, or susceptibility between childhood and adult onset.

In the current study, we analyze variations in copy numbers of both *TLR7* and *TLR8* genes, through newly optimized real-time quantitative PCR assays, in Caucasian patients with RA compared to healthy individuals. Analyses are done according to gender and age.

Methods

Study subjects

TLR7 and TLR8 CNVs were studied in DNA from 60 patients with RA (11 women and 49 men; median age 58 and 59) and 64 healthy controls (22 women and 42 men; median age 52.5 and 41), all Caucasians. All patients with RA satisfied the 2010 revised criteria of the American College of Rheumatology and the European League Against Rheumatism (16). All patients with RA were anti-citrullinated protein antibody-positive (ACPA+). Median age at the onset of disease was 42 for women and 48 for men. The selection criterion for healthy controls was no history of autoimmune disease in the family.

All participants signed informed consent according to the Declaration of Helsinki (17). The study is registered at the INSERM under the Biomedical Research Protocol number RBM-04-10 or as a collection under the number DC-2008-327.

Cell sorting from whole blood

Whole blood was drawn in EDTA or heparin vacuum tubes. Four mL of blood were processed by Ficoll Histopaque 1077 (Sigma-Aldrich, St Louis, MO, USA) gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC) and 6 mL processed with immuno-magnetic cell sorting (RoboSep™, STEMCELL™ Technologies; Vancouver, Canada) to separate cells into 4 fractions of interest: CD19+ (B cells), CD3+ (T cells), CD66b+ (granulocytes, particularly neutrophils) and CD19−/CD3−/CD66b− (all other subsets, including monocytes, macrophages, NK cells and dendritic cells). These fractions were then checked for purity by flow cytometry with the MACSQuant® device from Miltenyi Biotec (Bergisch Gladbach, Germany), using CD20-VioBleu®; CD4(VIT4)-FITC; CD8-PE and CD66abce-APC fluorescent antibodies, according to the protocol recommended by the manufacturer. Cell fractions with purity higher than 95% were kept for further analysis.

DNA isolation

DNA from PBMC and/or sorted fractions was extracted with EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) using a BioRobot EZ1 system (Qiagen, Hilden, Germany) according to the

manufacturer's instructions, and stored at –20°C for future investigation of TLR7 and TLR8 CNVs.

TLR7 and TLR8 Copy number estimation

We developed a real-time quantitative polymerase chain reaction (PCR) assay to accurately calculate the *TLR7* or *TLR8* (*TLR7/8*) gene CNVs. Determination of *TLR7/8* gene CNVs was performed by TaqMan® real-time quantitative PCR using LC FastStart DNA Master^{PLUS} HybProbe reaction kits (Roche Diagnostics GmbH; Mannheim, Germany) on a LightCycler® (LC) thermocycler machine and/or using LC480 Probes Master reaction kits (Roche Diagnostics GmbH; Mannheim, Germany) on a more recent model; the LightCycler®480 (LC480) instrument. All samples were tested in triplicates in a final volume of 20 µL (on the LC) or 10 µL (on the LC480) using 25 ng (± 6.6 ng) of genomic DNA. Each DNA sample was simultaneously amplified with a set of primers/probe designed for *TLR7* and *TLR8* and *HBD* (part of the β-globin locus, the reference gene in this study). Primers and probes used were as following: TLR7-forward: 5'-CAGTATTGTGCTGTCTTTGAAATGTA-3' ; TLR7-reverse: 5'-TGGTTGAAGAGAGCAGAGCA-3' ; TLR7-probe: 5'-(6-FAM)-TTGGGCCCATCTCAAGCTGATCTTG-(TAMRA)-3' ; TLR8-forward: 5'-GTGAGGCCACACAAGATGGA-3' ; TLR8-reverse: 5'-TTCCAGACCACTCCCTTTGC-3' ; TLR8-probe: 5'-(6-FAM)-CGCCCAAGTGTCCACCTAAACATGAGT-(TAMRA)-3' ; HBD-forward: 5'-AGATTCCTACTTTCAGCGTTGG-3' ; HBD-reverse : 5'-CAGCAGGGTTCAGGAAGATAAA-3' ; HBD-probe: 5'-(6-FAM)-CAACCTGGATCCACTTGCCCAGTG-(TAMRA)-3'. Thermal cycling conditions were 95°C for 10 min followed by 38 cycles at 95°C for 15 s and 60°C for 60 s. Data were analyzed using LightCycler® software version 3.5.3 or LightCycler®480 software version 1.5. CNV calculation was performed using two methods.

***The delta delta cycle at threshold (ΔΔCt) method*:** This method consists in amplifying *TLR7* and *8* genes (on the X chromosome), against the *HBD* gene (1 copy on each homologous chromosome 11). Differences of Ct between *TLR7/8* and *HBD* were then calculated. A male is expected to show amplification of *TLR7/8* one Ct later than *HBD* (1 copy versus 2 copies, respectively), whereas a female should show amplification of *TLR7/8* and *HBD* at the same Ct (2 copies for each gene). A healthy male control was systematically used as inter-assay control and calibrator for the normalized value of the relative copy number of *TLR7/8* versus *HBD*. In addition, the efficiency (E) of each Q-PCR assay was taken into account in this calculation method as follows: $(1+E)^{-\Delta\Delta Ct}$.

***The relative standard curve method*:** This method calculates the *TLR7/8* gene copy number versus *HBD* without assessment of Q-PCR efficiency (18). Each DNA sample was

amplified in parallel with *TLR7/8* specific Q-PCR versus *HBD* specific Q-PCR. The absolute quantification of copies for each gene was calculated according to their respective standard curves. A male healthy control was systematically used as inter-assay control (calibrator). To generate standard curves, a serial dilution was obtained from genomic DNA of the calibrator sample (66 – 4.125 ng; dilution factor: ×2). *TLR7/8* copy number was calculated using the following formula:

$$\text{TLR7/8 Copies} = \frac{\text{absolute TLR7/8 quantity}}{(\text{absolute HBD quantity}) \div 2}$$

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software (La Jolla, CA, USA). Normality of distribution of continuous variables was examined with the Shapiro-Wilk test. The non-parametric Mann-Whitney U test was used to compare the distribution of relative *TLR7* and *8* gene copy numbers as sample sets were not normally distributed or were less than 30. Continuous variables were represented as medians (with interquartile range). To examine correlations, Pearson's correlation test was used when data sets were normally distributed; otherwise Spearman's rank correlation test was used. For all tests, P values less than 0.05 were considered significant.

Results

Validation of the TLR7 and TLR8 Q-PCR assay

A Q-PCR method was designed and optimized to explore copy numbers of *TLR7* and *TLR8* on the human X chromosome normalized against the *HBD* gene on chromosome 11. Based on the ΔΔCt calculation method, DNA from peripheral blood mononuclear cells (PBMC) of 26 healthy men and 22 healthy women was assayed on the LC instrument. As expected, men had approximately 1 copy of *TLR7* or *TLR8* and women had 2 copies (**Figure 1A**), as differences in cycles at threshold (between target and reference genes) were respectively close to 1 and 0 (Example in **Figure 1B**).

Experiments on healthy men were compared with the two calculation methods (ΔΔCt and relative standard curve methods). Although data obtained with the relative standard curve method show narrower dispersion of copy numbers [0.65 – 1.42] than data obtained with ΔΔCt method [0.48 – 1.97], they showed concordant results for both *TLR7* and *TLR8* CNVs (respectively Spearman r = 0.86, p < 0.0001 and Spearman r = 0.57, p = 0.0004). These results provide confidence in the precision of Q-PCR measurements (**Figure 2**).

Age-mediated TLR7 and TLR8 CNV in PBMCs from men with RA and healthy men

Because median age was significantly different between healthy men and men with RA (41 versus 59, $p < 0.0001$; Mann Whitney U test), this parameter was considered in our analyses. We found that *TLR7* and *TLR8* copy numbers (relative to *HBD* copy numbers) show a steady increase with age in all men ($p < 0.0001$, **Figure 3**). The correlation was not specific of the disease. Indeed, a marginal positive correlation with age was observed for relative copy numbers of *TLR7* and *TLR8* in healthy controls ($r = 0.30$; $p = 0.06$ and $r = 0.25$; $p = 0.1$, respectively) as well as in men with RA (respectively, $r = 0.26$; $p = 0.07$ and $r = 0.27$; $p = 0.06$, Spearman's rank correlation test; data not shown). Furthermore individuals who had an increased *TLR7* copy number had a similar pattern for *TLR8* with a statistically significant correlation (Pearson's $r = 0.84$; $p < 0.0001$; **Figure 4**).

Such increase was not observed in women, be they healthy (N = 22, median age; 52.5) or with RA (N = 11, median age 58), but rather an opposite trend (**Figure 5**).

As patients with RA are older (onset around 50 years old) than healthy donors in blood banks, it was only possible to match 25 patients and controls one by one for age (16 men and 9 women). *TLR7/8* copy numbers were not significantly increased in RA patients when compared with controls (1.12 versus 1.00 and 1.10 versus 1.00 respectively for *TLR7* and *TLR8* in men; 2.26 versus 2.21 and 1.89 versus 1.67 respectively for *TLR7* and *TLR8* in women, data not shown).

Similar results in cell subsets.

As the increase observed in age-matched patients with RA when compared to controls was very slight and not significant when PBMCs were analysed, we checked whether one particular cell subpopulation could be affected and the effect hidden when observed as a global population of cells. A total of 31 men with RA and 18 healthy men were further screened for *TLR7* and *TLR8* CNV in 4 subpopulations: B cells, T cells, granulocytes and the depleted fraction of the former 3, containing monocytes, macrophages, NK cells, dendritic cells… However, none of the 4 subpopulations showed significant increase in copies in patients with RA when compared with healthy controls, rejecting the hypothesis of an increase restricted to a small population (data not shown).

Finally, all 4 cell subsets showed a tendency to higher *TLR7* and *TLR8* copy number with age (statistically significant for *TLR8* in all subsets except in B cells), reflecting what was observed in PBMC samples (**Figure 6**).

Discussion

We have presented evidence that copy number of X-linked *TLR7* and *TLR8* genes increases with age in peripheral blood cells of men, whether they have RA or not. Interestingly, such effect was not observed in women, suggesting a difference according to gender. *TLR7/8* copy number increases at very small amplitude to reach a mean of 1.22 *TLR7/8* copy numbers at the age of 80, according to the linear regression curves' best fit. Therefore, unlike in the Yaa mouse model, we do not observe a 2-fold increase.

Several mechanisms could explain the significant, but small variation observed with age in men. One of them is that somatic duplication affects some cells. This will progressively result in somatic mosaicism, defined as the presence of genetically distinct cell populations in a single organism. In other words, the increase in *TLR7/8* genes could be due to duplication of DNA segments encompassing at least the two genes tested and happening during mitosis. Two recent studies support the idea of somatic mosaicism for CNVs and suggest it is relatively common in normal human cells. The first shows that different CNVs occur among monozygotic twins (19), and the second reports the presence of different number of copies in different tissues of the same individual (20). In such context, the X chromosome stands as an interesting candidate for structural variations as it contains a relatively large amount of repetitive elements. For example, compared to autosomes, it harbours approximately 2 times more long interspersed element (LINE)-1 retrotransposable repetitive sequences (21), which are believed to confer alterations and instability on DNA with age (22).

As the possible duplication could occur in a restricted fraction of blood cells, we have investigated whether the slight increase of *TLR7* and *TLR8* copy numbers could come from particular cell subpopulations more prone to variations than others. We screened 4 major blood cell subsets: B cells, T cells, granulocytes and a cell fraction depleted from these three cell types containing in majority monocytes, macrophages, dendritic cells and NK cells. Candidates for CNVs are T and B cells for which translocation and mutagenesis are part of their normal maturation stages, which imply V(D)J recombination, class switch recombination and somatic hypermutation. Granulocytes are also interesting as they survive only few days (23) and their TLR7/8 copy number status closely reflects that of bone marrow stem cells. Our data showed that all cell subsets tested had similar tendency towards *TLR7* and *TLR8* copy number increase with age, suggesting the somatic event was more likely upstream in the hematopoietic stem cell compartment.

Then, why is increased copy number with age not observed in women? In an early report in 1963, it was shown that in normal human leukocytes the proportion of aneuploid cells increases with age in both sexes, but that the chromosome involved in aneuploidy was different according to sex (24). Males were more often missing the Y chromosome and

females a “medium sized” chromosome (likely one of the X). Since that time, many studies have found that aneuploidy in cells increases with age and with higher rate among women, for whom it involves the loss of an X chromosome (25-27). This loss could explain why women have an opposite tendency compared to men regarding CNV in X linked genes.

Another possibility is that the few cells responsible for the observed *TLR7/8* gene dosage increase in men come from the presence of female cells (with 2 X chromosomes). Such an acquisition happens during in utero life, where maternal cells pass the placenta barrier and persist in the growing child and adult (28). Interestingly, this phenomenon called maternal microchimerism has been observed in different cell subsets such as T lymphocytes, B cells, monocytes/macrophages and NK cells (29). However, we do not know whether maternal microchimerism levels could increase with age and/or hormonal changes. If this is the case, the phenomenon should only be visible in men (with the addition of 2 X chromosomes), which would explain the gender bias observed. Furthermore, we and others have shown that microchimerism could persist in immuno-competent individuals but be more important in patients with RA (30-32), which would explain the slight increase observed in RA when compared to healthy controls. Nevertheless, if microchimerism contributes to the increased copy number of *TLR7/8* genes observed in elder men, it would only be partial. Indeed, the highest reported levels of microchimerism are in patients with RA and represented 5% of host's cells (30), whereas the average increase in CNVs observed in our study is about 20%.

A potential role for TLR7 and TLR8 has been previously described in RA, where higher production of TNF-α, IL-6 or type I IFN through endosomal TLRs (including TLR7 and 8) was reported in synovial inflammation (33-35). We therefore thought that *TLR7/8* copy number would be increased in RA compared to healthy controls. However, our data do not show a difference between patients with RA and healthy controls. This absence of correlation with disease could be due to the reduced number of subjects analysed after age adjustment and this would merit to be addressed in a study including a larger number of subjects. Another possibility is that the slight increase in CNVs is not directly correlated with disease but would rather contribute to trigger RA in men already genetically and environmentally predisposed to disease. As *TLR7* and *TLR8* copy number increases with age in men, this would explain the later onset of RA in men compared to women (36).

In our study, we have designed a reliable Q-PCR method that allows quantification of CNVs of 2 X-linked genes. However, our findings could be part of a wider somatic CNV phenomenon affecting other DNA segments on the X chromosome. One expected future direction of this research is global analysis of CNVs on this particular chromosome, in relation to age, sex and disease.

Acknowledgements

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Figure legends

Figure 1. Validation of the *TLR7* and *TLR8* Q-PCR assay on a group of healthy individuals. (A) *TLR7* and *TLR8* relative gene copy number was normalized against *HBD* using ΔΔCt method of calculation, as described in the Methods section. Based on this method, healthy men (HLTH) had, as expected, approximately 1 copy of *TLR7* or *TLR8* and women had 2 copies. Bars represent medians with interquartile ranges and symbols represent individual subjects. **(B)** *TLR7* gene (like *TLR8*) is on the X chromosome, while the *HBD* gene is on chromosome 11. Therefore, Q-PCR amplification profiles differ when DNA sample comes from a man or a woman. At the exponential phase of amplification, in the male sample (top), amplification of *TLR7* (in red) appears at 1 cycle at threshold (Ct) after that of *HBD* (in blue). In the female sample (bottom), the two amplifications are superposed. The same is observed for *TLR8*.

Figure 2. Correlation of data obtained based with ΔΔCt and Relative Standard Curve methods (on LC480 instrument) to estimate *TLR7* (A) and *TLR8* (B) relative copy numbers. Symbols represent individual healthy men; N = 35; p < 0.0001 for *TLR7* and p = 0.0004 for *TLR8* with Spearman's rank correlation test.

Figure 3. *TLR7* and *TLR8* copy numbers increase with age in men. Variation of the relative *TLR7* and *TLR8* copy number in genomic DNA from peripheral blood mononuclear cells (PBMC) of men with rheumatoid arthritis (RA) and healthy (HLTH) men is positively correlated with age (p < 0.0001; Spearman's rank correlation test). *TLR7* and *TLR8* copy number was determined by real-time Q-PCR normalized against the *HBD* gene, using the Relative Standard Curve Method of calculation as described in the Methods section. Square and triangle symbols respectively correspond to *TLR7* and *TLR8* assays. Filled symbols (black) represent individual men with RA (N = 49) and unfilled symbols (white) represent control men (N = 42).

Figure 4. Correlation of *TLR7* and *TLR8* copy numbers normalized against *HBD* gene (with Relative Standard Curve method) obtained in DNA from PBMC samples of all men. Symbols represent individual men; N = 76; p < 0.0001 with Pearson's correlation test.

Figure 5. *TLR7* and *TLR8* copy numbers does not increase with age in women. *TLR7* and *TLR8* copy number in genomic DNA from peripheral blood mononuclear cells (PBMC) of women with rheumatoid arthritis (RA) and healthy (HLTH) women was determined by real-time Q-PCR normalized against the *HBD* gene, using the ΔΔCt method of calculation as described in the Methods section. Decrease of copy numbers with age is marginally significant for *TLR7* but not *TLR8* (Spearman's rank correlation test). Square and triangle symbols respectively correspond to *TLR7* and *TLR8* assays. Filled symbols (black) represent individual women with RA (N = 11) and unfilled symbols (white) represent control women (N = 22).

Figure 6. TLR7 and TLR8 copy number variations (CNVs) show same trend with age in blood subpopulations of men. *TLR7* and *TLR8* copy number is estimated in genomic DNA of B cells, T cells, Granulocytes (particularly neutrophils) and a fraction of cells excluding the former 3 types (mostly monocytes, macrophages, NK and dendritic cells) between men with rheumatoid arthritis (RA) and healthy men. *TLR7* and *TLR8* copy number was determined by real-time Q-PCR normalized against the *HBD* gene, using the relative standard curve method of calculation as described in the Methods section. In all cell subsets, a tendency towards an increase of copy number with age is observed (significant for *TLR8* in most cases; Spearman's rank correlation test). Square and triangle symbols respectively

correspond to *TLR7* and *TLR8* assays. Filled symbols (black) represent individual men with RA (N = 31) and unfilled symbols (white) represent control men (N = 18).

Figures

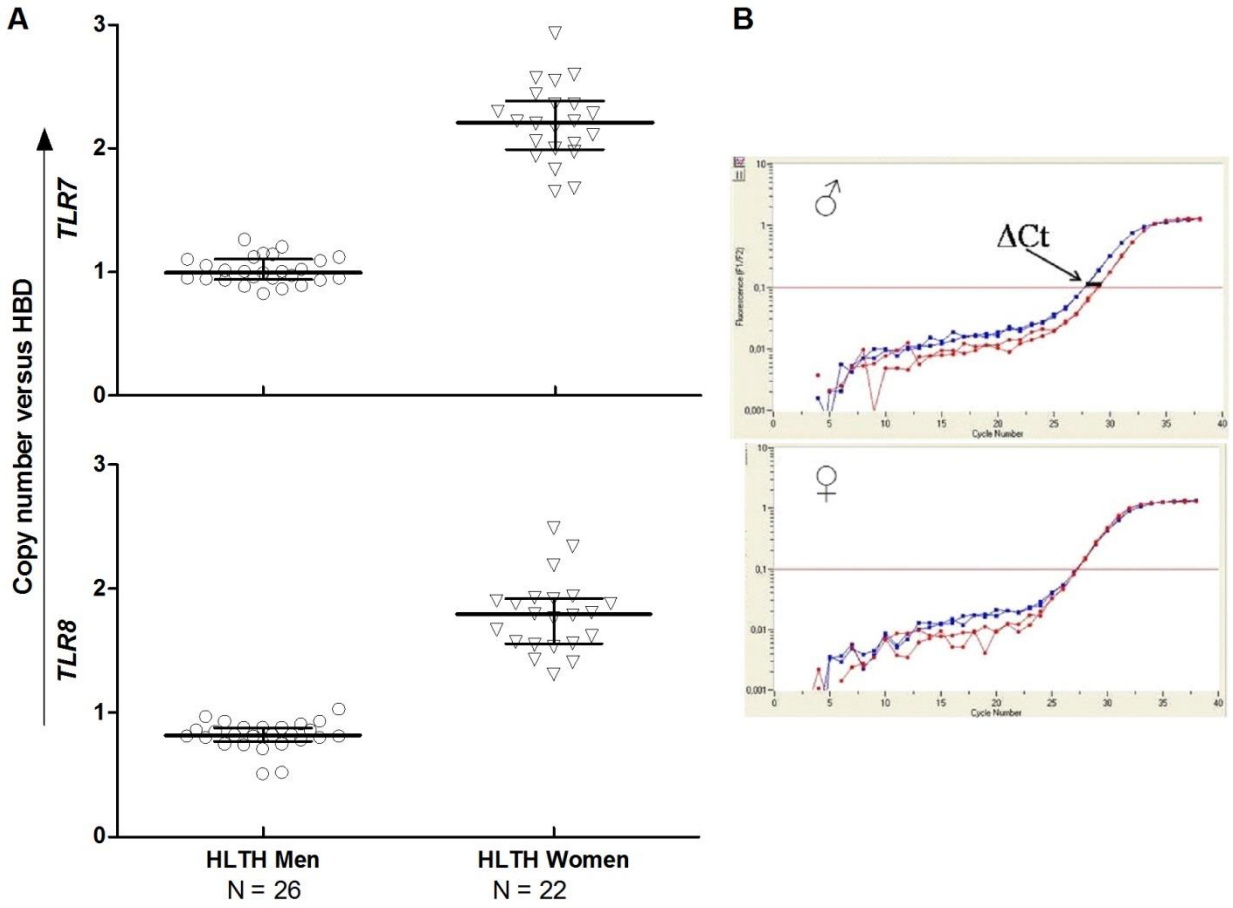

Figure 1

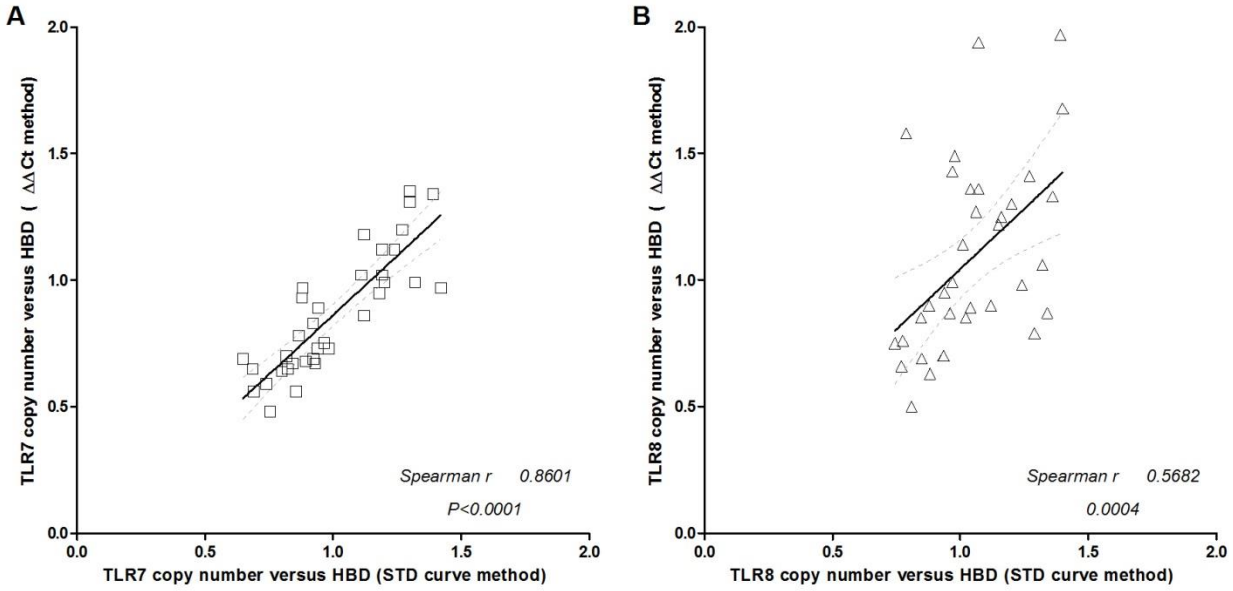
A
TLR7 copy number versus HBD (ΔΔCt method)
TLR7 copy number versus HBD (STD curve method)
Spearman r 0.8601
P<0.0001
B
TLR8 copy number versus HBD (ΔΔCt method)
TLR8 copy number versus HBD (STD curve method)
Spearman r 0.5682
0.0004

Figure 2

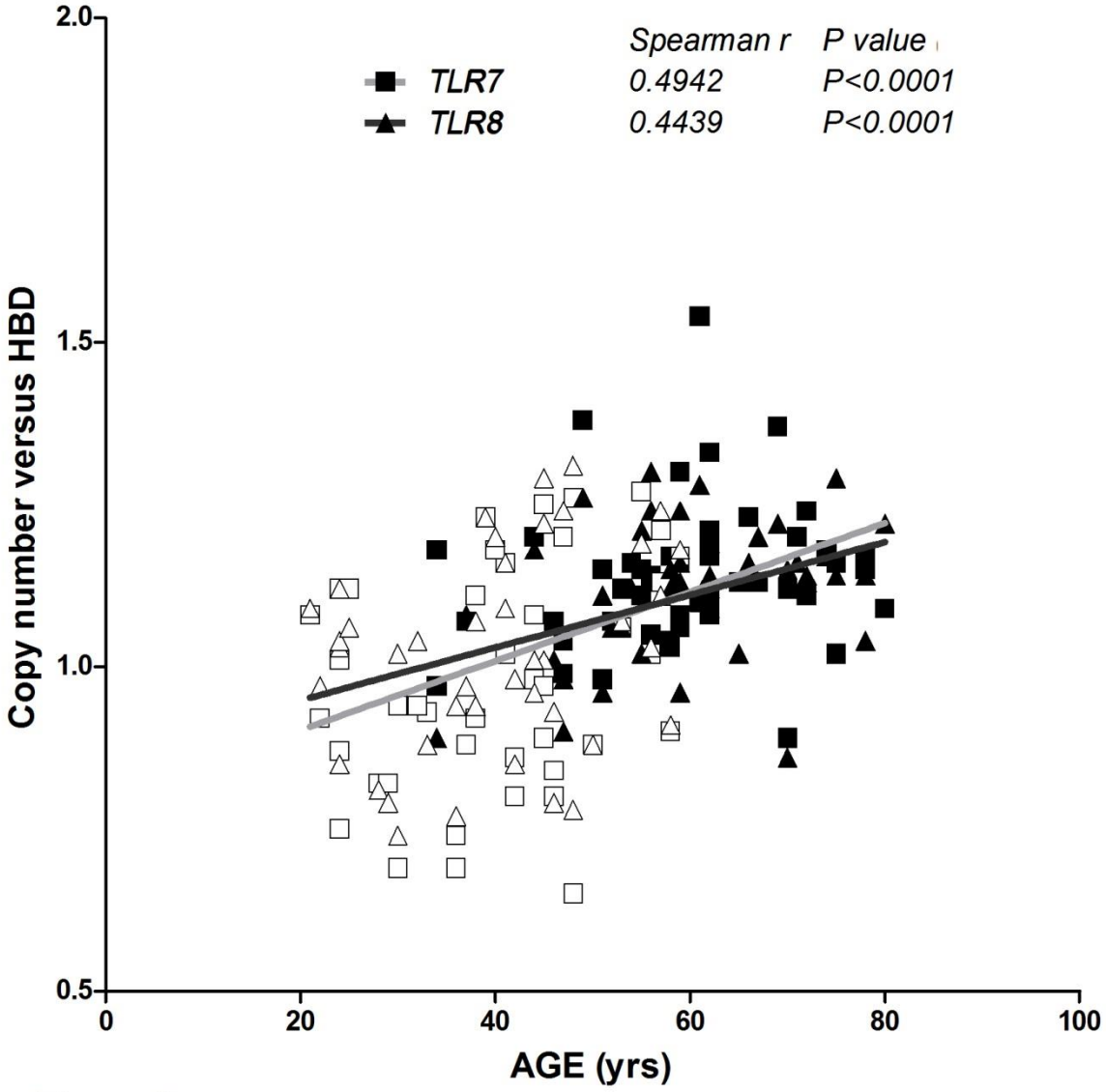
Spearman r
P value
TLR7
0.4942
P<0.0001
TLR8
0.4439
P<0.0001
Copy number versus HBD
2.0
1.5
1.0
0.5
0
20
40
60
80
100
AGE (yrs)

Figure 3

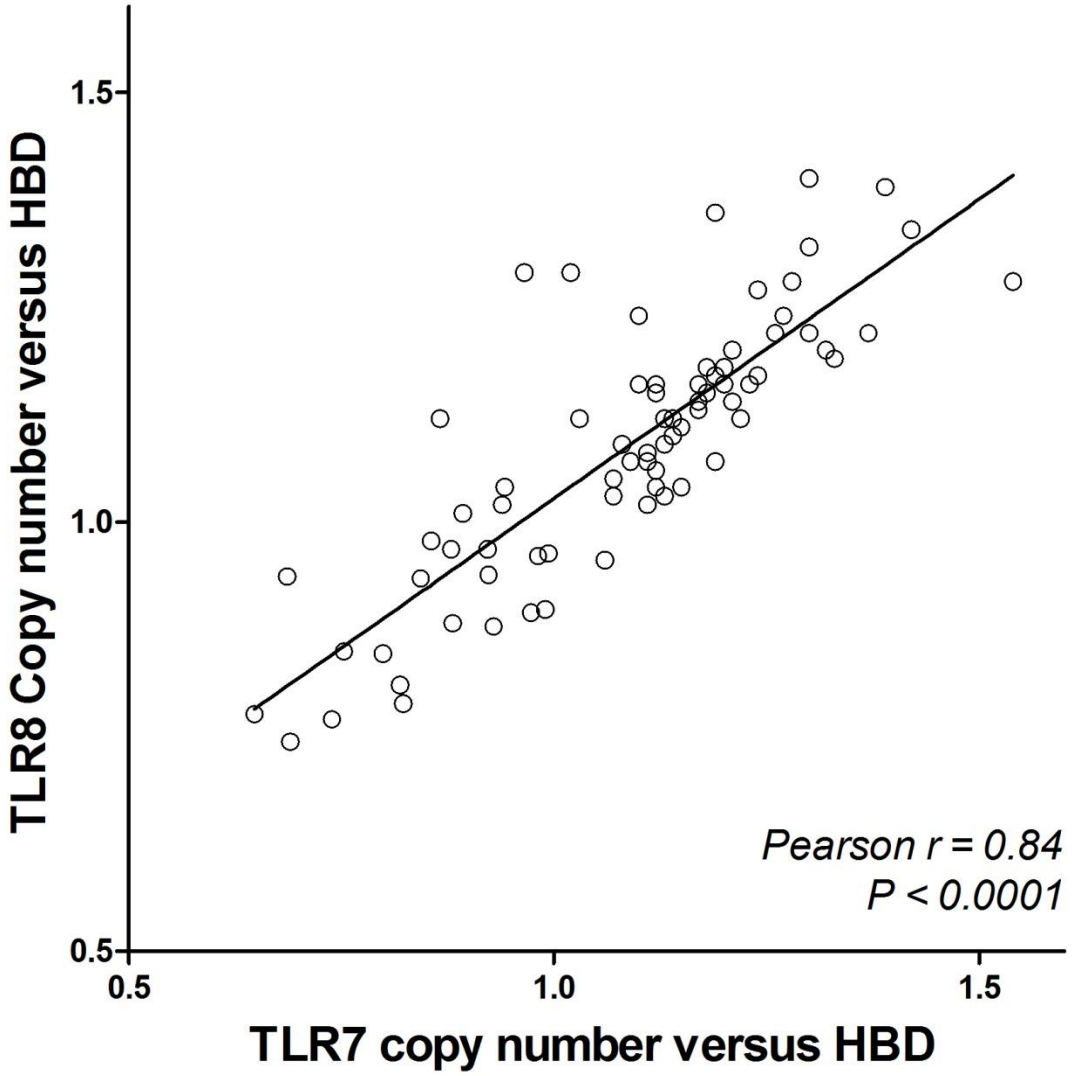
TLR8 Copy number versus HBD
1.5
1.0
0.5
0.5
1.0
1.5
TLR7 copy number versus HBD
Pearson r = 0.84
P < 0.0001

Figure 4

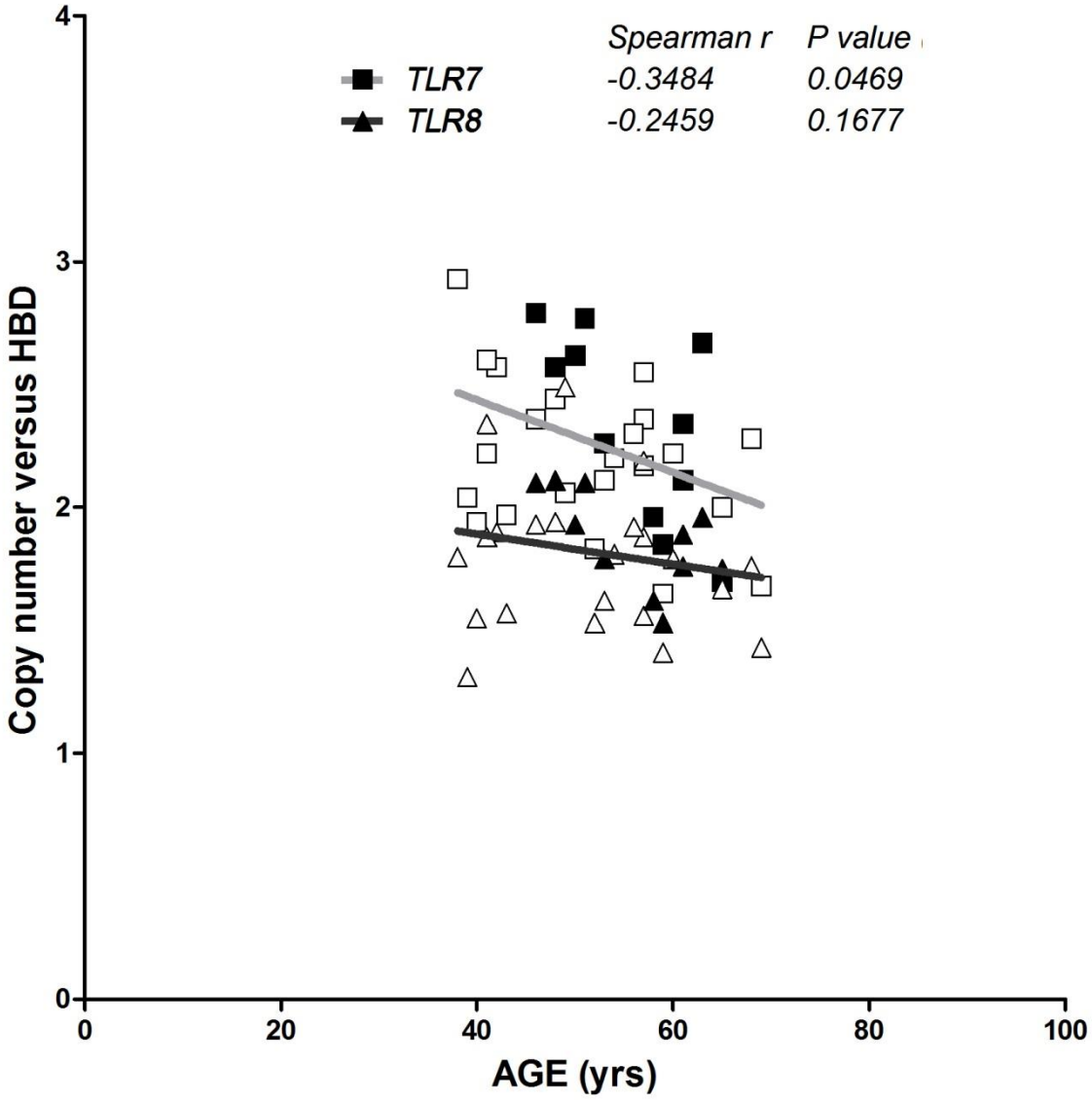
Spearman r
P value
TLR7
-0.3484
0.0469
TLR8
-0.2459
0.1677
Copy number versus HBD
AGE (yrs)
0
1
2
3
4
0
20
40
60
80
100

Figure 5

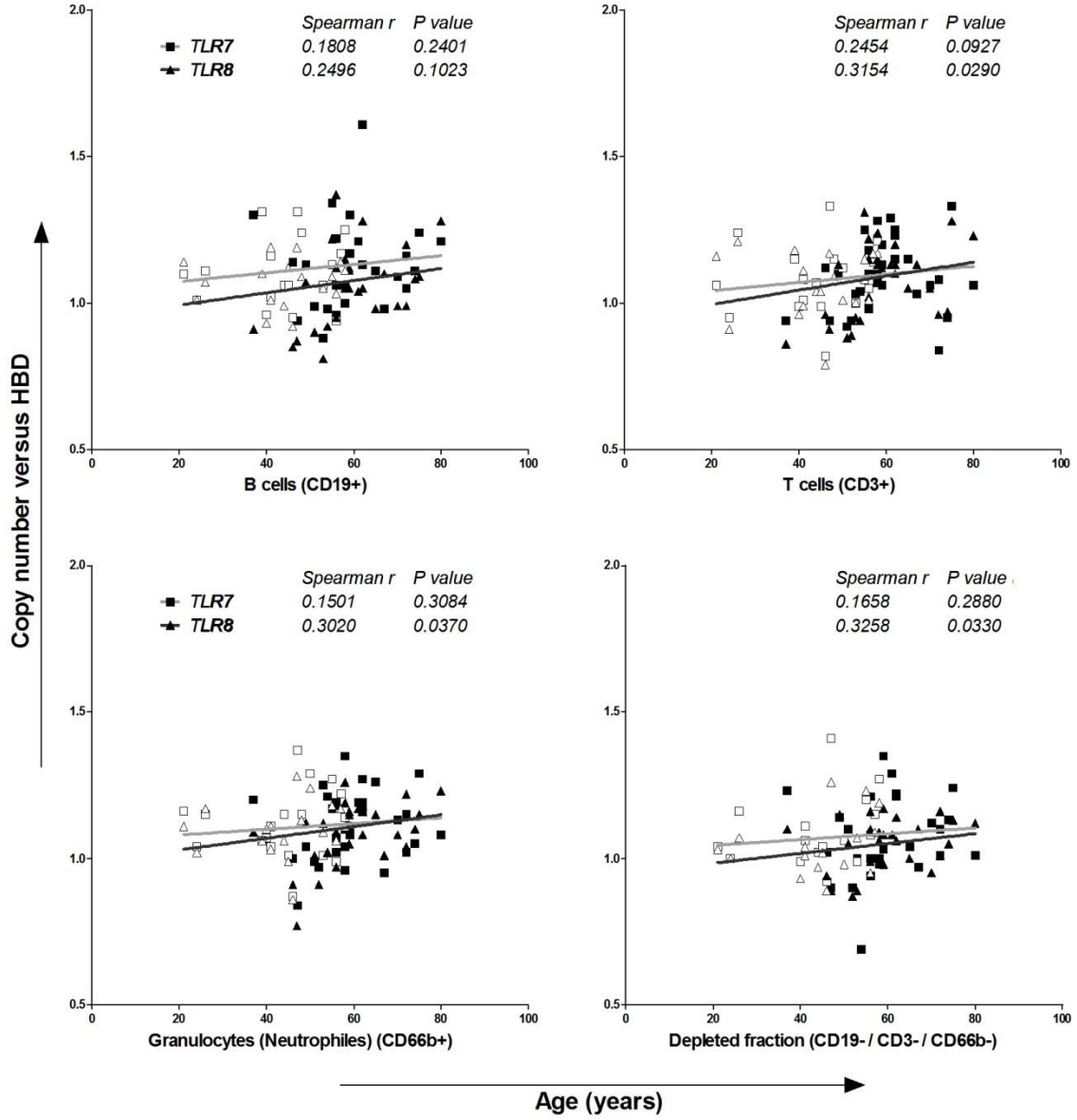
Spearman r P value
TLR7 0.1808 0.2401
TLR8 0.2496 0.1023
B cells (CD19+)
Spearman r P value
0.2454 0.0927
0.3154 0.0290
T cells (CD3+)
Spearman r P value
TLR7 0.1501 0.3084
TLR8 0.3020 0.0370
Granulocytes (Neutrophiles) (CD66b+)
Spearman r P value
0.1658 0.2880
0.3258 0.0330
Depleted fraction (CD19- / CD3- / CD66b-)
Copy number versus HBD
Age (years)

Figure 6

CHAPTER 3.2 – FEMALE MICROCHIMERISM'S PERSISTANCE IN A MALE HOST

The age- and sex-mediated variations in X-linked *TLR7* and *TLR8* gene copy numbers can be explained by somatically acquired duplications affecting some cells over time and resulting in an increase with age in men. Another explanation could be through female microchimeric cells carrying 2 X chromosomes among XY host cells and contributing to the apparent copy number increase of X-linked genes.

The following article describes the case of a man with a scleroderma-like syndrome, in whom female microchimeric cells are detected by cytogenetic and PCR-based methodologies. These cells are from an unrecognized "vanished" twin sister.

Natural sources of microchimerism for men are from the mother or from a twin during in utero life. This article suggests that even a vanished twin can represent potential source of microchimerism. Still, further studies are needed to determine the frequency of this source and the extent to which it might contribute to the increase of X chromosome copies in men and predispose them to autoimmunity.

3.2.1 ARTICLE 2 – [PUBLISHED]

CELLS FROM A VANISHED TWIN AS A SOURCE OF MICROCHIMERISM 40 YEARS LATER

Chimerism 1:2, 56-60; October/November/December 2010; © 2010 Landes Bioscience

Cells from a vanished twin as a source of microchimerism 40 years later

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Key words: chimerism, twin, scleroderma, gender, HLA-specific PCR

We report the case of a 40-year-old man diagnosed with a scleroderma-like disease. Clinical similarities with graft versus host disease prompted initial testing for chimerism employing fluorescence in situ hybridization (FISH). Female cells were observed within peripheral blood mononuclear cells from the patient.

Because maternal cells have been detected in healthy immunologically competent adults and patients with autoimmune conditions, we hypothesized that these cells were of maternal origin. Contrary to our expectations, HLA-specific quantitative PCR (QPCR) ruled out maternal microchimerism. However, HLA-specific QPCR testing was positive for the paternal HLA haplotype that the patient did not inherit. We reasoned that the most likely origin of chimerism with non-inherited paternal HLA alleles was from an unrecognized "vanished" twin. The patient had never received a blood transfusion.

This report suggests that cells from a vanished twin are a possible source of chimerism. The frequency of chimerism from this source is not yet known and whether the scleroderma-like disease observed in the patient is anecdotal or implies a potential association with autoimmune disease remains to be elucidated.

Introduction

Systemic Sclerosis (SSc) or scleroderma, from the Greek *skleros* (hard or indurated) and *derma* (skin), is an autoimmune disease characterized by skin induration and thickening accompanied by various degrees of tissue fibrosis and chronic inflammation involving numerous organs, functional and structural abnormalities of the vascular system and cellular immune alterations.

Female sex is one of the strongest risk factors for development of autoimmune disease. SSc does not escape this rule with a female:male ratio between 4:1 and 15:1.[1]

We and others have previously shown that microchimerism (Mc) arising from pregnancy may contribute to the pathogenesis of SSc in women.[2-4] Indeed naturally acquired fetal Mc enters the maternal circulation and can be found decades later, with higher frequencies and quantities observed in parous women with SSc compared to healthy matched controls.

Scleroderma also affects men, although to a lesser extent. Alternative sources of Mc that could affect males include cells from a blood transfusion, a twin or their mother.[5]

Maternal Mc has been demonstrated in cord blood samples wherein female cells were detected in male cord blood by fluorescence in situ hybridization and PCR methods.[6,7] Long-term persistence of maternal cells was first described in peripheral blood from infants with severe combined immunodeficiency,[8] and subsequently detected in immuno-competent individuals[5,9] and implicated in women with SSc. Microchimeric cells can also be transferred in utero from a twin and potentially from an unrecognized (vanished) twin. A vanished twin is relatively common in healthy pregnancies.[10] We present here the case of a 40-year-old male with a scleroderma-like disease who was studied for maternal Mc and for Mc from a vanished twin.

Patient History and Results

Patient history. The patient was a 40-year-old Belgian male with a history of professional exposure to hydrocarbons at the age of 20, who developed skin and lung inflammation within months after the toxic exposure. Rapidly progressive cutaneous inflammation associated with lung fibrosis led to the presumed diagnosis of environmental scleroderma, although the patient did not have Raynaud's phenomenon or antinuclear antibodies. Over the following years this condition evolved into a chronic inflammatory disease, reminiscent of graft-versus-host disease with lymphocytic infiltrates and involvement at other locations including small bowel, uvea and liver. His disease has been partially controlled by chronic immunosuppressive therapy with low-dose corticosteroids and azathioprine, and at the time of enrollment in the current

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DOI: 10.4161/chim.1.2.14294

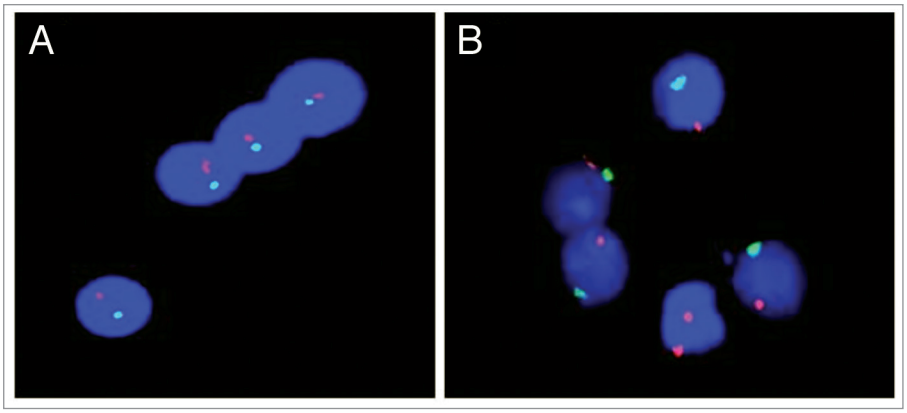

Figure 1. Fluorescence in situ hybridization of Y (green) and X (red) chromosomes in PBMCs from males. (A) Absence of female cells in a healthy male. (B) Presence of female cells in a male patient with "scleroderma-like" disease.

studies his physical findings included digital vitiligo, disseminated patchy erythematous lesions and nail involvement (20 of 20 nails).

The patient did not fulfill the American College of Rheumatology classification criteria for SSc,[11] i.e., major criterion (symmetrical thickening, tightening and induration of the skin of the fingers and the skin proximal to the metacarpophalangeal or metatarsophalangeal joints), or presence of two minor criteria (sclerodactyly, digital pitting scars/loss of substance of the finger pad and basilar pulmonary fibrosis). Therefore his disease is referred to as "scleroderma-like".

We investigated this man, who never received a blood transfusion, initially for Mc from his mother and subsequently for Mc that could have originated from a vanished twin and persisted 40 years after his birth.

Results

Studies for maternal Mc. We first investigated peripheral blood mononuclear cells (PBMC) from this male patient for female cells employing fluorescence in situ hybridization with X- and Y-chromosome specific probes (**Fig. 1**). Among 40,600 PBMC examined seven cells with two distinct red signals were found, corresponding to two X chromosomes within a clearly defined nucleus.

We next conducted HLA-specific quantitative PCR (QPCR) testing for a maternal HLA allele that was not transmitted to the patient. This is referred to as targeting the non-inherited maternal HLA allele (NIMA) (**Fig. 2**). For this purpose, we developed a new QPCR assay that was specific for HLA-A*01 (NIMA). However, we found no evidence of Mc from the mother as DNA from the patient's blood did not amplify, despite testing a total of 642,935 DNA cell equivalents in two different experiments. **Figure 3** illustrates one of the two experiments.

Studies for twin Mc. Female cells observed by FISH could not have originated from a blood transfusion as the patient had never been transfused. We then questioned whether the female cells could have originated from a female twin, although the patient was born as a singleton child.

Should this hypothesis be correct, the chance of detecting DNA from a vanished twin was 50% because if the twin had inherited the same paternal HLA haplotype as the patient, then Mc would not be distinguishable. Conversely, if the twin had inherited the other paternal haplotype, QPCR testing for non-inherited paternal HLA alleles (NIPA: DRB1*07-DQB1*02 haplotype) would allow detection of Mc by NIPA-specific QPCR.

We had previously developed QPCR assays for HLA-DRB1*07 and DQB1*02,[9,12] and employed each of these assays to test DNA extracted from the patient's PBMC. The patient's DNA amplified for both assays, confirming the NIPA haplotype and supporting the interpretation that the patient acquired cells from a vanished twin (**Fig. 4**). Both assays gave identical results with, respectively for DRB1*07 and DQB1*02 QPCR assays, 844 and 845 genome equivalent of cells (gEq) for a total of 160,000 gEq patient cells tested. These results suggest high levels of chimerism, reaching 5%. Testing by FISH identified lower levels of 0.02%.

Follow-up over time. We investigated twin Mc in a follow-up blood sample drawn 15 months later. HLA-DRB1*07 and HLA-DQB1*02 PCR were both negative. However, the two samples also had an important difference in that the first (positive for twin Mc) was obtained by cytapheresis [without Granlocyte Macrophage Colony Stimulating Factor (GM-CSF) mobilization], whereas the second (negative for Mc) was a standard blood sample drawn into heparinized vacutainer tubes. There was no difference in the patient's clinical status or treatment between the two dates.

Studies of the mother for fetal Mc from prior pregnancy with a vanished twin. A peripheral blood sample was obtained from the mother who was 60 years-old at the time of the blood draw. The same HLA-DRB1*07 and DQB1*02 QPCR assays were used to test DNA extracted from the mother's PBMC for evidence of fetal Mc from a prior pregnancy with a vanished twin. Results were negative for both assays (results not shown).

Discussion

We report here the case of a man presenting with marked lymphocytic inflammation involving the skin, lungs and digestive tract, who had a previous exposure to hydrocarbons. Although initially diagnosed as environmental scleroderma, diagnosis was revised in the absence of sufficient criteria for this disease to that of a scleroderma-like condition reminiscent of graft-vs-host-disease. These characteristics prompted initial studies employing FISH to assess for chimerism with female cells. The presence of cells with two X chromosomes among male PBMC suggested maternal Mc.

However, contrary to our expectations, female cells were not maternal in origin as the patient's DNA did not amplify by HLA-specific QPCR when tested for a non inherited maternal HLA allele (NIMA).

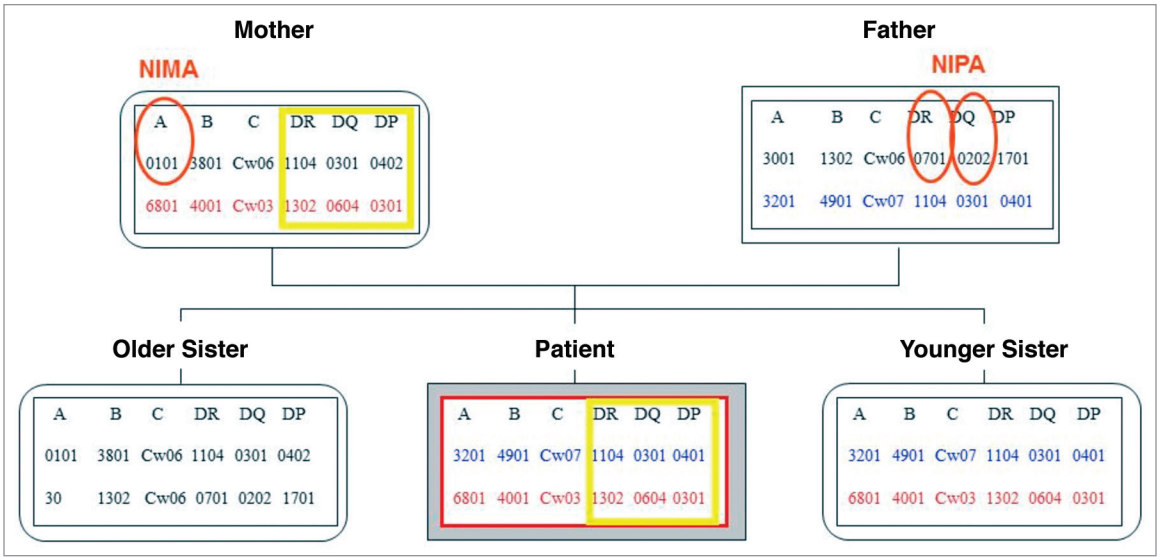

Figure 2. Familial HLA genotyping results. NIMA: non-inherited maternal HLA alleles of the patient; NIPA: non-inherited paternal HLA alleles of the patient. To the left of the patient's HLA-typing results are results for the patient's older sister and to the right a younger sister.

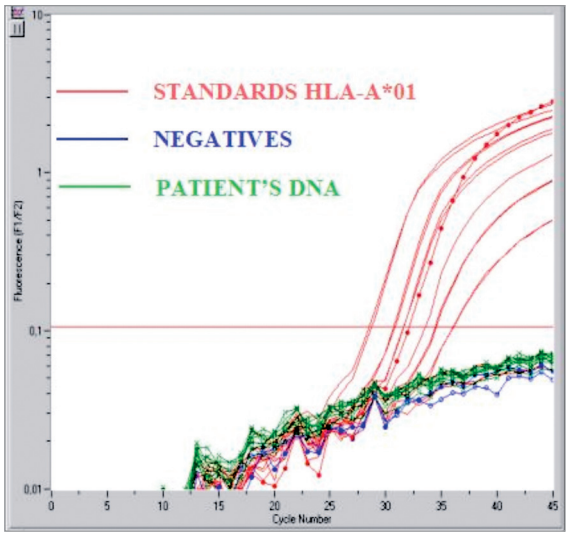

Figure 3. QPCR targeting HLA-A*01, representing an HLA allele that was not inherited by the patient, demonstrating the absence of maternal Mc.

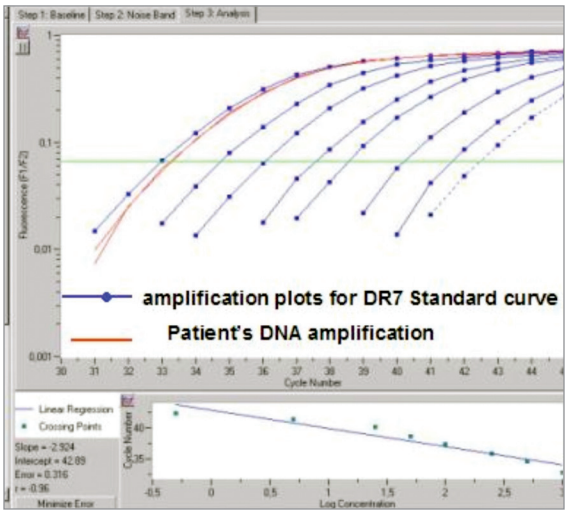

Figure 4. QPCR targeting HLA-DRB1*07, representing an HLA allele that was not inherited by the patient, demonstrating the presence of Mc.

We then hypothesized that the female cells were from an unrecognized vanished twin, as this phenomenon is relatively common in healthy pregnancies.[10] The patient tested positively for non inherited paternal HLA alleles (NIPA) with amplification for two different HLA class II alleles by QPCR, supporting this hypothesis. An alternative explanation could be Mc from an older sister passed via maternal blood to the patient during his fetal life or from a miscarriage (recognized or unrecognized) of the mother prior to the patient's birth. However, the patient tested negatively for HLA-A*01 and his older sister had inherited the maternal haplotype containing HLA-A*01. Additionally, the patient's mother had no history of miscarriage. Thus the most likely interpretation of our results is that this study represents the first report of persistent Mc from an unrecognized twin 40 years after birth.

We were unable to extend experiments to include testing for HLA class I NIPA (A*30 and B*13), because the remaining DNA was insufficient for Mc testing.

Quantitative assessment of Mc is difficult to evaluate in this patient as FISH results indicate that Mc represented 0.02% of total host cells, whereas QPCR showed levels as high as 5%. Two separate HLA-specific QPCR assays, DRB1*07 and HLA-DQB1*02, were concordant for Mc levels of 5%. Previous Mc studies have reported variations in Mc quantities depending on the method used, with FISH being reputedly less sensitive than PCR. The manufacturer's instructions (Abbott laboratories, Illinois, USA) report that the probe kit used for FISH offers a

limit of detection of 1% through a combination of CEP X and CEP Y fluorescently labeled DNA probes for specific regions of chromosome X and chromosome Y, respectively. In parallel HLA-specific QPCR offers a limit of detection of 0.005%, which could in part explain a difference in results. QPCR for additional HLA-specificities, such as HLA-A*30 or B*13, would have been useful but were not feasible in this case.

We also observed a difference in Mc in two different time points. While fluctuation of Mc levels has been described, including in transplant recipients, the difference in the two time points in our study could be due to the method of blood sampling, as the first time point (positive for Mc) was from a cytapheresis and the second time point (negative for Mc) from a standard blood test. Alternatively, changes in levels could reflect "physiological" fluctuations of cells depending on recruitment to tissues.

It has recently been shown in women undergoing surgery for suspected lung cancer that male stem cells, presumably of fetal origin, are present at sites of tissue injury.[14] These cells could be recruited from a natural niche, such as bone marrow or proliferate locally and either way could result in dissimilar amounts in peripheral blood at different time points. In our patient, clinical status and treatment were very similar between the two blood draws, arguing against variations in Mc related to inflammation and recruitment. Thus it seems most likely that technical aspects related to cytapheresis resulted in higher levels of Mc compared to a standard blood draw.

Interestingly, our experiments did not show evidence of fetal Mc from prior pregnancy with a "vanished fetus" in PBMC from the patient's mother. One could suggest, as previously described by our group, that the host's genotype might influence persistence of Mc, but in this particular case the patient and his mother had very similar HLA typing, at least for HLA class II, where they were HLA-identical. Besides genetic factors, chimerism in this case may be dependent on other factors such as inflammation, environmental exposure (e.g., hydrocarbons), timing or the method used to obtain and isolate PBMC. A decade ago, in a mouse model Christner et al.[15] showed marked proliferation of pre-existing microchimeric cells of fetal origin following intraperitoneal injections of vinyl chloride, associated with the development of fibrosis and a heavy mononuclear infiltration in the dermis. We suggest that a similar expansion and recruitment of microchimeric cells may have occurred in this scleroderma-like patient after hydrocarbon exposure, in contrast to his mother, who had no such exposure.

Parous women are potential hosts to at least two sources of Mc, maternal and fetal. We and others, proposed that this particular condition might contribute to female preponderance in auto-immune diseases.[16] This hypothesis has been most extensively tested for SSc, a condition resembling graft-versus-host-disease, the latter a condition of chimerism that clearly leads to inflammation. Higher levels of Mc in women with SSc compared to matched healthy women have reinforced this hypothesis.

Our current study suggests that a vanished twin represents an additional potential source of Mc that can occur in men as well as women. Further studies are needed to determine the frequency of vanished twin Mc and to elucidate whether the autoimmune-like disease in the current report is anecdotal or whether vanished twin Mc might predispose to autoimmunity.

Methods

Peripheral blood samples. PBMCs were obtained on two occasions for the current study: the first sample derived from cytapheresis without stem cell mobilization (March 2005) and the second sample was a standard heparinized blood sample (June 2006). At both time points, the patient was taking immunosuppressive medications including low-dose corticosteroids and azathioprine. The patient gave informed consent according to the Declaration of Helsinki.[13]

Familial HLA typing. HLA-A, B, DR, DQ and DP typing was done in the Laboratory of Immunology-Hematology-Transfusion of Erasme Hospital, in Brussels, Belgium.

Fluorescence in situ hybridization (FISH). To test for chimerism with female cells FISH was employed and the patient's PBMCs were probed, after Ficoll density separation, with CEP X/Y DNA Probe Kit according to manufacturer's instruction (Abbot laboratories, Illinois, USA). The CEP X/Y probe is a mixture of a Spectrum Orange labeled CEP X DNA probe and a Spectrum Green labeled CEP Y DNA probe specific for the alpha satellite centromeric region of chromosome X and the satellite III (Yq12) region of chromosome Y. The limit of detection sensitivity, according to the manufacturer's instructions, is 1%. A healthy male donor was tested in parallel as a control (**Fig. 1**).

HLA-specific quantitative PCR (QPCR). In order to identify and quantify maternal Mc, we first examined HLA typing results for the patient and his mother. The patient and his mother were HLA-DR and DQ identical (**Fig. 2**) so we could not employ any of the HLA class II specific QPCR assays that had previously been developed. We therefore designed a new QPCR assay that was specific for a maternal HLA class I allele that the patient did not inherit (NIMA), HLA-A* 01; the assay was validated with a sensitivity of 0.005%, as the DNA-equivalent of one HLA-A*01 cell was detectable in a background of the DNA-equivalent of 20,000 non HLA-A*01 cells (**Fig. 3**).

To investigate Mc from a vanished twin the patient samples were tested with QPCR assays that were specific to the patient's non-inherited paternal HLA alleles (NIPA). These experiments were performed employing previously developed QPCR assays for HLA-DRB1*07 and DQB1*02. [9,12]

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CHAPTER 3.3 – SHARED EPITOPE AND X CHROMOSOME INACTIVATION IN RA

The other part of my Ph.D. project is the study of the epigenetic phenomenon of X chromosome inactivation among women with RA. Generally, X-inactivation is random, and female tissues are mosaics of two cell lines expressing either the maternally-derived or the paternally-derived X chromosome. However, the prevalence of a non-random X-inactivation has been described in many autoimmune diseases, but often without revealing the reasons behind it.

Here, we investigate the patterns of X-inactivation in women with RA. Moreover, while analyzing different parameters of the disease, we discover that a skewed pattern is very likely influenced by a genetic background of susceptibility to the disease…

3.3.1 ARTICLE 3 – [DRAFT VERSION]

SKEWED X CHROMOSOME INACTIVATION IS ASSOCIATED WITH THE PRESENCE OF THE SHARED EPITOPE IN WOMEN WITH RHEUMATOID ARTHRITIS

Skewed X chromosome inactivation is associated with the presence of the shared epitope in women with rheumatoid arthritis

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SBK and OEO equally contributed to this work

Words count: Abstract: 279 words; Text: 2555 words; Figures: 3; Tables: 2; Supplementary material: 0

Keywords: X chromosome inactivation – Shared epitope – Rheumatoid arthritis – Female predominance

Conflict of Interest Disclosure: The authors have no competing financial interests.

Abstract

Objectives: Skewed X chromosome inactivation (XCI) has been reported in many female-predominant autoimmune diseases, but only once in rheumatoid arthritis (RA). In the current study, we propose to confirm biased XCI patterns in women with RA and further analyze them with respect to various disease parameters including autoantibody titration, disease duration, age of patients at disease onset and presence of shared-epitope (SE)-positive susceptibility alleles.

Methods: XCI profiles were performed in peripheral blood mononuclear cell from 161 women with RA and 100 healthy women by genotyping a polymorphic repeat in the androgen receptor (*AR*) gene, using a methylation sensitive enzyme (*HpaII*) followed by a PCR. All the patients were positive for the anti-citrullinated protein antibody (ACPA) and all women were HLA-DRB1 genotyped at the allelic level. XCI skewing was defined as having a ratio ≥ 80:20 of cells inactivating the same X chromosome.

Results: A total of 110 patients and 69 controls were informative for the *AR* polymorphism. Among them 40.9% of women with RA (45/110) had a skewed XCI compared to only 17.4% of healthy women (12/69) ($P = 0.0018$). A pattern of extreme skewing (≥ 90:10) was present in 26.4% of patients compared to only 2.9% of controls ($P = 0.0001$). When considering various parameters, XCI patterns did not significantly correlate with age of individuals, age of disease onset, disease duration or ACPA titrations. Interestingly, the presence of the SE was associated with a higher skewing among patients ($P = 0.002$) and such association was not observed in controls.

Conclusions: These results confirm previously described association of skewed XCI in autoimmunity. Furthermore, and for the first time, they show that XCI mosaicism may be influenced by HLA polymorphisms.

Introduction

Female predominance in autoimmune diseases is remarkable as approximately 80% of patients are women (1, 2). Rheumatoid arthritis (RA), the most prevalent rheumatismal autoimmune disease, does not escape to this rule with a women:men ratio of 3:1. Pathological features include chronic inflammation of the synovial membrane of peripheral joints and tendons gradually leading to destruction of bones and cartilage. RA is often characterized by the presence of autoantibodies, such as the highly specific anti-citrullinated protein antibodies (ACPA) (3), which can precede the clinical manifestation of RA by many years (4). Gene polymorphisms in the Human Leucocyte Antigen (HLA) locus account for the highest genetic risk in the development of RA. Several *HLA-DRB1* alleles (*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01 and *14:02) are implicated (5). They code for a shared motif of five amino-acid sequence ^{70}Q/R K/R R A A^{74}, the so-called *shared epitope* (SE), in the third hypervariable region of the DRβ1 molecule. The SE hypothesis predicts that RA-associated DRβ1 molecules bind the similarly charged peptide(s) and thus facilitate the development of autoreactive T cells involved in the pathogenesis of RA (6). However, despite the pivotal role of SE-mediated susceptibility, it only contributes partially to the disease and does not explain gender bias. It is therefore likely that X-linked risk factors have a role to play in disease onset and progression.

Recently, it has been reported that skewed X chromosome inactivation (XCI) patterns are found in peripheral blood of women with autoimmune diseases such as systemic sclerosis, autoimmune thyroiditis, juvenile idiopathic arthritis, and RA (7-11). XCI is a dosage compensation mechanism used by mammals to ensure that XX females and XY males equalize X chromosome gene expression (12). As a consequence, females are functional mosaics of two cell lines, one expressing the maternally-derived and the other the paternally-derived X-linked genes with a ratio close to 50:50 when XCI is random. Skewing then represents a deviation from the 50:50 ratio and is arbitrarily defined, often as a pattern were 80% or more of the cells inactivate the same X chromosome (13). This deviation is the result of i) genetic factors directly involved in the process of XCI, ii) genetic defects (mutations, rearrangements,…) on the X chromosome leading to a selective process, iii) tendency towards monoclonal expansion of cells related to aging, or iv) pure chance, due to the stochastic nature of the choice of which X chromosome to inactivate in the early stages of embryogenesis (14, 15).

A skewed XCI has the potential to make X-linked self-antigens escape presentation in the thymus, leading to the development of autoimmunity (16). This hypothesis, however, is speculative and a lot remains unexplained regarding reasons for biased XCI in autoimmunity.

In an attempt to uncover arguments of this skewing in disease pathogenesis, we propose to examine XCI patterns in women with RA, with respect to various disease parameters including autoantibody titration, disease duration, age of patients at diseaseonset and presence of SE-positive susceptibility alleles.

Methods

Study subjects

Among the 261 female subjects included in the study, 110 women with RA (median age: 58 years) and 69 healthy women (52 years) were informative for the XCI assay. All patients with RA satisfied the 2010 revised criteria of the American College of Rheumatology and the European League Against Rheumatism (17). All patients with RA were anti-citrullinated protein antibody-positive (ACPA+). Median age at the onset of disease was 48 and median disease duration was 6 years (Table 1). The selection criterion for healthy controls was no history of autoimmune disease in the family.

All participants signed informed consent according to the Declaration of Helsinki (18). The study is registered at the INSERM under the Biomedical Research Protocol number RBM-04-10 or as a collection under the number DC-2008-327.

PBMC and plasma isolation and DNA extraction

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from 8 mL of whole blood (drawn in EDTA or heparin vacutainer tubes) using Ficoll Histopaque 1077 (Sigma-Aldrich, St Louis, MO, USA) gradient centrifugation. Plasma was stored at −80°C. Genomic DNA was extracted from PBMCs with EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) using a BioRobot EZ1 system (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and stored at –20°C.

Anti-citrullinated protein antibodies

Positivity for ACPA was used to define mainstream RA because of its well-recognized diagnostic and predictive value (3). ACPA were detected by anti-CCP2 Enzyme-linked immuno-sorbent assay (ELISA) (Immunoscan RA, Euro-Diagnostica, Arnhem, the Netherlands). Positivity was defined by a cut-off value of 25 Unites/mL at a dilution of 1/50 of patients' plasma.

***HLA-DRB1* genotyping**

HLA-DRB1 typing was carried out at the Etablissement Français du Sang (Marseille, France) and/or in our laboratory, using sequence-specific oligonucleotide (SSO) typing kits for generic typing and/or sequence-specific primer (SSP) typing kits for allelic typing (SSO Dynal, Invitrogen, Carlsbad, CA, USA; SSO LABType, One Lambda Inc., CA, USA; SSP Olerup HLA-DRB1*04, Genovision, Vienna, Austria).

X chromosome inactivation assay

Genotyping of a polymorphic site in the human androgen receptor (*AR*) gene was performed and quantified based on the use of radioactive α-^{33}P-dCTP (NEN, Boston, MA, USA) to assess the XCI patterns as previously described (7, 19). DNA methylation occurs on the inactive X, and prevents a methylation-sensitive *HpaII* restriction enzyme to cleave on its specific site located on exon 1 or *AR* gene. When the genomic DNA is cleaved with *HpaII* prior to PCR, only the methylated *AR* allele, which represents the inactive X-chromosome, is amplified. A polymorphic CAG repeat located within the amplified region is used to distinguish between the two alleles (Figure 1). For each patient and control two separate polymerase chain reactions (PCRs), with or without *Hpa*II treatment, were performed using the same set of primers. Male DNA with verified 46XY karyotype was used as control for complete digestion. Densitometric analysis of the alleles was performed at least twice for each sample using the MultiAnalyst version 1.1 software (Bio-rad, Hercules, California, USA). A corrected ratio was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the non-predigested sample for normalization of the ratios that were obtained from the densitometric analyses. The use of corrected ratio compensates for preferential amplification of the shorter allele when the number of PCR cycles increases (20). A skewed population is defined when a cell population represents 80% or higher of one of the *AR* alleles (mosaicism ratio of ≥ 80:20) and extremely skewed when mosaicism is at a ratio ≥ 90:10.

Statistical analyses

The results from control and RA groups in XCI assays were compared by X^2 test (with Yates' correction in case of 2 × 2 contingency), or by $r \times c$ Fisher's exact test (r and c represent respectively numbers of rows and columns equal or greater than 2) (21). Odds ratios were calculated using Woolf's method at 95% confidence interval. To examine correlations, Spearman's rank correlation test was used. P values of 0.05 or less were considered significant.

Results

Women with RA have a skewed XCI pattern

Individuals for whom paternally-derived and maternally-derived androgen receptor gene alleles could not be distinguished were not included in the analysis and considered as non-informative. XCI status was informative for 110 of the 161 RA patients (68.3%) and 69 of the 100 healthy women (69.0%).

Overall, XCI patterns significantly differed between patients and controls (P = 0.0016; X^2 test; Table 2). Skewed XCI ratios (≥ 80:20) were found in 40.9% of women with RA (45/110) compared to only 17.4% of healthy women (12/69) (P = 0.0018; X^2 with Yates' correction; Odds ratio with 95% confidence interval (OR): 3.3 [1.6 – 6.8]). More importantly, extremely skewed XCI patterns (≥ 90:10), were seen in 26.4% of patients (29/110), versus only 2.9% of healthy women (2/69) (P = 0.0001; X^2 with Yates' correction; OR: 12.0 [2.6 – 52.1]) (see Table 2).

XCI patterns do not correlate with ACPA titration, age at disease onset, or disease duration

In order to investigate reasons for a skewed XCI in women with RA, we proposed to analyze whether age of the patients, concentration of ACPA detected in plasma, age at the onset of RA, or disease duration (Table 1) had an influence on XCI patterns. XCI patterns did not significantly correlate with age of individuals, whether they were healthy or with RA (respectively, Spearman r = +0.08 and +0.14; P = 0.52 and 0.16; Figure 2A), neither with age of disease onset, disease duration or ACPA titrations (respectively, Spearman r = +0.03, +0.09 and +0.12; P = 0.75, 0.37 and 0.25; Figure 2B, C and D) in women with RA.

XCI is skewed with the presence of the SE among women with RA

Another important parameter to study in the context of its potential influence on XCI patterns is the presence of *HLA-DRB1* susceptibility alleles carrying the SE motif. A total of 170 informative women were *HLA-DRB1* typed. The presence of the SE, as expected, was more frequent among women with RA. Indeed, SE was present in double dose (SE+/+) in 20.8% (21/101) and in single dose (SE+/−) in 51.5% (52/101) of RA patients, compared to only 7.2% (5/69) and 27.6% (19/69) respectively, in healthy women (Table 1).

We observed that prevalence of skewed XCI increased with SE doses in women with RA ($r \times c$ Fisher's exact test, P = 0.002; Figure 3). Extreme skewing (≥ 90:10) was also marked by a higher prevalence among RA patients when their number of SE doses increased from 0 to 2 (compared to population of "not extremely skewed" XCI [50:50 → 89:11]; $r \times c$ Fisher's exact test; P = 0.002). On the other hand, no statistically significant association in XCI patterns could be observed with the presence of the SE among healthy women ($r \times c$ Fisher's exact test, P = 0.29; Figure 3).

Altogether, skewed XCI (≥ 80:20) was observed in 35 of 73 RA women with at least 1 dose of SE (47.9%), compared to 7 of 28 RA women with 0 dose of SE (25%) (Fisher's exact test; $P = 0.04$; OR: 2.8 [1.1 – 7.3]). Healthy women do not follow this pattern as 5/24 (20.8%) had a skewed XCI pattern with at least 1 dose of SE (versus 7/45 (15.6%) with 0 dose) (Fisher's exact test; $P = 0.74$; OR: 1.4 [0.4 – 5.1]).

Discussion

We have demonstrated a higher prevalence of skewed XCI in peripheral blood cells of women affected with RA compared to healthy women ($P = 0.0016$). This prevalence is even more significant for extremely skewed XCI, as proportionally 9 times more RA women have an extremely skewed status compared to controls ($P = 0.0001$). Our results agree with a previously published study about XCI in RA (11). Moreover, XCI patterns found in our group of healthy women match those described in large cohorts of controls (22).

For the first time we show that skewing is strongly associated with the presence of *HLA-DRB1* alleles carrying the susceptibility-conferring shared epitope (SE) in women with RA ($P = 0.002$). In other words, a patient with RA is likelier to have a skewed or extremely skewed pattern when she carries SE-positive alleles. Importantly, this association between SE and skewing is not noticed among healthy women ($P = 0.29$). Although we used the $r \times c$ Fisher's exact test; robust with small counts and calculating exact P values, we remain limited in the number of counts, which is small in both healthy women with extreme skewing and healthy women with a double dose of the SE. However, using another control group of women with systemic sclerosis (N = 86), previously analyzed for XCI patterns (23) and for whom HLA-genotype is known, we confirmed what was observed in healthy women. Similarly to healthy women, women with SSc who carried the RA-specific SE did not have more often a biased XCI pattern (data not shown). This indicates that the observed influence of RA -HLA specific alleles on XCI patterns is peculiar to RA.

A skewed XCI has been observed in higher frequencies in many autoimmune diseases and has been proposed as an X-linked risk factor in the development of autoimmunity (7-11). In this perspective, our findings suggest that skewed inactivation of the X chromosome and HLA polymorphisms on HLA genes from chromosome 6 are synergic risk factors in RA, and they are somehow characteristically and mechanistically related in disease pathogenesis. However, if this hypothesis is true, a skewed XCI would be more commonly found in subjects carrying the SE, even without RA. This assumption is ruled out by the observation that this is not the case in our group of healthy controls or in the group of women with scleroderma for which the SE do not confer susceptibility.

The SE is the main genetic susceptibility marker associated with RA pathogenesis. It is also a marker of severe disease activity, cartilage erosion and bone destruction (24-26). In such context of chronic inflammation, peripheral blood mononuclear cells would have an accelerated rate of turnover, and a skewed pattern would occur as a result of clonal selection. Such phenomenon, leading to a gradual skewed XCI status with age, is already known in the normal population (27, 28), although it is not significantly visible in our groups, because number of individuals is not very extended in groups of young age (respectively for RA women and controls, N = 24 and 28 at ≤ 49 years) and mostly concentrated in an older range of age ([50 – 80] years). Collectively, our findings of a strong correlation of skewed XCI with the presence of the SE and the lack of correlation with disease duration suggest that such an acceleration in inflammatory-mediated clonal selection may occur at the very early stages of disease, possibly even before the establishment of a diagnosable RA. Although we do not have direct evidence to confirm it in the current study, this hypothesis remains plausible. Finally, our data do not show a correlation between ACPA titration and XCI patterns, which does not contradict our hypothesis as ACPA titration has never been considered as a good marker of disease severity (29).

From here, future perspectives in understanding XCI skewing in autoimmune disease can take at least two directions. Firstly, investigating the nature of the inflammatory-mediated clonal selection should provide clues on X-linked genes responsible for giving survival advantage in an inflammatory context. One can start by looking for which specific cell subpopulation is more affected then others with biased XCI, and what could be the eventual cellular and molecular mechanisms leading to such clonal selection. A study on cell subpopulations has already been addressed in scleroderma without finding a difference in skewing between the immune cell subsets (30). However, this study showed a deregulation in Forkhead box P3 (Foxp3)-positive regulatory T cells when XCI skewing was present in women with scleroderma (30). Such elements would be interesting to be verified in RA. Secondly, one should consider analyzing XCI patterns in a large cohort of females genotyped for their HLA and with no history of autoimmunity at the time of blood draw. Following them up over a long period of time should give more insights into the view of skewing as being consequence or cause of the disease. This will increase our understanding on the relevance of X-inactivation in autoimmunity. XCI patterns would have predictive value, as skewing would happen before the onset of symptoms, indicating a potential patient with RA and/or possibly other autoimmune diseases.

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Figure legends

Figure 1.

Example of X chromosome inactivation status in 5 samples. Polymerase chain reaction products from the androgen receptor (*AR*) methylation assay shows random X chromosome inactivation (50 – 79%) in samples A and C, and skewed X chromosome inactivation (80 – 89%) in sample E. Samples B and D have a non-informative status. For each sample, DNA was either undigested (−) or digested (+) with the methylation-sensitive restriction enzyme HpaII. Marker (331-bp and 242-bp) fragments are visible.

Figure 2.

Correlation plots of X chromosome inactivation (XCI) skewing status with (A) age of individuals, (B) age at disease onset, (B) disease duration, and (C) ACPA titration. (A) r = +0.08 and +0.14 (*P* = 0.52 and 0.16) for healthy women and women with RA, respectively; **(B)** r = +0.03 (*P* = 0.75); **(C)** r = +0.09 (*P* = 0.37); **(D)** r = + 0.12 (*P* = 0.25). Symbols represent individuals. Unfilled (white) symbols represent healthy females and filled (black) symbols represent females with rheumatoid arthritis (RA). Correlation was examined with Spearman's rank correlation test.

Figure 3.

Comparison of X chromosome inactivation patterns and the presence of the shared epitope in patients and controls. Among women with rheumatoid arthritis (RA), X chromosome inactivation patterns, from random (50:50 → 79:21) to skewed (80:20 → 89:11) and extremely skewed (90:10 → 100:0), are significantly different according to the presence of *HLA-DRB1* susceptibility alleles carrying the shared epitope (SE) in 0, 1 or 2 doses ($r \times c$ Fisher's exact test, $P = 0.002$). Such effect is not statistically significant among healthy women ($r \times c$ Fisher's exact test, $P = 0.29$). Numbers in columns represent number of individuals (N).

Tables

Table 1. Patients' and controls' characteristics

	RA patients	**Female controls**
Individuals	161	100
Informative in the *AR* assay (% of total)*	110 (68.3%)	69 (69.0%)
Median age (with IQR)	58 (51 – 66)	52 (46 – 58)
Median age at diagnosis (with IQR)	48 (41 – 57)	NA
Median disease duration (with IQR)	6 (1 – 17)	NA
Median ACPA titration (Units/mL) (with IQR)	643 (232 – 866)	NA
SE−/− (% of total)	28 (27.7%)	45 (65.2%)
SE+/− (% of total)	52 (51.5%)	19 (27.6%)
SE+/+ (% of total)	21 (20.8%)	5 (7.2%)

ACPA: anti-citrullinated protein antibody; *AR*: Androgen receptor gene; IQR: interquartile range; NA: not applicable; RA: rheumatoid arthritis; SE: shared epitope; XCI: X chromosome inactivation. *All following characteristics account for individuals informative in the *AR* assay.

Table 2. Proportions of X chromosome inactivation patterns in patients and controls informative for the androgen receptor (*AR*) assay

XCI patterns (% of total)†	**RA patients (N = 110)**	**Female controls (N = 69)**
50 – 59%	36 (32.7%)	35 (50.7%)
60 – 69%	15 (13.6%)	12 (17.4%)
70 – 79%	14 (12.7%)	10 (14.5%)
80 – 89%	16 (14.6%)	10 (14.5%)
≥ 90%	29 (26.4%)	2 (2.9%)

†For patients-versus-controls comparisons of X chromosome inactivation (XCI) patterns by X^2 test: P = 0.0016 (differences in the 5 degrees of skewing), P = 0.0018 (skewed [≥ 80%]

versus random [50 – 79%]), *P* = 0.0001 (extremely skewed [≥ 90%] versus not extremely skewed [50 – 89%]).

Figures

Figure 1.

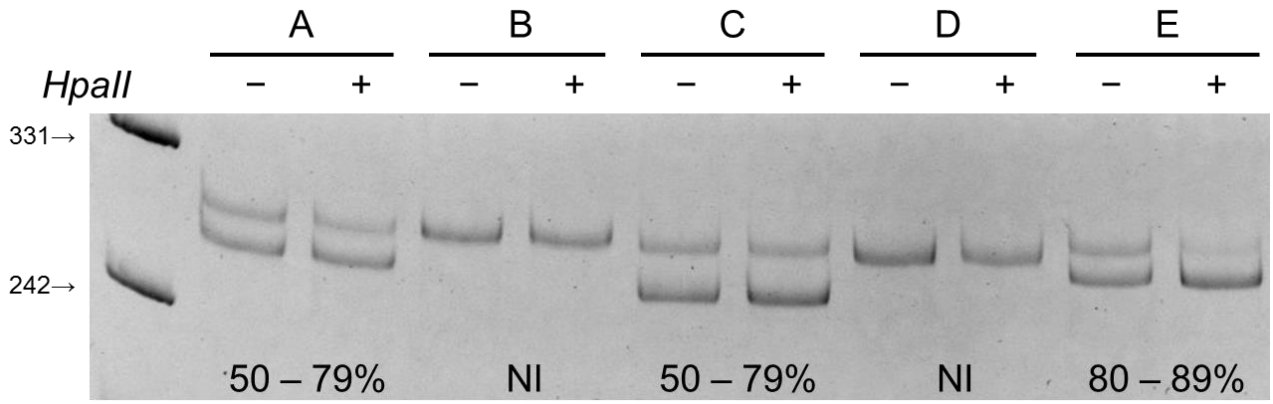


Figure 2.

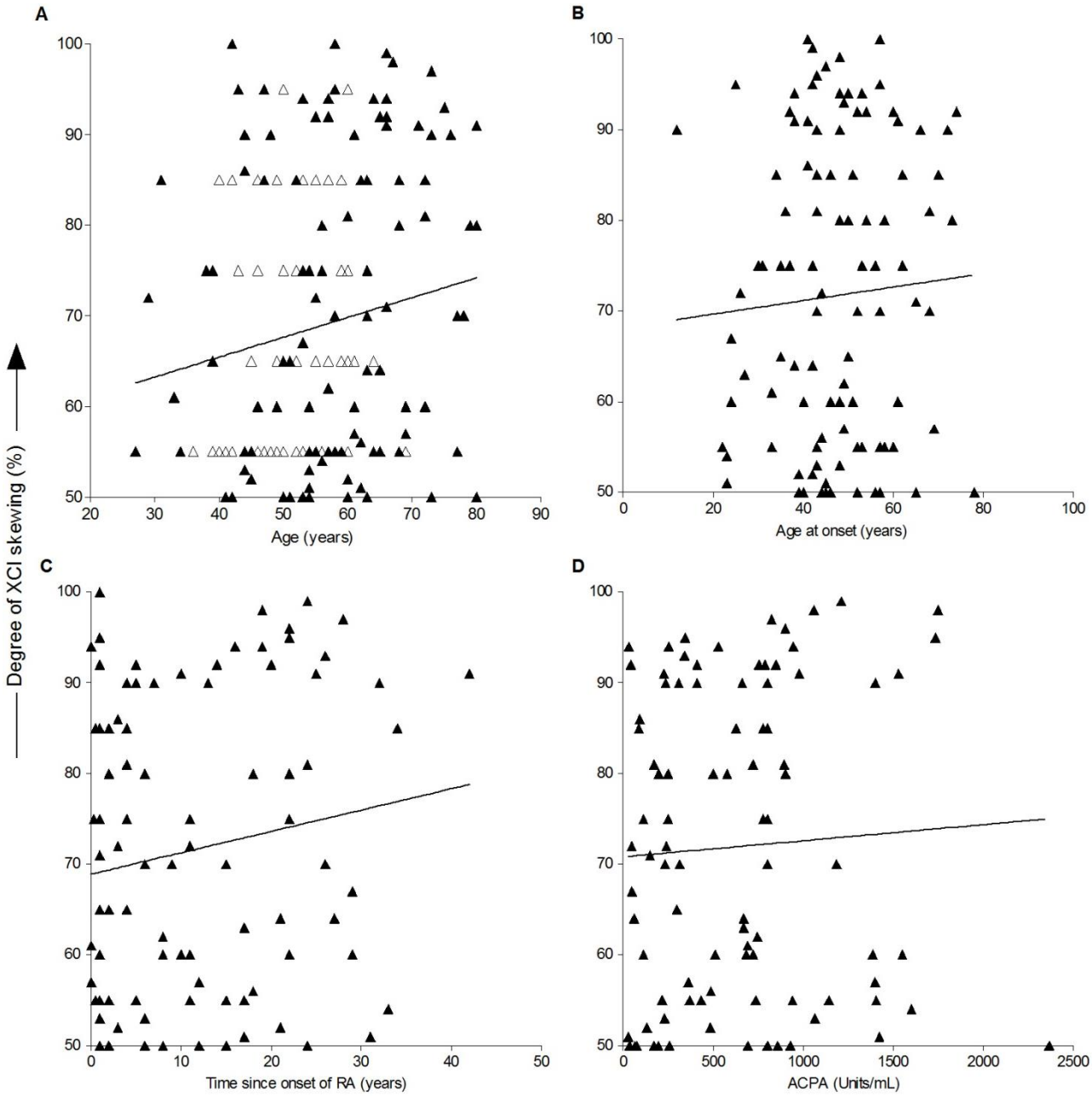
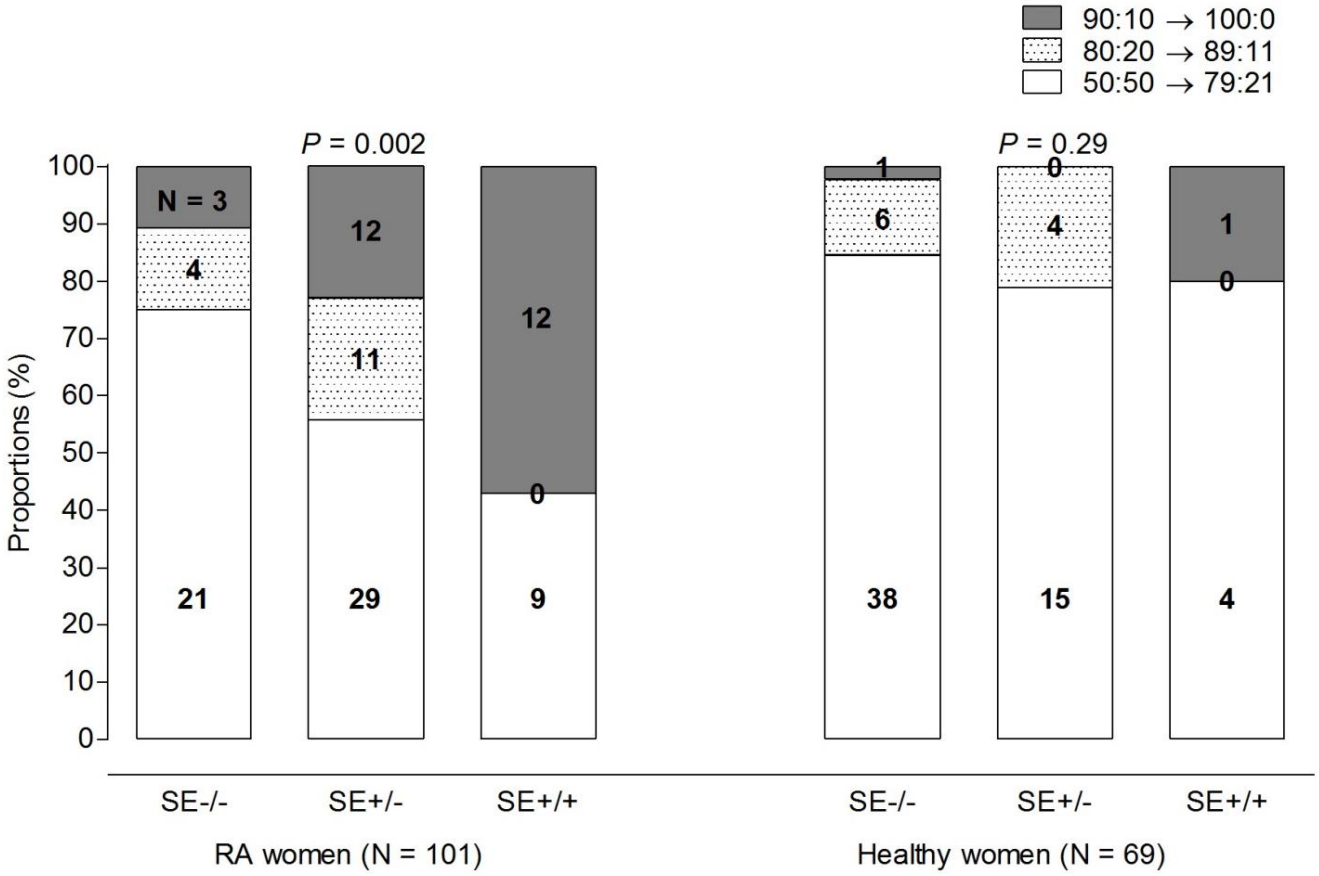

A
B
C
D
Degree of XCI skewing (%)
Age (years)
Age at onset (years)
Time since onset of RA (years)
ACPA (Units/mL)

Figure 3.


90:10 → 100:0
80:20 → 89:11
50:50 → 79:21
P = 0.002
P = 0.29
Proportions (%)
100
90
80
70
60
50
40
30
20
10
0
N = 3
4
21
12
11
29
12
0
9
1
6
38
0
4
15
1
0
4
SE-/-
SE+/-
SE+/+
SE-/-
SE+/-
SE+/+
RA women (N = 101)
Healthy women (N = 69)

CHAPTER 3.4 – X CHROMOSOME INACTIVATION PATTERNS IN SCLERODERMA

Scleroderma (SSc) is an autoimmune disease of unknown etiology and is characterized by a higher female predominance with up to 9 times more patients being women [5]. A few studies have already shown that skewed X chromosome inactivation (XCI) is a marker of the disease and may be involved in its pathogenesis [172, 177, 178]. However, these studies lacked information about XCI patterns' correlations with different characteristics of the disease.

Here we present data on XCI patterns in our cohort of French SSc patients, and analyze their effects in respect of clinical (diffuse versus limited cutaneous form) and serological (anticentromere antibodies [ACA] and antitopoisomerase antibodies [ATA]) differences, and the presence of *HLA-DRB1* and *HLA-DQB1* susceptibility alleles, known to carry a shared amino-acid sequence on their beta-chains, respectively ^{67}FLEDR^{71} and ^{71}TRAELDT^{77} [95].

Out of 96 women with SSc included in the analysis, 68 were informative for the XCI assay (median age and interquartile range: 55 [48 – 54] years), and compared to the previously analyzed 110 women with rheumatoid arthritis (RA) and 69 healthy women (*Chapter 3.3* of this manuscript). All patients with SSc met the requirements of LeRoy [43]. Thirty six were diagnosed with the diffuse cutaneous form of the disease, and 31 had the limited cutaneous form. Information about the presence of autoantibodies was available in 67 patients. Twenty six were positive for anti-topoisomerase antibody (ATA+), and 20 were positive for anti-centromere antibody (ACA+). Sixty six patients were genotyped for *HLA-DRB1* and *-DQB1* alleles as previously described [95]. X chromosome inactivation experiments were carried on DNA extracted from peripheral blood mononuclear cells (PBMC). The highly polymorphic CAG repeat on the first exon of the androgen receptor (*AR*) gene was genotyped by the *HpaII*-PCR assay to determine the XCI status in patients, as previously described [172, 174, 250]. The results from test groups in XCI analyses were compared by X^2 test (with Yates' correction) and $r \times c$ Fisher's exact test (when contingency cell counts were fairly small).

Skewed XCI, defined as a ratio of ≥ 80:20, was observed in DNA from PBMCs in 25 of 68 informative patients (36.8%), compared to 12 of 69 informative healthy controls (17.4%) ($P < 0.02$; X^2 test with Yates' correction; **Figure 13**). Extreme skewing (≥ 90:10) was marked by a

higher prevalence among women with SSc as it was present in 20 of the patient group (29.4%) compared to only 2 of healthy controls (2.9%) ($P < 0.0001$; X^2 test with Yates' correction; **Figure 14**). Among women with SSc, however, no differences in XCI patterns were detected according to disease duration, disease type (limited or diffuse), autoantibodies (ATA versus ACA), or the presence of susceptibility alleles with FLEDR or TRAELT (**Table 5**). Because higher prevalence of skewing was detected among women with RA when they carried susceptibility "shared epitope" QKRAA motif (*Chapter 3.3* of this manuscript), this latter was added in the present analysis as a control.

We have shown that skewed XCI mosaicism is higher in frequencies among women with SSc compared to healthy controls. These findings are consistent with previous studies [172, 177, 178]. We have also noted that, unlike results in women with RA (*Chapter 3.3* of this manuscript), this skewing is not associated with the presence of SSc-specific HLA susceptibility alleles. Moreover, no correlation is observed with disease characteristics described herein. However, absence of evidence does not always mean evidence of absence, and studies on larger cohorts are needed to confirm these observations.

Our results show that factors leading to XCI skewing are present in a significant proportion of female patients with SSc, and questions regarding causes and consequences of this phenomenon are still raised. Non-random XCI patterns can be the consequence of various factors, including pure chance, genetic alteration directly involved in XCI process or located on other loci leading to a differential expression patterns and providing selective disadvantage [251]. These factors can contribute to the skewing observed in patients. In conclusion, we have confirmed that perturbations in XCI patterns correlate with SSc in females. Nevertheless, a skewed X-inactivation does not occur in all women with SSc, suggesting that it would be part of a multitude of factors associated with disease pathogenesis, all working in a convenient predisposing genetic background.

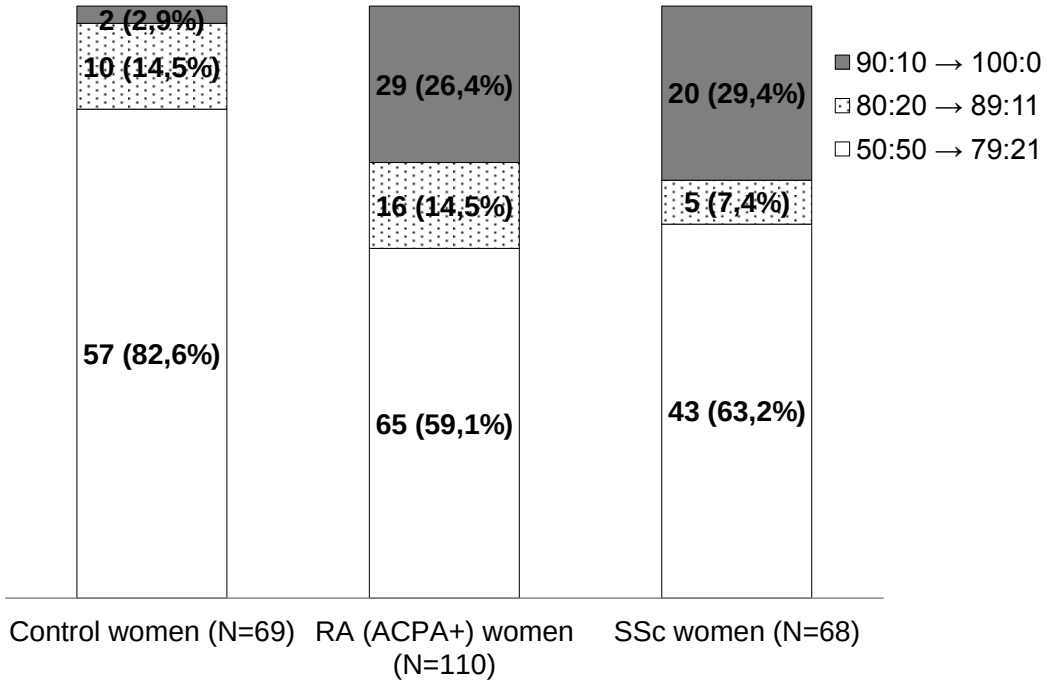


Figure 14. Comparison of X chromosome inactivation (XCI) patterns between women with scleroderma (SSc) and previously analyzed healthy women (controls) and women with rheumatoid arthritis (RA).
XCI is skewed (80:20 → 100:0) among women with SSc as compared to healthy controls (36% versus 17.4%) ($P < 0.02$; X^2 test with Yates' correction). Extreme skewing (90:10 → 100:0) is also higher in frequencies among SSc women compared to healthy controls (29.4% versus 2.9%) ($P < 0.0001$; X^2 test with Yates' correction). No differences in skewing patterns are detected between women with SSc or women with RA ($P = 0.35$; X^2 test). Analyses on XCI in RA and controls are from *Chapter 3.3* of this manuscript.

Table 5. Contingency analyses of X chromosome inactivation patterns in regard to various characteristics of scleroderma (SSc).

	Total counts	**Random XCI patterns (50:50 → 79:21)**	**"Moderate" skewing (80:20 → 89:11)**	**Extreme skewing (90:10 → 100:0)**	***P* value (*r* × *c* Fisher's exact test)**
SSc women	68	43 (63.2%)	5 (7.4%)	20 (29.4%)	
Limited cutaneous	31	21 (67.7%)	3 (9.7%)	7 (22.6%)	*0.47*
Diffuse cutaneous	36	21 (58.3%)	2 (5.6%)	13 (36.1%)	
ATA					
ATA-positive	26	15 (57.7%)	2 (7.7%)	9 (34.6%)	*0.85*
ATA-negative	41	27 (65.9%)	3 (7.3%)	11 (26.8%)	
ACA					
ACA-positive	20	11 (55.0%)	3 (15.0%)	6 (30.0%)	*0.31*
ACA-negative	47	31 (66.0%)	2 (4.2%)	14 (29.8%)	
Disease duration					
0 – 9 (years)	45	26 (57.8%)	4 (8.9%)	15 (33.3%)	
10 – 19 (years)	15	10 (66.7%)	1 (6.7%)	4 (26.6%)	*0.70*
≥ 20 (years)	8	7 (87.5%)	0 (0.0%)	1 (12.5%)	
FLEDR dose					
0	15	9 (60.0%)	1 (6.7%)	5 (33.3%)	
1	34	19 (55.9%)	3 (8.8%)	12 (35.3%)	*0.71*
2	17	13 (76.5%)	1 (5.9%)	3 (17.6%)	
TRAELDT dose					
0	9	6 (66.7%)	0 (0.0%)	3 (33.3%)	
1	32	17 (53.1%)	4 (12.5%)	11 (34.4%)	*0.58*
2	25	18 (72.0%)	1 (4.0%)	6 (24.0%)	
QKRAA dose*					
0	50	31 (62.0%)	2 (4.0%)	17 (34.0%)	
1	13	8 (61.5%)	3 (23.1%)	2 (15.4%)	*0.13*
2	2	1 (50.0%)	0 (0.0%)	1 (50.0%)	

ACA: anticentromere antibody – ATA: antitopoisomerase antibody – SSc: scleroderma – XCI: X chromosome inactivation. *QKRAA represents the motif carried by "shared epitope"-positive *HLA-DRB1* alleles that predispose to rheumatoid arthritis (RA). Unlike FLEDR and TRAELDT motifs, it does not confer susceptibility to SSc. However, it is added in the present analysis to serve as a control for the XCI study in RA (*Chapter 3.3* of this manuscript).

CHAPTER 3.5 – X CHROMOSOME INACTIVATION PATTERNS AND TELOMERE LENGTH IN RHEUMATOID ARTHRITIS

In previous chapters, we have shown that skewing of X chromosome inactivation (XCI) is more frequently present in women with rheumatoid arthritis (RA) compared to healthy controls. Moreover, this skewing is associated with *HLA-DRB1* susceptibility alleles carrying the shared epitope (SE) among women with RA but not among healthy women or a control group with women with SSc.

It is very difficult to interpret how RA- specific HLA polymorphisms on chromosome 6 could influence XCI patterns and only in women with RA.

Interestingly, Schönland *et al.* showed an association between the presence of the SE (carried by *HLA-DRB1*04* alleles) and premature immunosenescence, characterized by shortened telomeres, in peripheral blood of patients with RA [252]. Moreover, they showed that DR4-positive healthy individuals had significantly shorter telomeres compared to DR4-negative subjects. Another team later extended this study on a larger cohort (176 RA patients versus 1151 controls), including all other SE-encoding *HLA-DRB1* alleles. They reported that the presence of SE-positive allele was associated with reduced telomere length in RA cases, but not in controls [253].

The potential link between telomere length and XCI is suggested by the findings that telomere shortening is accelerated on the human inactive X chromosome [254] and that telomeric non-coding RNAs are enriched near the inactive X chromosome in mammals [255].

In such context, we sought to check whether women with RA had shortened telomere length and whether this could influence XCI mosaicism.

We have included in this analysis 108 women with RA (median age and interquartile range: 57 [50 – 65] years), all positive for anti-citrullinated protein antibodies (ACPA+) and 89 healthy women (54 [46 – 60] years). Patients satisfied the 2010 criteria of the American College of Rheumatology and the European League Against Rheumatism [256] and healthy subjects had no history of disease. Among them, 73 women with RA and 69 controls were known for their

XCI status. All individuals were genotyped for HLA-DRB1 alleles as previously described [85]. Relative telomere length was estimated by real-time Q-PCR with primers described by Cawthon [257, 258], using the $2^{-\Delta\Delta Ct}$ method, on DNA from peripheral blood mononuclear cells. The difference between Telomere-specific PCR and *36B4*-specific PCR (a single copy gene, coding for the acidic ribosomal phosphoprotein PO) was evaluated for each sample (ΔCt) then compared to a specific control sample systematically used in each assay (ΔΔCt). Q-PCR experimental conditions were adapted for the LightCycler® thermocycler instrument and were as previously described [258]. Briefly, samples were tested in triplicate PCR reactions, using 35 ng of DNA in 5 µL in each reaction, in a 20 µL final volume, carried out in the LightCycler FastStart DNA Master SYBRGreen kit (Roche Diagnostics, Mannheim, Germany), with $MgCl_2$ added to a final concentration of 3 m*M*. Primers for telomeres and *36B4* were added to final concentrations of 0.2 µ*M* and 0.3 µ*M* respectively. The primer sequences are FWD-Tel: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'; REV-Tel: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' [258]; FWD-36B4: 5'-CAGCAAGTGGGAAGGTGTAATCC-3'; REV-36B4: 5'-CCCATTCTATCATCAACGGGTACAA-3' [257]. Denaturation was carried at 95°C for 10 min, followed by 35 cycles of 95°C for 5 s, 58°C for 10 s, and 72°C for 40 s for the *36B4* reaction, or 25 cycles of 95°C for 5 s, 56°C for 10 s, and 72°C for 60 s for the telomere reaction. All transition rates were set to 20°C/s with the exception of the annealing transition rate in the telomere reaction, which was 4°C/s.

Results show that women with RA have a decreased telomere length with age (Spearman r = −0.32; *P* = 0.0007). Such effect was not detected in healthy women (Spearman r = −0.06; *P* = 0.59) (**Figure 15**). This could be the effect of a stronger senescence profile in RA patients, already visible on small number of patients but not on small number of controls.

Nevertheless, contrary to findings from Schönland *et al.* we did not find an association between telomere length and the presence of SE-positive HLA alleles (**Figure 16**). Contradiction could be explained by insufficient number of subject tested in our case, or by the difference in experimental approach, as they analyzed telomeric restriction fragment length by Southern blotting method.

Furthermore, in women with RA, shorter telomere length did not correlate with XCI mosaicism (N = 73; Spearman r < 0.01; *P* = 0.99; **Figure 17**), in agreement with a study that found XCI patterns to be independent from telomere length [259]. It was neither correlated with disease duration (N = 91; Spearman r = −0.14; *P* = 0.19; **Figure 18**). The latter is in agreement with findings showing no correlation between duration of RA and telomere loss in CD4+ T cells [260].

This study attempted to explain the observation of a significantly higher prevalence of skewed XCI associated with the presence SE-positive genotype in women with RA (*Chapter 3.3*) by finding a common associated factor. Telomere loss, reflecting immunosenescence, seemed a good candidate as it was accelerated in SE-positive individuals. Nevertheless, our study failed to be conclusive in this direction.

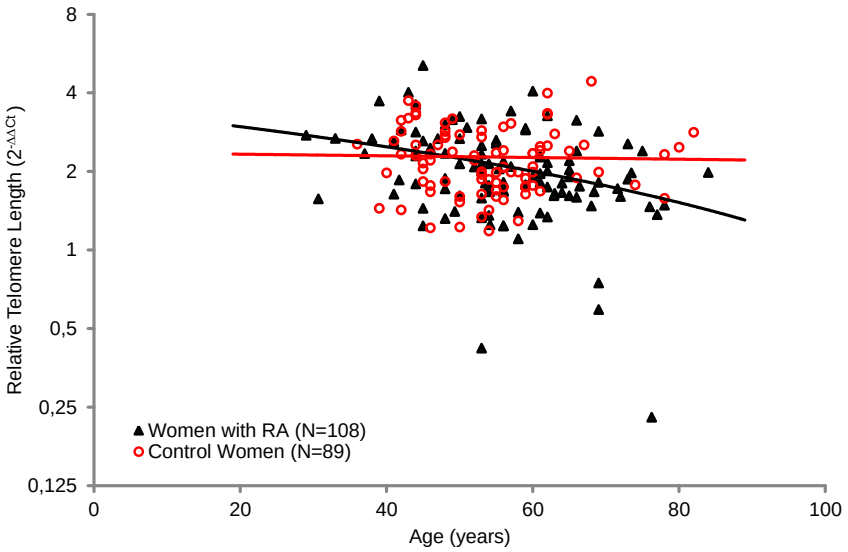


Figure 15. Telomere length decreases with age in rheumatoid arthritis (RA) patients, but not in healthy controls.
Women with RA: N = 108, Spearman r = −0.32, *P* = 0.0007; Healthy women: N = 89, Spearman r = −0.06; *P* = 0.59. Symbols represent individuals.

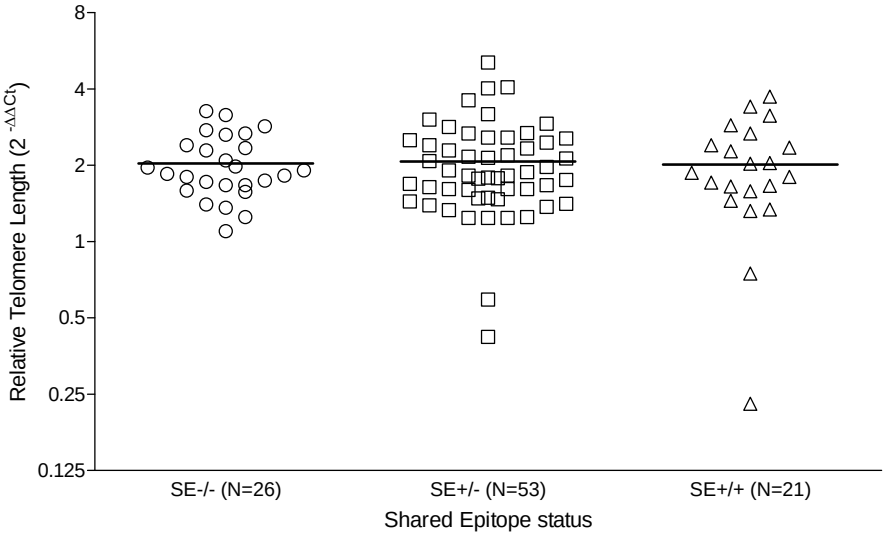


Figure 16. No association between the presence of the shared epitope (SE) and telomere length in women with rheumatoid arthritis (RA)

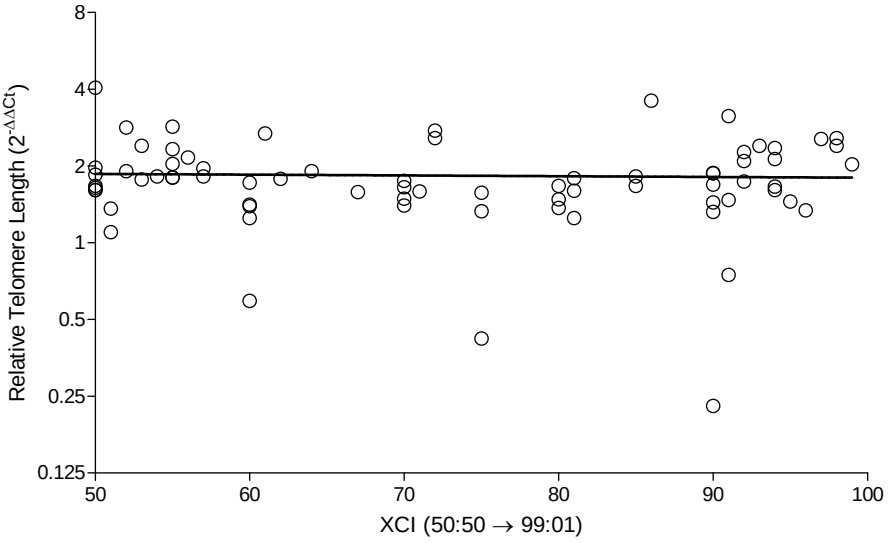


Figure 17. Telomere length is not correlated with skewed X chromosome inactivation (XCI) status.
N = 73; Spearman r < 0.001; *P* = 0.99

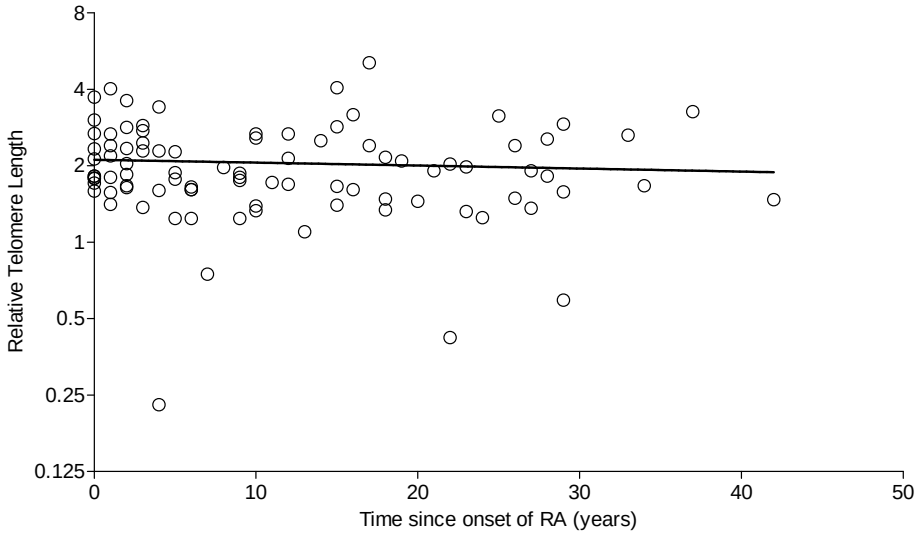


Figure 18. Telomere length is not correlated with duration of rheumatoid arthritis (RA).
N = 91; Spearman r = −0.14; *P* = 0.19

3.5.1 ARTICLE 4 – [PUBLISHED ABSTRACT]

DOES TELOMERE SHORTENING IN WOMEN WITH RHEUMATOID ARTHRITIS PREDICT X CHROMOSOME INACTIVATIONBIAS? [A7.9]

Materials and Methods We tested, using a 5 methyl cytosine (5MeCyt) ELISA, global DNA methylation in long-term cultured salivary gland epithelial cells (SGEC), peripheral T cells and B cells from eight SS patients. DNA methylation/demethylation partners were assessed by real time quantitative PCR (DNA methyl transferase (DNMT)1, DNMT3a/b, PCNA, UHRF1, MBD2, MBD4, and Gadd45-alpha). Immunofluorescence was conducted on labial salivary gland biopsy. Co-culture experiments were performed associating the human salivary gland cell line (HSG) and B cells.

Results Global DNA methylation was reduced in SGEC from SS patients (5MeCyt: 36.3 ± 3.2% in SS *versus* 43.1 ± 3.3% in controls, $P = 0.01$), while no difference was observed in T and B cells. SGEC demethylation in SS patients was associated with a 7-fold decrease of DNMT1 and a 1.8-fold increase of Gadd45-alpha expression. The other DNA methylation/demethylation partners tested were not differently expressed when compared to controls. Interestingly, SGEC demethylation may be attributed to the B cell infiltrate as DNA methylation increased in salivary gland biopsy after rituximab (anti-CD20 antibody) treatment. Such hypothesis was confirmed using co-culture experiments (HSG cells and B cells) revealing an alteration of the PKC-delta/ERK/DNMT1 pathway. Finally, DNA methylation was associated with the overexpression of several SGEC genes such as ICAM-1 and human endogenous retrovirus (HERV).

Conclusions SGEC dysfunction in SS may be linked to epigenetic modifications and this tissue specific defect may be ascribed in part to infiltrating B cells. This observation opens new therapeutic perspectives in SS.

A7.9 DOES TELOMERE SHORTENING IN WOMEN WITH RHEUMATOID ARTHRITIS PREDICT X CHROMOSOME INACTIVATION BIAS?

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Background Rheumatoid Arthritis (RA), like most auto-immune diseases, is a female predominant disease. As a possible explanation for gender bias, we have previously shown that women with RA have non-random X chromosome inactivation (XCI) that could trigger autoimmunity (article in preparation). Intriguingly, this bias in XCI correlates with presence of the shared epitope (SE) and with disease duration.

Also associated with presence of the SE, premature immunosenescence, characterised by shorter telomere length, has been described in peripheral blood cells from patients with RA [1]. Moreover, telomeric non coding RNAs have been reported to be enriched near the inactive X chromosome in mammals [2] indicating a potential link between telomere length and XCI.

Objectives In this context, we propose to test whether women with RA have shortened telomere length and whether that could influence the epigenetic mechanism of XCI.

Methods A total of 73 women with RA and 48 healthy women with no history of autoimmune diseases, who had previously been tested for XCI and HLA-genotyped, were evaluated for telomere length. The relative telomere length was estimated by real-time PCR as originally described by Cawthon [3] with the $2^{-\Delta\Delta Ct}$ method.

Results Preliminary results show that women with RA have smaller telomere length than healthy women, although the difference is modest ($p = 0.07$) and has to be adjusted for age on a larger cohort. Contrary to expectations, shorter telomere length is not correlated with skewed XCI status, disease duration or the presence of shared epitope in our small cohort.

Conclusions This preliminary study seems to confirm that women with RA have shorter telomeres than healthy women. Further telomere length measurements have to be done on a larger group of patients with RA and healthy controls, as well as HLA-genotyping them and evaluating their XCI status. This will be a step forward in understanding the relationship between immune senescence, female predisposition and genetic risk (SE) in RA.

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A7.10 GENETIC VARIANTS IN THE *IL-4* AND *IL-4* RECEPTOR GENES IN ASSOCIATION WITH THE SEVERITY OF JOINT DAMAGE IN RHEUMATOID ARTHRITIS: A STUDY IN SEVEN COHORTS

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Objective The progression of joint destruction in rheumatoid arthritis (RA) is determined by genetic factors. Changes in *IL-4* and *IL-4R* genes have been associated with RA severity but not replicated. We studied the association between *IL-4* and *IL-4R* tagging SNPs and progression rate of joint damage in RA in a multi-cohort candidate gene study.

Methods *IL-4* and *IL-4R* tagging SNPs (8 and 39, respectively) were genotyped in 600 RA-patients of whom 2,846 sets of hands and feet X-rays were collected during 7 years follow-up. Subsequently, significantly associated SNPs were genotyped and studied in relation to 3,415 X-rays of 1,953 RA-patients; these included data-sets from Groningen (NL), Lund (SE), Sheffield (UK), NARAC (USA), Wichita (USA) and NDB (USA). The relative increase in progression rate per year in the presence of a genotype was determined in each cohort. An inverse variance weighting meta-analysis was done on the six datasets that together formed the replication-phase.

Results In the discovery-phase none of the *IL-4* SNPs and seven of the *IL-4R* SNPs were significantly associated with joint damage progression rate. In the replication-phase, two SNPs in *IL-4R* gene were significantly associated with joint damage progression rate (Rs1805011, $p = 0.02$ and Rs1119132, $p = 0.001$).

Conclusions Genetic variants in *IL-4R* were identified and independently replicated to associate with progression rate of joint damage in RA.

A7.11 GENETIC VARIATION IN PROMOTER SEQUENCE OF B-CELL-ACTIVATING FACTOR OF THE TNF FAMILY (BAFF) IN PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHIES (IIM)

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PART 4 – *DISCUSSION*

CHAPTER 4.1 – KEY MESSAGES

In this work, we have analyzed specific genetic and epigenetic factors on the X chromosome, which may contribute to the increased risk of developing autoimmunity in women compared to men. The autoimmune disease mainly studied is rheumatoid arthritis (RA), although a comparison has been made with systemic sclerosis (SSc). Genetic influence is assessed through gene copy number analysis of Toll-like receptor (*TLR*)*7* and *TLR8*, located on the X chromosome. Epigenetic influence is studied through the analysis of X chromosome inactivation patterns among women with and without disease.

1. On the genetic level, we have presented for the first time, evidence that copy numbers of X-linked *TLR7* and *TLR8* are influenced by age and gender. Indeed, *TLR7* and *TLR8* copy numbers increase with age in peripheral blood cells of men only, regardless of RA. Women have a rather opposite pattern with a tendency to decreased *TLR7/8* copy numbers with age.

2. Feminine microchimerism could contribute to the increased pool of X-linked genes in men. This hypothesis is backed up by the case report of a man, in whom we show the presence of female microchimeric cells by fluorescence *in situ* hybridization and HLA-specific quantitative PCR. Whereas in women, the observed decrease could come from X chromosome monosomy previously reported to increase with age.

3. On the epigenetic level, we report a higher prevalence of non-random X chromosome inactivation in peripheral blood cells of women with RA and women with SSc compared to healthy women. Moreover, the skewing correlates with the presence, among women with RA, of *HLA-DRB1* susceptibility alleles carrying the shared epitope, marker of disease severity. This correlation is interestingly absent in controls, whether they are healthy women or women with SSc.

CHAPTER 4.2 – CAUSE AND CONSEQUENCE OF TLR7 AND TLR8 GENOMIC DYSREGULATION

Dysregulated gene dosage on the X chromosome may lead to autoimmunity. The Yaa mouse model constitutes the best example for such copy number dysregulation [191, 192]. This strain of mice harbors in its genome an unbalanced translocation from the X to the Y chromosome that includes the innate immunity genes *Tlr7* and *Tlr8*. The translocation thus causes a 2-fold increase in copy numbers of those genes in affected males. Furthermore, gene dosage effect of the X chromosome on autoimmune pathogenesis is highlighted by the findings that mice with 2 X chromosomes (XX females or XX/*Sry*+ males) are more susceptible to experimentally induced autoimmune syndromes, compared to mice with a single X chromosome (XY^{Sry-} females or XY^{Sry-}/*Sry*+ males), regardless of the hormonal and gonadal background [261]. This suggests that double dosage of X chromosome confers susceptibility to autoreactivity.

We have tested the possibility of a copy number alteration similar to the one described in the Yaa mouse in humans affected with RA. We have observed, in men, an increased *TLR7* and *TLR8* copy number mediated by age and independent of disease, whereas such phenomenon is reversed in women. Nevertheless, the copy number increase in men is of small amplitude and represents an average of +20% at the age of 80. Therefore, unlike in the Yaa model, we do not observe a 2-fold increase. Furthermore, as we check, in men, for *TLR7* and *TLR8* copy number increase that could come from a particular cell subpopulation, we observe that tendency towards increase with age is paralleled in B cells, T cells, granulocytes and the rest of cell subsets, suggesting an event occurring upstream in the hematopoietic stem cell compartment.

Similar analyses are being conducted in our laboratory on men with SSc, in order to see whether copy numbers of *TLR7* and *TLR8* vary, compared to controls. Results are still preliminary, and progress at slow pace as men represent nearly 8% of patients in a disease that is already rare [5].

4.2.1 HOW TO EXPLAIN GENE COPY NUMBER VARIATIONS DIFFER BETWEEN SEXES?

Several mechanisms can explain such copy number increase with age in men (summarized in **Figure 20**). One of them is somatic duplication that begins to affect cells and progressively

results in somatic mosaicism in male tissues. In other words, DNA segments' duplications encompassing at least *TLR7* and *TLR8* genes could happen during mitosis. This kind of somatic mosaicism for copy number variations (CNVs) is supported by the finding that different CNVs occur among monozygotic twins [262] and that the presence of different CNVs in different tissues of the same individual [263].

On the other hand, the opposite tendency in women can be explained by somatic X chromosome monosomy. Such phenomenon was first suggested in an early report by Jacobs *et al.* who showed that in normal human leukocytes the proportion of aneuploid cells increases with age in both sexes, but that the chromosome involved in aneuploidy was different according to sex [264]. Males were more often missing the Y chromosome and females a "medium sized" chromosome (likely one of the X) in some of their cells [264]. Later, it has been confirmed that acquired aneuploidy is common and increases with age, hypoploidy more often than hyperploidy, and the lost chromosome is often the X chromosome in females and the Y chromosome in males [265-267]. The latest of these studies has also shown that X chromosome loss is much higher than that of Y chromosome, and that autosomal mosaicism (with chromosomes 1 and 17) is not affected by age [267]. In this context, an increase of *TLR7* and *TLR8* would be hidden by a more important loss of the whole X chromosome in women and explain why they have an opposite tendency compared to men regarding CNV of those genes.

4.2.2 Is there other X-linked genes involved?

Our findings suggest that somatic mosaicism involves CNVs of X-linked genes, and is different depending on the gender. We have analyzed copy numbers of *TLR7* and *TLR8* based on a sensitive real-time quantitative polymerase chain reaction (Q-PCR) normalized against an autosomal gene of reference, in the β-globin locus on chromosome 11. We have reasons to speculate that the X chromosome is an excellent candidate for such structural variations. For instance, it contains a large amount of repetitive elements (like "long interspersed element-1") compared to autosomes [268], which are known to confer DNA instability with age [269].

Nevertheless, a very important perspective to this study is the choice of at least one other control gene on another autosomal chromosome, and several other X-linked genes, from

different segments of the X chromosome, to have a wider view on structural variations involving this chromosome. Choice of X-linked genes to analyze can include genes with and without immune functions, in order to evaluate the impact of such genomic variations on the immune system and eventually give insights into the importance of the X chromosome in immunity.

4.2.3 DOES MICROCHIMERISM CONTRIBUTE TO *TLR7* AND *TLR8* OVERALL COPY NUMBER INCREASE?

Another explanation for men with X-linked gene copy increase could come from the presence of female microchimeric cells. Maternal cells arising from *in utero* cell trafficking are known to persist in adults [116]. These cells, carrying 2 X chromosomes would contribute to *TLR7* and *TLR8* copy number increase only in XY men (indistinguishable in XX women), and therefore would explain gender difference observed for this phenomenon. Another source of female cells can be from a twin sister. One interesting example illustrating this hypothesis is described in the case of a man in whom we detect female cells derived from a twin sister by fluorescence *in situ* hybridization within peripheral blood mononuclear cells [122].

Nevertheless, it is still unclear whether female chimerism varies with age and/or with hormonal changes. Furthermore, although microchimerism is present in healthy individuals, it is often more important in patients with autoimmune diseases such as RA [85, 86, 117]. It would be interesting to investigate whether feminine microchimerism contributes to TLR7/8 copy increase with time in men, and whether it predisposes them, later with age, to RA.

Such project has been already initiated in our group. Microchimerism is assessed in our laboratory by targeting the non-inherited HLA genotype using HLA-specific quantitative-PCR, as previously described [117]. In the case of maternally-derived microchimerism, the non-inherited maternal antigen (NIMA) is targeted. In the case of fraternally-derived microchimerism, the twin has equal chance to carry either the non-inherited paternal antigen (NIPA) or the NIMA. Only a NIPA-positive result has the ability to distinguish twin-derived from maternal microchimerism (**Figure 19**). Recruitment and HLA-genotyping of case and control subjects with their family members (required for NIMA and NIPA identification) as well as the development of new HLA-specific Q-PCR assays is currently in progress.

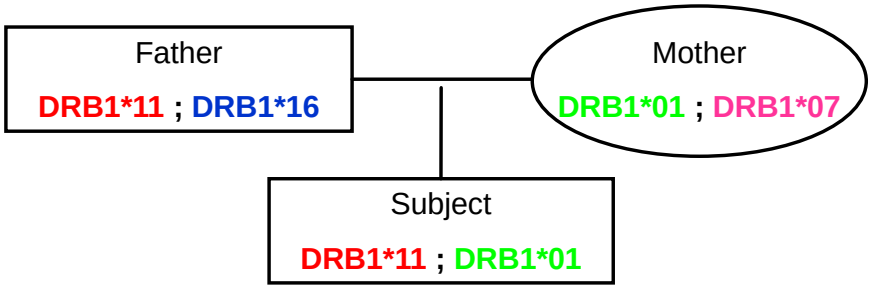

Figure 19. Pedigree diagram showing inheritance of *HLA-DRB1* genotype and possibility of microchimerism assay.
For the subject, non-inherited maternal antigen (NIMA) is the *HLA-DRB1*07* allele and reveals maternal microchimerism when positive in HLA-specific quantitative PCR in subject. Non-inherited paternal antigen (NIPA) is *HLA-DRB1*16* and reveals twin-derived microchimerism when tested.

4.2.4 COMBINATION OF FACTORS WITH GENDER EFFECTS IN AUTOIMMUNITY

Our initial hypothesis has been to investigate *TLR7* and *TLR8* copy number variations in RA, in a parallel manner to the Yaa autoimmunity mouse. This hypothesis is supported by potential role of those TLRs in RA (higher production of TNF-α, IL-6 or type I IFN in synovial tissues [215, 224, 270]). However, our results did not show a difference between patients with RA and healthy controls, possibly due to reduced numbers when matching for age. It is therefore clear that a larger number of age and sex matched subjects is required in order to answer this question. If this hypothesis is true, the observed copy number increase with age of those X-linked genes would contribute to trigger RA in men that are already genetically and environmentally predisposed. This would explain the later onset of RA in men compared to women [14]. Indeed, not only RA differs between the sexes by a later onset in men, but also in term of disease progression. Men have more frequently an erosive form of RA, and more severe rheumatoid nodules and lung disease [271]. Moreover, men need stronger genetic susceptibility to develop RA, as exemplified by considerable higher risk of disease, compared to women, when strong susceptibility *HLA-DRB1*04:01/*04:04* genotype is present [83].

Other predisposing factors are sex hormones, different between men and women. Indeed, it has been recently demonstrated that estrogens influence TLR7-mediated response of plasmacytoid dendritic cells [272]. Such observation supports the view of an earlier onset of

disease in women during their reproductive years. One can wonder whether such phenomenon has an effect on age- and sex-mediated CNVs of TLR7/8 genes and/or on microchimerism. Investigations on the influence of age related changes in sex hormone levels would allow answering this question.

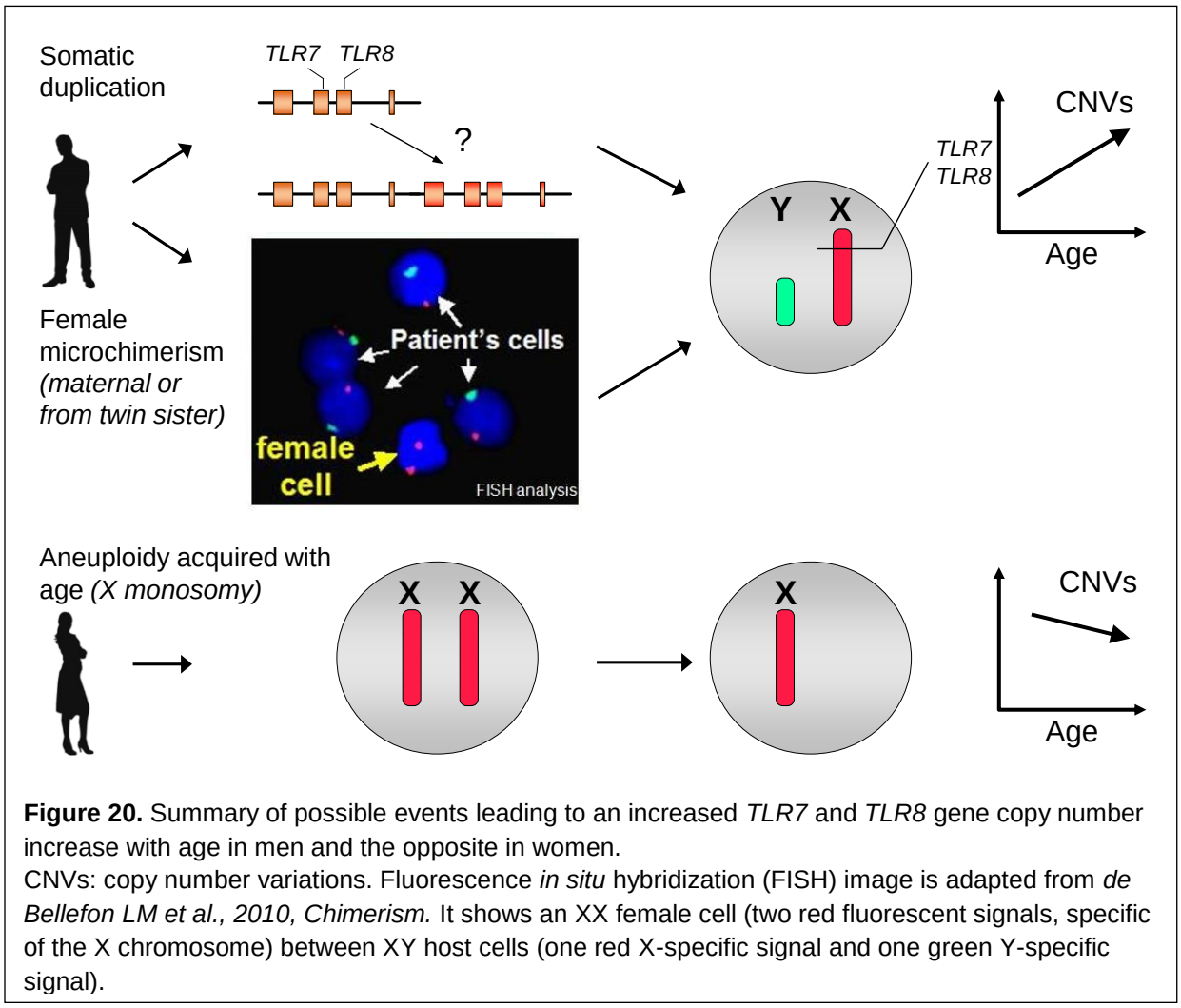

Figure 20. Summary of possible events leading to an increased *TLR7* and *TLR8* gene copy number increase with age in men and the opposite in women.
CNVs: copy number variations. Fluorescence *in situ* hybridization (FISH) image is adapted from *de Bellefon LM et al., 2010, Chimerism.* It shows an XX female cell (two red fluorescent signals, specific of the X chromosome) between XY host cells (one red X-specific signal and one green Y-specific signal).

CHAPTER 4.3 – LOSS OF X-INACTIVATION MOSAICISM AND THEIR ROLE IN AUTOIMMUNITY

Loss of mosaicism hypothesis states that disturbances in random X chromosome inactivation (XCI) may lead to autoimmunity. This hypothesis is supported by findings showing a higher prevalence of skewed X chromosome inactivation in females with many autoimmune diseases, including autoimmune thyroid diseases, scleroderma, RA, and juvenile idiopathic arthritis [172-178].

Non-random XCI can be explained by several mechanisms. The first is normal random X inactivation. Indeed, tissues of healthy women usually have 50:50 ratio of cells expression either the maternally derived or paternally derived X chromosome. But in some cases, the inactivation pattern can be skewed (≤ 20:80 or ≥ 80:20) purely by chance, when the choice of which parentally-derived X chromosome to inactivates happens at the blastocyst stage, on a then small number of embryonic cells.

One more way of losing X chromosome mosaicism is random clonal loss, gradually acquired with age. For example, a skewed pattern is more often observed in peripheral blood of elderly [171, 273]. This would be reflection of a change in their stem cell usage, with gradual loss of some of the original members of stem cell lineages, counterbalanced by the expansion of others, thus maintaining hematopoiesis [273].

Another mechanism leading to unbalanced XCI involves a genetic alteration or a mutation on one of the X chromosomes that affects cell survival, conferring a growth disadvantage for cells with an active X chromosome harboring this alteration and resulting in overrepresentation of cells with that chromosome silenced. Therefore, a highly skewed inactivation ratio may be indicative of carrier status for many X-chromosome disorders, such as X-linked Mendelian diseases or balanced X-chromosome/autosome translocations [251, 274].

A fourth mechanism could be a defect in the X-inactivation itself. For example, a mutation has been reported in the *XIST* promoter region [275]. Although the direct effect of this mutation is not clear, it has resulted in a skewed pattern of XCI in concerned females, with a preferential inactivation of the X carrying the mutation [275].

Skewing of XCI is often tissue specific [169, 276]. In the case of X-linked mutations, it can occur in the tissues sensitive for the mutant gene. This depends on the role of the gene in survival, proliferation and/or differentiation of the various cell lineages. Therefore, immune cells with a functional advantage may become overrepresented in the bone marrow, blood or other organs involved in immune responses. An example can be found in the Wiskott-Aldrich syndrome (a life-threatening X-linked immunodeficiency, characterized by thrombocytopenia in affected men, but no clinical abnormalities in carrier females). Hematopoietic precursor cells in female carriers are subject to selection against the mutated gene, resulting in loss of X-linked mosaicism and preferential expression of the normal allele in T cells, B cells and granulocytes [277].

When skewing occurs among immune cells, Stewart has proposed that it may lead to autoimmunity [278]. In his model, women would host a large population of cells expressing one X chromosome and a smaller population expressing the other X chromosome. Cells from the smaller population would fail to be sufficiently represented in the thymus and some auto-reactive lymphocytes would escape negative selection. Nevertheless, this hypothesis remains to be confirmed as no evidence of autoimmunity directed against X-encoded antigens has been yet reported. A direct link between skewing of X chromosome inactivation and autoimmune disease in women remains elusive.

In this work, we show that skewing of X chromosome inactivation is more frequent in peripheral blood cells of females with RA and SSc, compared to healthy controls. These findings agree with previously published data in both diseases. Moreover, this skewing is strongly associated with *HLA-DRB1* susceptibility alleles carrying the shared epitope (SE) among women with RA but not among healthy women or women with SSc.

The SE is the main genetic susceptibility marker for RA. Also, it is often considered a marker of disease severity, cartilage erosion and bone destruction [83, 279, 280]. In such context, a skewed pattern would occur as a result of a higher clonal selection caused by chronic inflammation.

We also report that skewing is already established at the time women are diagnosed with RA. This indicates that this inflammatory-mediated clonal selection may already have begun before the establishment of a diagnosable RA, in a way similar to the occurrence of the characteristic anti-citrullinated protein antibodies years before RA manifestation [17].

Our results give rise to a new model in which a genetic background conferring more severe form of disease and chronic inflammation would contribute to the loss of mosaicism in X chromosome inactivation (**Figure 21**).

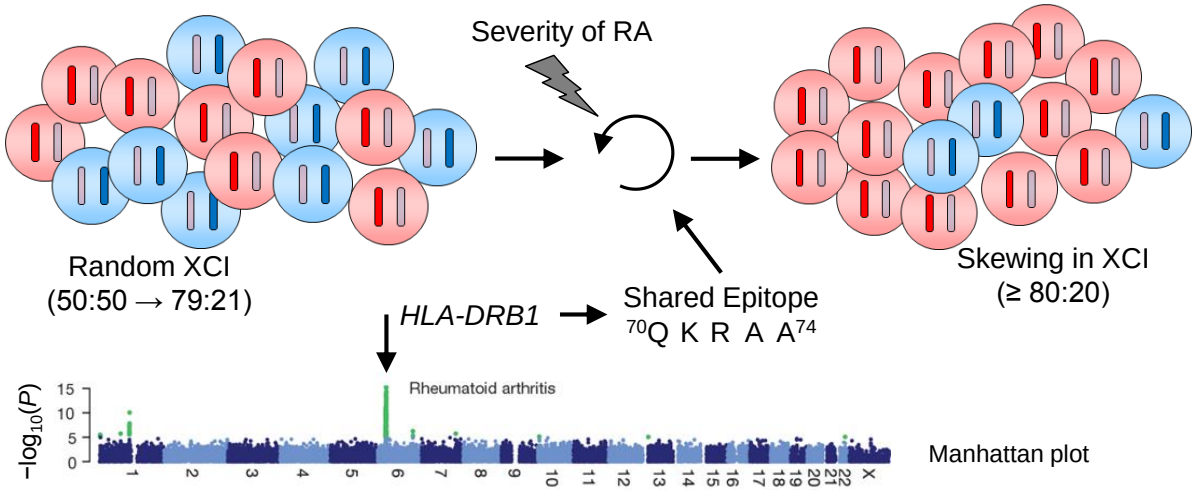

Figure 21. A proposed model where genetic background confers susceptibility (in this case the shared epitope) and chronic inflammation in autoimmune disease (in this case rheumatoid arthritis; RA), contributing to the loss of X chromosome inactivation (XCI) mosaicism.
The Manhattan plot is retrieved from *Wellcome Trust Case Control Consortium, 2007, Nature,* and indicates susceptibility loci. The lowest *P* value (highest $-\log_{10}(P)$) corresponds to the HLA locus on chromosome 6.

4.3.1 DOES SEVERITY AFFECT SKEWING IN OTHER AUTOIMMUNE CONDITIONS?

After we showed the influence of the shared epitope in RA, next step is to check whether such pattern is followed in the other autoimmune diseases where skewing is prevalent. No association between different parameters of SSc and skewing are detected in our group of women with SSc. However, SSc is a complex and heterogeneous disease divided into two diseases according to clinical and serological factors, with different HLA susceptibility genes.

Nevertheless, our sample of SSc females being too small for fine analysis, we have added (with permission) a previously described SSc cohort from Seattle [177] and have confirmed our analysis.

4.3.2 IS THERE A PARTICULAR TYPE OF CELLS MORE CONCERNED WITH SKEWING?

So, skewing would be a reflection of clonal selection of immune cells. It is interesting to investigate whether a particular cell subset would be more affected by this clonal selection. Interestingly, a study in scleroderma shows that purified T cells (CD3+), B cells (CD19+), monocytes (CD14+), plasmacytoid dendritic cells (BDCA+), and myeloid dendritic cells (CD1c+) have patterns of inactivation similar to those observed in peripheral blood mononuclear cells, suggesting that skewing takes place at the hematopoietic precursor level [178].

4.3.3 DOES SKEWING HAPPEN BEFORE THE ONSET OF SYMPTOMS?

Another perspective is to analyze X chromosome inactivation patterns in a large cohort of females with no history of autoimmunity at the time of blood draw, and to follow them up in order to test the hypothesis whether skewing is predictive of autoimmunity or rather a consequence of inflammation. All women should be HLA-genotyped. Such study would investigate the possibility of skewing occurring before disease symptoms and give insights into the relationship between the SE and skewing. However, its cost would be important as in the general population only 2–3% of women have a skewed XCI profile. Therefore, in order to obtain enough results for statistical analyses, at least 1000 women would need to be followed up, HLA-typed, and analyzed for their XCI patterns.

4.3.4 IS SKEWING ASSOCIATED WITH A SHORTENING IN TELOMERE LENGTH?

Parallel to our findings of the relationship between higher skewing and the SE, two studies showed a correlation between loss of telomeres and the presence of the SE [252, 253]. Telomeres consist in the TTAGGG DNA repeat sequence found at the end of chromosomes. They have an essential role in preventing inappropriate DNA repairs and end-to-end fusion by turning the last part of DNA sequence of a chromosome into a folded loop. Telomeres are eroded with each cell division by 30 to 200 base pairs [281]. Many cell types, such as stem

cells or (pathologically) cancer cells, have an active mechanism of telomere extension, notably the enzyme telomerase. Nevertheless, telomeric shortening is responsible of limiting the life duration of a cell clone in human somatic tissues, and therefore is considered a biomarker of biological age.

The SE-positive HLA alleles were reported to be associated with T cell immunosenescence characterized by telomere erosion in patients with RA [252]. Although we have established a link between the SE and skewed XCI, we have not demonstrated that such link can exist between XCI patterns and telomere erosion, as the two events seem to change with time in an independent manner.

CHAPTER 4.4 – WHEN X CHROMOSOME CONFERS ADVANTAGES THAT COULD TURN OUT AS DISADVANTAGES

It has been long recognized that women live longer and healthier than men. Their immune system is stronger against infection. Already from birth, male newborns are more prone to septicemia, meningitis and tuberculosis (up until adulthood) [282, 283]. The X chromosome carries many genes which are involved in immune functions. The presence of two X chromosomes in females confers more allelic diversity and better chances to survive deleterious gene mutations and to face new immunological challenges, compared to males. Nevertheless, their stronger immune system seems to be more aggressive and thus the prevalence of most autoimmune diseases is higher among them. It is widely accepted that the X chromosome contributes in the hyperresponsiveness of their immune system [149].

Characteristics that make the X chromosome unique, compared to autosomes, are numerous. It has a highly conserved genetic content in mammals [146], indicating that there is a barrier to the exchange of genes between the X chromosome and the autosomes. It contains 2-times more long interspersed element (LINE)-1 retrotransposable repetitive sequences [268]. It undergoes epigenetic silencing like no other autosome, affecting up to 85% of its 1100 genes [150]. Organisms can tolerate aneuploidies affecting the X chromosome[G] much better than autosomes, and Klinefelter's syndrome (47,XXY) is the most common human aneuploidy condition [284]. Age-mediated somatic mosaicism involving chromosomal aneuploidy affects sex chromosomes significantly more often than autosomes, and the most frequent of such aneuploidy corresponds to the X chromosome monosomy in females [267].

The pathogenesis of autoimmune diseases has been associated with many of the X chromosome's features mentioned above. On one hand, the mouse Yaa phenotype, caused by a duplication of Tlr7 [191, 195] provides an excellent example of X-linked gene

[G] And affecting the Y chromosome as well. In fact humans are much more able to tolerate sex chromosome than autosome aneuploidies. Aneuploidies involving the Y chromosome have much less effect on humans, mainly because of its relatively low number of functional genes, not to mention that exactly half the human population live quite happily without it.

dysregulation leading to autoimmunity. On the other hand in humans, men with Klinefelter's syndrome have 14 times higher risk of SLE than men with normal karyotype [285]. Evidence for a relatively strong association of Klinnefelter's syndrome with other rheumatic autoimmune diseases including RA, Juvenile Idiopathic Arthritis and SSc is implied by the numerous case reports published in the last decades [10]. Women with SSc, primary biliary cirrhosis and autoimmune thyroid disease is reported with higher X-chromosome monosomy in their blood cells compared to age-matched controls [11]. Loss of X chromosome inactivation mosaicism is linked to autoimmune thyroid diseases, scleroderma, RA, and juvenile idiopathic arthritis [172-178].

Genome-wide association studies have identified few disease-associated genes located on the X chromosome and responsible for autoimmunity (such as *TIMP1* and *IRAK1*). However, it is unlikely that mutations on X-linked genes would be responsible for higher susceptibility of females to autoimmunity. If this was the case, roles would be reversed and we would expect (just like in X-linked primary immunodeficiencies) a higher prevalence of autoimmune disease among males that only have one X chromosome. In such context, we propose to look for disease-associated polymorphisms on the X chromosome, which is often neglected in genome-wide association studies, exclusively in male samples. Men rarely have autoimmune disease, but often with a more severe form [101]. It would then be interesting to see whether X-linked polymorphisms contribute to their predisposition.

In this work, we have presented additional elements that highlight the importance of the X chromosome in the development of autoimmunity. Two X-linked genes have been found to vary in their copy numbers according to gender and age. Loss of X-inactivation mosaicism is frequent in autoimmunity and, in some cases, influenced by genetic susceptibility. However, complete mechanistic explanations are still lacking. We still have ahead of us a lot to learn from this fascinating chromosome, which did not reveal all of its facets. It is clear that the X chromosome can have great influence in immunity, and that it can confer advantage and disadvantage to either sex depending on the immune context. A better understanding will certainly give clues on female predominance in autoimmune disorders, but also on their perspective in immunity and life expectancy.

PART 5 – *BIBLIOGRAPHY*

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APPENDIX: LIST OF SELECTED PUBLICATIONS

Appearing in the RESULTS section:

de Bellefon LM, Heiman P, **Kanaan SB**, Azzouz DF, Rak JM, Martin M, Roudier J, Roufosse F, Lambert NC. *Cells from a vanished twin as a source of microchimerism 40 years later.* Chimerism, 2010. 1(2): p. 56-60.

Kanaan SB, Onat OE, Balandraud N, Azzouz DF, Roudier J, Ozcelik T, Lambert NC, *Does telomere shortening in women with rheumatoid arthritis predict X chromosome inactivation bias?,* Ann Rheum Dis 2013;72(Suppl 1):A1–A88.

Other selected publications

Picard C, Di Cristofaro J, Azzouz DF, **Kanaan SB**, Roudier J, Lambert NC. *Analyzing HLA-G polymorphisms in children from women with scleroderma*. Hum Immunol. 2013 Apr;**74**(4):468-72.

Azzouz DF, Rak JM, Fajardy I, Allanore Y, Tiev KP, Farge-Bancel D, Martin M, **Kanaan SB**, Pagni PP, Hachulla E, Harle JR, Didelot R, Granel B, Cabane J, Roudier J, Lambert NC. Comparing HLA *Shared Epitopes in French Caucasian Patients with Scleroderma*. PLoS One 2012;7(5):e36870.

Kanaan SB, Azzouz DF, Karlmark KR, Francophone Group of Scleroderma Research, Rak JM, Roudier J and Lambert NC, *Do men with Scleroderma or men with Rheumatoid Arthritis have a greater magnitude of maternal and/or fraternal microchimerism than women with similar diseases?,* Chimerism 4:2 Supplement, 37–59; April/May/June 2013.

Azzouz DF, Balandraud N, **Kanaan SB**, Auger I, Martin M, Arnoux F, Roudier J, Lambert NC. *Allograft inflammatory factor 1 (aif1) polymorphisms in french caucasians with rheumatoid arthritis.* Ann Rheum Dis 2013;72(Suppl 1):A1–A88.

Azzouz DF, Di Cristofaro J, **Kanaan SB**, Cabane J, Farge D, Allanore Y, Hachulla E, Harlé JR, Granel B, Martin M, Roudier J, Picard C, Lambert NC. *HLA-G a putative susceptibility gene in scleroderma, but only in women*. Ann Rheum Dis A58 2012;71(Suppl 1):A1–A93.

Azzouz DF, Onat OE, Balandraud N, **Kanaan SB**, Roudier J, Ozcelik T, Lambert NC, *Skewed X chromosome inactivation in rheumatoid arthritis women.* Ann Rheum Dis, 2011. 70(Suppl 2): p. A88.

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Analyzing HLA-G polymorphisms in children from women with scleroderma

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ABSTRACT

Embryos during pregnancy and organs during transplantation, express high levels of soluble HLA-G (sHLA-G) molecules for successful implantation and protection against maternal immune cells or recipient's cells. We and others have shown that women with scleroderma (SSc) carry cells/DNA arising from pregnancy, so-called fetal microchimerism (Mc) more often and in higher quantities than healthy women decades after delivery.

We hypothesized that high levels of fetal Mc were the consequence of a fetus with a high sHLA-G profile, therefore that children from women with SSc would have this profile more often than children from healthy women.

High sHLA-G secretor profile is influenced by at least two variations in the HLA-G 3′ untranslated region (UTR): a 14bp deletion in exon 8 and the presence of cysteine (C) in position +3142 and by one variation in the 5′ Upstream Regulatory Region (URR) at position −725.

By a previously developed three-step multiplex PCR SNaPshot method, we evaluated 16 HLA-G polymorphisms in DNA samples from the first-born children of 39 women with SSc and 32 healthy women.

Contrary to expectations, children from women with SSc did not have a high sHLA-G profile, but rather the opposite. We discuss possible reasons for this result and future orientations for HLA-G studies in SSc.

1. Introduction

To ensure successful embryo implantation, extravillous trophoblasts, which are in close contact with maternal tissue, express HLA-G molecules, encoded by the non-classical HLA-G gene [1,2]. This presence prevents them from being attacked by maternal uterine natural killer (NK) cells and is essential for trophoblast invasion during placentation [3]. Conversely, a low secretion of soluble HLA-G (sHLA-G) is predictive of a low implantation rate and higher miscarriage risks after in-vitro fertilization [4,5].

Studies in the transplantation field have also shown that the expression of HLA-G in serum and allografts is associated with allograft acceptance (for review [2]).

We hypothesized that a particular HLA-G profile would favor or not the passage of graft cells into the host (fetal cells in the mother, donor cells in the recipient). Since a high secretor profile fetus during pregnancy allows a stronger implantation, we further hypothesized that it would also trigger higher trafficking of fetal cells during pregnancy.

Indeed during pregnancy, the placenta is not the hermetical barrier it is often thought to be. A feto-maternal traffic of cells appears as early on as the 5th week of pregnancy. This bi-directional exchange leaves a residual chimerism in respective hosts, decades after delivery, known as fetal and maternal microchimerism (Mc).

Interestingly, we, and other groups, have shown that women with systemic sclerosis (SSc) carry persistent fetal Mc more often and in higher quantities (for review [6]). Long-term persistence of fetal Mc has been suggested as a possible contribution to the pathogenesis of autoimmune diseases and in particular SSc, partly explaining female predominance in such diseases [7]. Increased levels of persisting fetal Mc in the host (patient) is probably due to increased passage during pregnancy.

Based on previous observations, we therefore hypothesized that children from women with SSc would have a different HLA-G profile than children from healthy women.

Several HLA-G polymorphisms have been reported to influence the expression of HLA-G transcripts and protein in the 5′ Upstream Regulatory Region (URR) and in the 3′ untranslated region (UTR). Regarding the 5′ URR, it is mainly a Guanine Single Nucleotide polymorphism (SNP) at position −725 that results in a relatively

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higher level of HLA-G expression [8]. In the 3′UTR, several studies show that a 14bp insertion/deletion triggers respectively reduced/increased levels of HLA-G mRNA and soluble HLA-G protein in serum [9–13] while a G/A SNP at position 3172 in exon 8 has been reported to increase the affinity of specific microRNAs to the HLA-G mRNA and consequently decrease HLA-G expression [14].

In the current study, we propose to investigate the HLA-G genotyping status of children from women with SSc and compare it with children from healthy women.

2. Patients and methods

2.1. Subjects

We arbitrarily selected the first-born child from 39 women with SSc and 32 healthy women. Patients were enrolled in collaboration with seven French hospitals from Paris, Marseille and Lille and fulfilled the criteria of LeRoy [15] for SSc. Healthy women and women with SSc were matched for age and ethnicity. Their mean age was, respectively 53 and 54 years old and they were, respectively 95% and 97% Caucasian.

The earliest onset of disease in women with SSc was after the birth of their first child. Children's ages ranged from 5 to 35 years (mean 21 years) for healthy women and from 13 to 57 years (mean: 31 years) for women with SSc. The women with SSc included 23 women with limited cutaneous SSc (lc-SSc) and 16 with diffuse cutaneous SSc (dc-SSc). Among women with lc-SSc, 18 had anti-centromere antibodies (ACA), 1 had anti-topoisomerase antibodies (ATA) and 4 had neither type. Among women with dc-SSc, 10 had ATA and 6 were negative for ATA and ACA.

Children signed informed consent forms as did family members according to Biomedical Research Protocol number 204–060.

2.2. DNA isolation from buccal cells

Children sent us mouthwashes (Eludril®, a generous gift from Fabre laboratory, France) from which DNA was subsequently extracted with a High Pure PCR Template Preparation Kit (Roche) and used for HLA-G polymorphism evaluations.

2.3. Multiplex PCR amplification and probe primer extension SNaP shot reaction

A previously described three-step method was developed to simultaneously analyze 16 SNPs (Table 1) defining 8 HLA*G alleles and 4 SNPs in the 5′ URR and 3′UTR regions [16,17]. The first step consisted in a multiplex PCR using primers flanking several SNPs. Five genomic fragments ranging from 83 to 940 bp were generated in a single reaction. Three fragments encompassed two target SNPs, one fragment encompassed 4 SNPs and one fragment encompassed 6 SNPs (Table 2). Multiplex PCR amplification was performed from 100ng of genomic DNA in a final volume of 25 µl containing 12.5 µl of Master Mix (Mutliplex PCR kit, Qiagen) and a defined concentration of each primer (for final concentration see Table 2). Amplification was carried out as follows: 95 °C for 15 min for 1 cycle; 95 °C for 30 s, 62,4 °C for 90 s, 72 °C for 60 s for 30 cycles; 72 °C for 7 min for 1 cycle. Multiplex PCR amplicons were controlled on 3% (w/v) agarose gel before treatment with Exonuclease-I/Shrimp Alkaline Phosphatase master mix containing 10 U/µl of Exo and 0.5 U/µl of SAP (Euromedex, Souffelweyersheim, France) to remove unincorporated primers and dNTPs. PCR product (10 µl) was incubated with 3.3 µl of Exo/SAP master mixture for 1 h at 37 °C followed by 15 min at 80 °C for enzyme inactivation. The second step consisted of a multiplex single-base primer extension reaction using the SNapShot kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. The final reaction volume was 10 µl containing 3 µl of the treated first-step PCR reaction, 5 µl of SNap Shot ready reaction premix containing fluorescent ddNTPs (*A* = dR6G, green; *C* = dTAMRA, black; *G* = dR110, blue; *T* = dROX, red), and probe primers (for final concentration see Supplementary Table S1). The reaction was carried out as follows: 95 °C for 10 sec, 50 °C for 10 sec, 60 °C for 30 sec, for 25 cycles. An aliquot of SNap Shot extension reaction (10 µl) was then treated with 1 unit of SAP for 1 h at 37 °C followed by 15 min at 80 °C for enzyme inactivation, before the third step of capillary electrophoresis.

Table 1
SNPs analyzed in the genotyping assay.

SNP	Region	Allele 1	Allele 2	Allele 3	Position
−725–CTG	5′URR	C-wt	G-mt	T-mt	5′URR
−716–GGC	5′URR	G-wt	T-mt		5′URR
−201–GTT	5′URR	G-wt	A-mt		5′URR
−56–CCT	5′URR	C-wt	T-mt		5′URR
13–TCC (Ser → Phe)	2	C-wt	T-mt		110
31–ACG(Thr → Ser)	2	A-wt	T-mt		163
54–CAG (Gln → Arg)	2	A-wt	G-mt		233
110–CTC (Ile → Leu)	3	C-wt	A-mt		400
130–CTG (Leu → Frameshift)	3	C-wt	del-T		460
159–TAC (Tyr → His)	3	T-wt	C-mt		547
219–CGG (Arg → Trp)	4	C-wt	T-mt		727
258–ACG (Thr → Met)	4	C-wt	T-mt		845
Insertion 14 pb	3′UTR	T-del	A-ins		3′UTR
3142–GCA	3′UTR	G-wt	C-mt		3′UTR
3187–ATA	3′UTR	A-wt	G-mt		3′UTR
3196–CTT	3′UTR	C-wt	G-mt		3′UTR

URR, Upstream Regulatory Region; UTR, Untranslated Region; wt, wild type; mt, mutant.

2.4. PCR and probe primer design

All primers were designed using the ≪Primer 3≫ program (http://frodo.wi.mit.edu/primer3/). The ≪Integrated DNA technology≫ program (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx) was used to test for potential primer-dimer conflicts and secondary hairpin structures. Multiplex PCR primers were selected according to following three criteria: (i) similar Tm value, (ii) calculated ΔG between 0 and −10 kcal per mol and (iii) PCR products length less than 1000 bp. Forward and reverse primers used in the 5-pair multiplex reaction for HLA* G gene amplification are shown in Table 2. Nearby SNPs were co-amplified in the same PCR fragment. Primers used as probes were designed to anneal immediately adjacent to the SNP site on either the sense or anti-sense DNA strand. Probe primer sequences were designed not to interact with each other ($\Delta G > -10$ kcal) and their lengths differ by at least 5 bases by addition of a polyT tail to the 5′-end to distinguish them from each other by capillary electrophoresis. Forward and reverse extension primers used in the multiplex extension reaction are shown in Table 3. All probe primers were synthesized by Eurogentec (Seraing Belgium) and purified by HPLC to remove incomplete synthesis products.

2.5. Capillary electrophoresis conditions

The fluorescence and size of extended products were determined by capillary electrophoresis on an ABI PRISM 3130XL genetic analyzer (Applied Biosystem) using POP-7 polymer and a 36-cm length capillary. Prior to loading onto the genetic analyzer, an aliquot of treated SNap Shot multiplex extension reaction (0.5 µl) was mixed with 9.25 µl of deionized formamide (Applied Biosystems) and 0.25 µl of size standard (GeneScan-120 LIZ ladder, Applied Bio-

Table 2
Forward and reverse primers used in the 5-pair multiplex reaction for HLA*G.

Region (SNPs)		Primer sequence (5′ > 3′)	Final C° (μM)	Amplicon size
5′URR (−725, −716)	F:	TGGGAAAGTGAAACTTAAGAGC	0.2	83 pb
	R:	ATGTGACTTTGGCCCGTTG	0.2	
5′URR (−201, −56)	F:	ACAAGAGTAGCGGGGTCAG	0.2	250 pb
	R:	TGAGCGAGGACTTTAGAACCA	0.2	
Ex 2 et 3 (13, 31, 54, 110, 130, 159)	F:	GGGTCGGGCGGGTCTCAA	0.3	940 pb
	R:	GGATTCCTCTCCCTCAGGAC	0.3	
Ex 4 (219 et 258)	F:	CCATGAGAGATGCAAAGTGCT	0.3	360 pb
	R:	TGCTTTCCCTAACAGACATGAT	0.3	
Ex 8(ins/del, +3142, +3187 and +3196)	F:	TGTGAAACAGCTGCCCTGTGT	0.6	358 pb
	R:	GTCTTCCATTTATTTTGTCTCT	0.6	

C, concentration; F, forward primer; R, reverse primer.

Table 3
Allele frequencies and genotype distribution of the HLA-G 14 bp Ins/Del gene polymorphism in first-born children from women with SSc compared with first-born children from healthy women.

	Children from SSc women		Children from healthy women	
	n	%	*n*	%
Allele[a]				
Ins	34	43.6	22	35.5
Del	44	56.4	40	64.5
Total	78		62	
Genotype[b]				
Ins/Ins	8	20.5	6	18.2
Del/Del	13	33.3	15	45.5
Ins/Del	18	46.2	10	30.3
Total	39		31	

[a] Alleles, $p = 0.33$, χ^2 test.
[b] Genotypes, $p = 0.4$, χ^2 test.

system). The sample was injected in 16 s at 1.2 kV and electrophoresis lasted 20 min at 15 kV and 5 μA at 60 °C. The laser was set at a constant power of 9.9 mW.

2.6. Data interpretation

Data were analyzed using GeneMapper 4.0 with specific detection parameters (see Supplementary Table S2). Using an in-house computer program, output files (.txt) exported from GeneMapper 4.0 were automatically formatted into files readable by the 'Phenotype' application of the Gene (Rate) computer tool package (http://geneva.unige.ch/generate) to determine the coding allele. Phenotype uses two input files (i.e. a kit description file -see Supplementary Table S3- and a reaction data file) [18]. To determine whether a genotype distribution or an allele frequency was significantly higher in children from women with SSc than children from healthy women, *p* values were calculated using the x^2 test.

3. Results

3.1. The 14-bp Ins/Del polymorphism

The 14-bp Ins/Del polymorphism has been the most studied HLA-G 3′UTR polymorphism and a deletion would favour mRNA stability and protein production [9,11]. We hypothesized that children from women with SSc would have this deletion corresponding to a high secretor sHLA-G profile more often compared to children from healthy women. Contrary to expectations, neither the HLA-G 14 bp allele frequencies nor the HLA-G 14 bp genotype distribution differed between the two groups of children ($p = 0.3$ and $p = 0.4$, respectively, Table 3). The polymorphism was in Hardy-Weinberg equilibrium ($p > 0.05$) in both groups of children (data not shown).

Not only was our hypothesis not confirmed, but a tendency towards the opposite result was observed, as the 14 bp Ins allele (low secretor profile) was present in 43.6% of children from women with SSc compared to 35.5% of children from healthy women. Furthermore, only 33.3% of children whose mother had SSc, were homozygous for the 14 bp deletion (high secretor profile) compared to 45.5% of children whose mother was healthy, although the difference was not significant ($p = 0.2$ when comparing Del/Del between the two groups, Table 3).

Therefore, on the contrary to what we initially thought, children from women with SSc may have a low secretor associated HLA-G polymorphism such as observed in children from pre-eclamptic women and this particular genetic factor may, through an as yet unknown mechanism, trigger higher fetal Mc transfer.

3.2. Other polymorphisms

However, the role of the 14-bp Ins/Del polymorphism in the magnitude of HLA-G expression is still not yet fully defined and other polymorphisms could influence HLA-G mRNA stability and protein production.

The presence of cysteine (C) at position +3142 of HLA-G 3′UTR, or the presence of guanine (G) at position −725 of HLA-G 5′URR result in a relatively higher level of HLA-G expression when compared with a G for the former position and a C or a T in the latter position [8]. For both polymorphisms, children from women with SSc had a tendency to carry the low secretor associated polymorphisms: the G allele at position +3142 and the C or T allele at position −725, (respectively, $p = 0.4$, Table 4 and $p = 0.17$, Table 5) without however reaching significance. We did not find differences between the two groups of children for any of the other polymorphisms (see Supplementary Tables S4 and S5).

3.3. Discussion

In conclusion, contrary to our initial hypothesis, children from women with SSc did not carry HLA-G polymorphisms involved with high sHLA-G secretion more often than children from healthy women. And this, despite higher fetal Mc quantities and frequencies previously described in this particular cohort of women with SSc.

A tendency to the opposite result, a low secretor profile, although without reaching significance, was even noticeable in children from women with SSc. Interestingly, the HLA-G 14 bp Del polymorphism associated with a low secretor profile has been observed in women with pregnancy complications including pre-

Table 4
Allele frequencies and genotype distribution of the HLA-G +3142 (3′UTR) polymorphism in first-born children from women with SSc compared with first-born children from healthy women.

+3142 SNP	Children from SSc women		Children from Healthy women	
	n	%	*n*	%
Allele[a]				
C	35	44.9	32	51.6
G	43	55.1	30	48.4
Total	78		62	
Genotype[b]				
C/C	9	23.1	8	25.8
G/C	17	43.6	16	51.6
G/G	13	33.3	7	22.6
Total	39		31	

[a] Alleles, $p = 0.4$, χ^2 test.
[b] Genotypes, $p = 0.6$, χ^2 test.

Table 5
Allele frequencies and genotype distribution of the HLA-G −725 (5′URR) polymorphism in first-born children from women with SSc compared with first-born children from healthy women.

	Children from SSc women		Children from healthy women	
−725 SNP	n	%	n	%
Allele[a]				
G	5	6.4	3	4.8
C	69	88.5	59	95.1
T	4	5.1	0	0.0
Total	78		*62*	
Genotype[b]				
G/C	5	12.8	3	9.7
C/C	30	76.9	28	90.3
C/T	4	10.3	0	0.0
Total	39		31	

[a] Alleles, $p = 0.17$, χ^2 test.
[b] Genotypes, $p = 0.16$, χ^2 test.

eclampsia in some studies, but not in all (for review [19]). Significantly reduced levels of sHLA-G have been reported in maternal serum and in the placenta in pre-eclampsia when compared with controls [20]. Moreover, from the fetal point of view, few studies agree with the consensus that the fetal Ins/Ins HLA-G genotype is not significantly associated with pre-eclampsia in the mother, but rather that the Del/Del genotype is less observed in pre-eclamptic placenta [10,21,22]. This is very similar to what we observed in children from women with SSc.

Pre-eclampsia is characterized by reduced trophoblast invasion and consequently poor placentation. Interestingly, a recent case control study has shown an increased incidence of pregnancy complications, including hypertension, pre-eclampsia, miscarriage and intra-uterine growth restriction, in women who later develop scleroderma [23]. Moreover pregnancy complications lead to increased trafficking of fetal cells [24] and we and others have described fetal microchimerism (Mc), a sequel of pregnancy, as a possible player in the increased incidence of SSc [6].

Otherwise, several reasons can explain why our initial hypothesis has not been validated and direct future HLA-G studies in SSc:

A possible explanation could be due to the arbitrary choice of the first-born child. An interesting analysis would be to test fetal Mc from all the children in women with SSc and healthy women and test whether persistent sources were from children with a high secretor genetic profile.

Another explanation could be that the number of children in both groups is still not sufficient and the significance of our data in children from women with SSc would benefit from larger numbers. Nevertheless, this first pilot study, although small, should initiate further analyses in the field.

A third and not necessarily exclusive explanation is that the initial hypothesis is incorrect. Having a high secretor profile does not allow more fetal-to-mother chimerism traffic. Along these lines, a recent study looking for genetic factors in HIV vertical transmission in Brazilian children indicates that the presence of Del/Del (high secretor) genotype is associated with a protective effect against HIV perinatal infection in exposed children [25]. In other words, high HLA-G secretor children would be less permissive to mother-child transmission. On the contrary, children from women with SSc who have a tendency for a genetic background corresponding to a low secretor profile would be more permissive to vertical transmission, including maternal cell transfer. Moreover, one could imagine that if they have a tendency for such a 'low secretor' profile, this is due to a maternal inheritance. It would in this case be extremely interesting to test whether women with SSc have a 14 bp Ins HLA-G polymorphism more often, as suggested by pregnancy complications in SSc women. This would explain a permissive phenotype for feto-maternal trafficking of cells in women with SSc. We are aware that further studies are needed to fully understand genetic factors predisposing feto-maternal transmission. Furthermore a limitation of this study is the absence of protein quantification as plasma samples from children were not available. Nevertheless, our pilot study raises questions never asked before and initiates new tracks to be followed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2012.11.030.

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Comparing HLA Shared Epitopes in French Caucasian Patients with Scleroderma

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Abstract

Although many studies have analyzed HLA allele frequencies in several ethnic groups in patients with scleroderma (SSc), none has been done in French Caucasian patients and none has evaluated which one of the common amino acid sequences, ^{67}FLEDR^{71}, shared by HLA-DRB susceptibility alleles, or ^{71}TRAELDT^{77}, shared by HLA-DQB1 susceptibility alleles in SSc, was the most important to develop the disease. HLA-DRB and DQB typing was performed for a total of 468 healthy controls and 282 patients with SSc allowing FLEDR and TRAELDT analyses. Results were stratified according to patient's clinical subtypes and autoantibody status. Moreover, standardized HLA-DRß1 and DRß5 reverse transcriptase Taqman PCR assays were developed to quantify ß1 and ß5 mRNA in 20 subjects with HLA-DRB1*15 and/or DRB1*11 haplotypes. FLEDR motif is highly associated with diffuse SSc ($\chi^2 = 28.4$, $p < 10-6$) and with anti-topoisomerase antibody (ATA) production ($\chi^2 = 43.9$, $p < 10-9$) whereas TRAELDT association is weaker in both subgroups ($\chi^2 = 7.2$, $p = 0.027$ and $\chi^2 = 14.6$, $p = 0.0007$ respectively). Moreover, FLEDR motif- association among patients with diffuse SSc remains significant only in ATA subgroup. The risk to develop ATA positive SSc is higher with double dose FLEDR than single dose with respectively, adjusted standardised residuals of 5.1 and 2.6. The increase in FLEDR motif is mostly due to the higher frequency of HLA-DRB1*11 and DRB1*15 haplotypes. Furthermore, FLEDR is always carried by the most abundantly expressed ß chain: ß1 in HLA DRB1*11 haplotypes and ß5 in HLA-DRB1*15 haplotypes. In French Caucasian patients with SSc, FLEDR is the main presenting motif influencing ATA production in dcSSc. These results open a new field of potential therapeutic applications to interact with the FLEDR peptide binding groove and prevent ATA production, a hallmark of severity in SSc.

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Introduction

Systemic Sclerosis (SSc) or Scleroderma, is a chronic autoimmune disease with unknown aetiology, characterized by fibrosis, vascular alterations and autoantibodies. Scleroderma is stratified by clinical criteria into two subtypes: limited cutaneous SSc (lcSSc) mainly affecting the hands, arms and face, and diffuse cutaneous scleroderma (dcSSc), affecting a large area of the skin, at increased risk of cardiac disease, interstitial lung disease, renal crisis and early death. Specific autoantibodies correlate with subtypes. Anti-centromere antibodies (ACA) and anti-topoisomerase antibodies (ATA) are respectively a hallmark of lcSSc and dcSSc, although not always detected and sometimes observed in each other group. Other autoantibodies, such as anti-RNA polymerase, antifibrilllin (AFA) or anti-U3RNP associate with particular clinical manifestations [1,2,3,4].

Disease subtypes and auto-antibody profiles are strongly associated with HLA-DRB and DQB alleles. The most frequently reported associations with dcSSc are HLA-DRB1*11, HLA-DQB1*03 among European and North American Caucasians and Africans [3,5,6]. However autoantibody specificities are different depending upon the HLA-DRB1*11 allele: HLA-DRB1*11:04 is associated with ATA in Caucasians and HLA-DRB1*1101 with AFA in Africans. HLA-DRB1*15:02- HLA-DQB1*06:01 haplotypes are commonly found among Asians with dcSSc and ATA [7]. Less commonly mentioned are associations of HLA-DRB1*0802 with dcSSc in Japanese and HLA-DRB1*0804 with ATA positive patients in American blacks [3,7,8].

Most common HLA-DR associated with dcSSc (HLA-DRB1* 11:01, *11:04, *15:01, *08:02…) have in common an amino acid sequence ^{67}FLEDR^{71} on their ß chain (**Table 1**). Similarly, most common HLA-DQB1 susceptibility alleles, DQB1*03:01, *03:02, *04:01, *04:02, *06:01 and *06:02, code for a common

Table 1. Shared amino acid sequences of the most common DRß chains.

DRB1 alleles	Amino acid number				
	67	68	69	70	71
*01:01, *01:02, *04:03–08, *14:02	L	L	E	Q	R
*01:03, *04:02, *11:02, *13:01, *13:02, *13:04	I	–	–	D	E
*15:01-:03	I	–	–	–	A
*15:04	F	–	–	–	A
16:01, *16:03, *16:04, *16:08	***F***	–	–	***D***	–
*16:02, *14:03	–	–	–	D	–
*16:05, *16:07, *12:01, *12:03–05, *07:01, *0703, 0803	I	–	–	D	–
*03:01–03:11, *04:01	–	–	–	–	K
11:01, *11:04–06, *11:09	***F***	–	–	***D***	–
*11:03	F	–	–	D	E
*11:07	–	–	–	–	K
12:02, *13:05	***F***	–	–	***D***	–
*13:03	I	–	–	D	K
*14:01, *14:04	–	–	–	R	–
08:01, *08:02, *08:04–09	***F***	–	–	***D***	–
*09:01	F	–	–	R	–
*10:01	–	–	–	R	–
DRB3 alleles					
*01:01–03, *02:01–08, *03:01–03	–	–	–	–	K
DRB4 alleles					
*01:01-:05	–	–	–	R	–
DRB5 alleles					
01:01,*01:02,*01:04,*01:05	***F***	–	–	***D***	–
*01:03	–	–	–	D	T
*01:06,*02:02,*02:03	I	–	–	–	A
*01:07	I	–	–	D	–
*01:09	F	–	–	N	–
*02:04	F	–	–	–	A

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^{71}TRAELDT ^{77} motif on their ß chain (**Table 2**). Reveille et al. have proposed that additionally to TRAELDT, an amino acid, tyrosine at position 30 (^{30}Y) present on HLA-DQB1*03 and DQB1*06:01-:03 and *6:05-:07 alleles (see **Table 2**) strengthens the association of ATA with Caucasians and African Americans [9].

HLA-DR molecules are heterodimers composed of a non-polymorphic α chain, encoded by DRA and a highly polymorphic ß chain (ß1 to ß5) encoded by DRB1*, DRB3*, DRB4* or DRB5* genes. In HLA-DR15 molecules, the α chain associates either with a ß1 or ß5 chain coded respectively by DRB1*15 or DRB5*01. The ^{67}FLEDR^{71} motif is only expressed on the ß5 chain. Conversely, in HLA-DR11 molecules, the ^{67}FLEDR^{71} motif is expressed on the ß1 chain encoded by DRB1*11 and not on the co-expressed ß3chain encoded by DRB3*02. Consequently, two heterodimeric HLA-DR molecules are co- expressed at the cell surface. For HLA-DR15, α chain associates with ß1 or ß5 and for DR11, α chain associates with ß1 or ß3. But often only heterodimers with ß1 chains are taken into consideration for antigen presentation purpose, as other chains are often considered as accessory chains. If indeed protein expression for ß1 chains is higher, a person carrying the FLEDR shared epitope on the HLA-DR15 haplotype would have less expression and a deficient FLEDR restricted antigen presentation compared to a person carrying it on the HLA-DR11 haplotype.

In the current study, we evaluated for the first time the relative risk conferred by one or two ^{67}FLEDR^{71} and/or one or two ^{71}TRAELDT^{77} motives among our Caucasian French patients with SSc. As we know, from previous studies, classification by clinical subtypes and autoantibody profiles could influence results, we conducted a complete analysis with both classifications to determine whether FLEDR and/or TRAELDT motives influence susceptibility to the limited or diffuse form of SSc and/or to autoantibody subsets. We further evaluated the importance of the ß chain carrying the FLEDR shared epitope by quantifying levels of ß chain transcripts.

Methods

Patients and Controls

We included 282 Caucasian patients with SSc with no overlapping disease, all with defined SSc type (94 diffuse SSc; 188 limited SSc) and auto-antibody status known for 243 of them (80 without ATA or ACA, 89 with ACA and 74 with ATA). Patients were enrolled in collaboration with 7 French hospitals from Paris, Marseille and Lille and fulfilled the criteria of LeRoy for SSc [10]. Altogether 235 women and 47 men were analysed for HLA-DR and DQ allele frequencies. Mean age at diagnosis was 49.3 years $\pm$14.3 (mean $\pm$ SD).

In parallel, 468 healthy Caucasian controls were recruited at the Centre d'Examen de Santé de l'Assurance Maladie (CESAM), Marseille, France (N = 154, mean age at the inclusion was 52.5 years $\pm$ 7.5 [mean $\pm$ SD]) and at Claude Huriez Hospital in Lille, France (N = 314, mean age at inclusion was 35.4 years $\pm$ 10.2 [mean $\pm$ SD]). None of the controls had any symptom or familial history of autoimmune disorder.

Ethics Statements

Controls from Marseille are registered at INSERM under the Biomedical Research Protocol number RBM-04-10.

Controls from Lille were drawn from a DNA bank created in the biological laboratory in 1993. Written consent forms obtained according to the Declaration of Helsinki were signed [11].

HLA-DRB1 and DQB1 Typing

Genomic DNA was extracted from peripheral blood by standard methods and HLA genotyping was performed either by using PCR-RFLP, as previously described for samples received in Lille or sequence-specific oligonucleotide (SSO) HLA-DRB1 and HLA-DQB1 typing kit, (RELI SSO, Dynal, Invitrogen, Bromborough, Wirral, UK) according to manufacturer's protocol as previously described for samples received in Marseille [12,13]. Allelic typing for DRB1 was done at Etablissement Français du Sang (EFS, Marseille, France) for samples received in Marseille.

RNA Preparation and cDNA Synthesis

Total RNA was extracted from PBMC using GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich, St Louis MO, USA). For each RNA sample, DNase I digestion was included as recommended (Sigma-Aldrich, St Louis MO, USA).

Table 2. Shared amino acid sequences of the most common HLA-DQß chains.

	Amino acid number								
DQB1 alleles	**30**	**…**	**71**	**72**	**73**	**74**	**75**	**76**	**77**
*05:01, *05:02, *05:03	H	…	A	R	A	S	V	D	R
*05:04	***Y***	…	D	–	–	–	–	–	
****03:01-*03:04,*06:01/2/5/7/9***	***Y***	***…***	***T***	**–**	**–**	***E***	***L***	**–**	***T***
****06:03, 06:04***	***H***	***…***	***T***	**–**	**–**	***E***	***L***	**–**	***T***
*02:01	S	…	K	–	–	A	–	–	–
****03:05***	***Y***	***…***	***T***	**–**	**–**	***E***	***L***	**–**	***T***
*04:01, *04:02	***Y***	…	D	–	–	–	–	–	T

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Concentration of RNA was spectrophotometrically measured and quality was ascertained with Agilent Analyser (Agilent RNA 6000 Nano Kit, Agilent Technologies, Germany). Total RNA was reverse-transcribed using Enhanced Avian HS RT-PCR kit (Sigma-Aldrich, St Louis MO, USA) according to manufacturer's recommendations.

Quantitative Comparison of mRNA from HLA-DRB1*15/HLA-DRB5*01 and HLA-DRB1*11/HLA-DRB3*02

For HLA-DRB1*15/DRB5*01 assays, we used previously described real-time PCR assay from Prat et al. with some modifications in primers sequences [14]. For HLA-DRB1*15 assay, the forward primer was modified: 5′-GCTCCCCACTGGCTTTGT-3′, a new reverse primer and probe were designed: 5′-GGCTGTTCCAGTACTCAGCG-3′ and 5′FAM- ACCACGTTTCCTGTGGCAGCCTAAGAG-TAMRA3′, respectively. For HLA-DRB5*01 assays, only the forward primer was modified: 5′- TGGAGGTTCCTACATGG-CAA-3′.

We designed HLA-DRB1*11 and HLA-DRB3*02 assays. To ensure that genomic DNA was not amplified, forward primers for each assay were designed to span Exon 1-Exon 2 borders. HLA-DRB1*11 forward primer, reverse primer and probe had respectively the following sequences: 5′-GGACACCAGAC-CACGTTTCT-3′, 5′-CGCACGTACTCCTCTTGGTTATA-3′ and 5′FAM-ATTGAAGAAATGACACTCAGACGTAGAG-TACTCC-TAMRA3′. HLA-DRB3*02 primers and probe design was as follow: forward primer 5′-GGACACCCGACCACGT-3′, reverse primer 5′-CGCGTACTCCTCCTGGTTAT-3′ and probe 5′FAM-CTTGGAGCTGCTTAAGTCTGAGTGT-CATTTC-TAMRA3′.

As quantitative comparisons between mRNAs are not trustable since no perfect gene reference exist, we cloned PCR products from each assay (DRB1*15, DRB5*01, DRB1*11 or DRB3*02) in pCR4-TOPO plasmids (pCR4-TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) to calibrate standards curves from these constructions, as explained below. After bacterial transformation and plasmid extraction (QIAprep Spin Miniprep kit, Qiagen), each construct was checked by sequencing (Cogenics, Grenoble France). Plasmids were then linearized with NcoI restriction enzyme (Invitrogen, Carlsbad, CA) and concentrations for the 4 constructs were adjusted by using a common set of primers and probe specific to the plasmid sequence and designed as follows : forward primer 5′-CAGAATTAACCCTCACTAAAGGGACT-3′, reverse primer: 5′-ATAGGGCGAATTGAATTTAGCG-3′ and probe 5′FAM-TCCTGCAGGTTTAAACGAATTCGCC-TAMRA3′. The 4 standards were then serially diluted. Normalization of concentrations between the 4 constructs was possible because inserts were very similar in size (210 bp and 193 bp for HLA-DRB1*15 and –DRB5*01 respectively and 114 bp and 111 bp for HLA-DRB1*11 and –DRB3*02 respectively). Standards were run with their own set of primers and probes. Adjustment for PCR efficiency of each curve was done and relative comparison between quantities of HLA-DRB1*15/DRB5*01 mRNA and HLA-DRB1*11/DRB3*02 mRNA was calculated.

Statistical Analysis

Pearson chi-square tests with the adjusted standardized residual method [15] were used to compare frequencies and give an indication of the strength of the association for each shared epitope (FLEDR and TRAELDT) between different groups. Indeed the $\chi 2$ test indicates whether there is an association between two categorical variables. However, it does not in itself give an indication of the strength of the association. In order to identify the cells (one dose, two doses…) that have the larger differences between the observed and expected frequencies, we used the adjusted standardized residuals. These differences are referred to as residuals, and they can be standardized and adjusted to follow a Normal distribution with mean 0 and standard deviation 1 [2]. The adjusted standardized residuals, dij, are given by: $dij = \frac{Oij - Eij}{\sqrt{Eij\left[1-\frac{ni}{N}\right]\left[1-\frac{nj}{N}\right]}}$.

Where *Oij* is the observed frequency in the cell in row *i* and column *j* and *Eij* is the expected frequency in the cell in row *i* and column *j*, where *ni* is the total frequency for row *i*, *nj* is the total frequency for column *j*, and *N* is the overall total frequency. The larger the absolute value of the residual, the larger the difference between the observed and expected frequencies, therefore the more significant the association between the two variables.

Adjusted standardized residual >1.96 indicates that the number of cases in that cell is significantly larger than would be expected if the null hypothesis were true, with a significance level of 0.05. An adjusted residual < -2.0 indicates that the number of cases in that cell is significantly smaller than would be expected if the null hypothesis were true.

Results

FLEDR Motif is Highly Associated with dcSSc (Table 3)

HLA-DRB1 allelic typing was performed on 468 controls and 282 patients with SSc (94 patients with dcSSc and 188 patients with lcSSc) to identify the FLEDR motif coded by some HLA-

Table 3 Prevalence of FLEDR and TRAELDT in patients with SSc divided by clinical subtypes.

		DcSSc			LcSSc			healthy controls		
		N = 94			N = 188			N = 468		
Motif	Doses	#	%	Adj. Std Resd[a]	#	%	Adj. Std Resd[a]	#	%	Adj. Std Resd[a]
FLEDR[b]	2	21	22.3	**3.7**	21	11.2	0.0	42	9.0	*−2.5*
	1	50	53.2	**2.3**	84	44.7	0.7	184	39.3	*−2.2*
	0	23	24.5	*−4.6*	83	44.1	−0.7	242	51.7	**3.8**
TRAELDT[c]	2	44	46.8	**2.9**	54	28.7	−1.7	155	33.1	−0.5
	1	42	44.7	−1.4	101	53.7	0.7	244	52.1	0.4
	0	8	8.5	−1.8	33	17.6	1.3	69	14.7	0.1

[a]adjusted standardized residual >1.96 indicates that the number of cases in that cell is significantly larger than would be expected if the null hypothesis were true (represented in bold), with a significance level of.05. An adjusted residual < −2.0 indicates that the number of cases in that cell is significantly smaller than would be expected if the null hypothesis were true (represented in italic).

[b]when comparing different subgroups for FLEDR association: DcSSc/LcSSc/healthy controls: $\chi^2 = 29.1$, $p<10^{-5}$; LcSSc/healthy controls: not significant; DcSSc/healthy controls: $\chi^2 = 28.4$, $p<10^{-6}$.

[c]when comparing different subgroups for TRAELDT association: DcSSc/LcSSc/healthy controls: $\chi^2 = 10.2$, $p = 0.029$; LcSSc/healthy controls: not significant; DcSSc/healthy controls: $\chi^2 = 7.2$, $p = 0.027$.

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DRB alleles (**Table 1 and Table S1**). Similarly HLA-DQB1 allelic typing was performed on the same number of controls and patients to identify the TRAELDT motif coded by some HLA-DQB1 alleles and the amino acid present at position 30 (**Table 2 and Table S2**). The presence of FLEDR or TRAELDT on one haplotype is noted: 1 dose, on both haplotypes: 2 doses and on none: 0 dose.

When patients are divided by clinical subtypes (**Table 3**), standardized adjusted residuals give an indication of association for each motif, as explained above. Presence of 2 doses of FLEDR is the most significant risk to develop dcSSc compared to a single dose (respective adjusted standardized residuals: 3.7 and 2.3, see Methods). For TRAELDT having 2 doses is also the most significant risk to develop dcSSc compared to a single dose (respective adjusted standardized residuals: 2.9 and −1.4).

However if we compare which one of the two motives is associated with the highest risk to develop dcSSc, FLEDR motif is at higher risk than TRAELDT (respectively $\chi^2 = 28.4$, $p<10^{-6}$ and $\chi^2 = 7.2$, $p = 0.027$).

The increase of FLEDR motif in patients with dcSSc is mostly due to the higher frequency of HLA-DRB1*11 and DRB1*15 alleles (**Table S1**).

Although TRAELDT motif is increased among patients with dcSSc, none of the TRAELDT positive alleles (HLA-DQB1*03 and *06) are statistically increased (**Table S2**).

As it had been previously proposed that tyrosine at position 30 (^{30}Y) could strengthen the TRAELDT association, we analysed this possibility. Indeed, the ^{30}Y residue added to TRAELDT slightly strengthens the TRAELDT association with dcSSc ($\chi^2 = 10.5$, $p = 0.0015$, data not shown) but remains weaker than FLEDR association ($\chi^2 = 28.4$, $p<10^{-6}$).

FLEDR Motif is Highly Associated with Patients with ATA

Patients were divided in three subgroups according to their autoantibody status (**Table 4, Tables S3 and S4**): patients without ACA or ATA (Abneg); patients with ACA (ACApos); patients with ATA (ATApos).

FLEDR motif is highly associated with ATA positive patients ($\chi^2 = 43.9$, $p<10^{-9}$) whereas TRAELDT has a weaker association ($\chi^2 = 14.6$, $p<10^{-3}$, **Table 4**).

Double dose of FLEDR motif is significantly increased among patients with ATA (28.4%) compared with healthy controls (9.0%). The risk to develop ATA positive SSc is higher when double dose FLEDR is present (adjusted standardized residuals: 5.1) than when only one dose is present (adjusted standardised residuals: 2.6). The increase of FLEDR motif in patients with ATA is, like for patients with dcSSc, mostly due to the higher frequency of HLA-DRB1*11 and DRB1*15 alleles (**Table S3**). The risk to develop ATA positive SSc is higher when double dose TRAELDT is present (adjusted standardized residuals: 3.6) than when only one dose is present (adjusted standardised residuals: −1.2). The increase of TRAELDT motif in patients with ATA is mostly due to the higher frequency of HLA-DQB1*03 alleles (**Supplementary Table S4**).

Again, the ^{30}Y residue added to TRAELDT slightly strengthens the TRAELDT association with ATA positive SSc ($\chi^2 = 19.7$, $p = 0.00005$, data not shown) without being higher than FLEDR association ($\chi^2 = 43.9$, $p<10^{-9}$).

Prevalence of FLEDR in ATA Positive dcSSc and Not ATA Negative dcSSc

Although ATA is a hallmark of dcSSc, not all patients with dcSSc have ATA, we wondered whether the FLEDR association was mostly with the clinical subtype (dcSSc ATA+ or −) or the autoantibody profile (**Table 5**). Among the 94 patients with dcSSc we were able to obtain information for autoantibody status for 85 who divided into 52 ATA positive and 33 ATA negative. When both groups (dcSSc ATA pos and dcSSc ATA neg) are compared to healthy controls, the only remaining association is with the group positive for ATA. Again FLEDR is the most prevalent shared epitope in this group ($\chi^2 = 35.2$, $p<10^{-7}$) compared with TRAELDT ($\chi^2 = 9.4$, $p = 0.009$). For both motives, having 2 doses confers the highest risk to develop dcSSc with ATA as they have the highest standardized adjusted residuals (respectively for FLEDR and TRAELDT: 4.8 and 2.6).

The ^{30}Y residue added to TRAELDT slightly strengthens the TRAELDT association with ATA positive dcSSc ($\chi^2 = 13.4$, $p = 0.0012$, data not shown) without being higher than FLEDR association ($\chi^2 = 35.2$, $p<10^{-7}$).

Table 4 Prevalence of FLEDR and TRAELDT in patients with SSc divided by antibody status.

		SSc Ab neg			SSc ACA pos			SSc ATA pos			Healthy controls		
		N = 80			N = 89			N = 74			N = 468		
Motif	**Doses**	#	%	Adj. Std Resd[a]	#	%	Adj. Std Resd[a]	#	%	Adj. Std Resd[a]	#	%	Adj. Std Resd[a]
FLEDR[b]	**2**	7	8.8	−0.7	8	9.0	−0.6	21	28.4	**5.1**	42	9.0	*−2.4*
	1	41	51.3	1.6	37	41.6	−0.2	42	56.8	**2.6**	184	39.3	*−2.6*
	0	32	40.0	−1.2	44	49.4	0.6	11	14.9	*−5.7*	242	51.7	**4.0**
TRAELDT[c]	**2**	29	36.3	0.5	18	20.2	*−2.9*	39	52.7	**3.6**	155	33.1	−0.6
	1	36	45.0	−1.1	50	56.2	1.0	33	44.6	−1.2	244	52.1	0.8
	0	15	18.8	1.0	21	23.6	**2.4**	2	2.7	*−3.1*	69	14.7	−0.4

[a]adjusted standardized residual >1.96 indicates that the number of cases in that cell is significantly larger than would be expected if the null hypothesis were true (represented in bold), with a significance level of.05. An adjusted residual < −2.0 indicates that the number of cases in that cell is significantly smaller than would be expected if the null hypothesis were true (represented in italic).

[b]when comparing different subgroups for FLEDR association: Ab neg/ACA pos/ATA pos/healthy controls: $\chi^2 = 48.5$, $p<10^{-8}$; Ab neg/healthy controls: not significant; ACA pos/healthy controls: $\chi^2 = 0.17$, $p = 0.9$; ATA pos: $\chi^2 = 43.9$, $p<10^{-9}$.

[c]when comparing different subgroups for TRAELDT association: Ab neg/ACA pos/ATA pos/healthy controls: $\chi^2 = 27.6$, $p = 0.00013$; Ab neg/healthy controls: not significant; ACA pos: $\chi^2 = 7.9$, $p = 0.02$; ATA pos/healthy controls: $\chi^2 = 14.6$, $p = 0.0007$.

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TRAELDT Association is Not Solely due to Linkage Disequilibrium with FLEDR

DRB1*11 alleles (generally ''FLEDR positive'' except DRB1*11:02 and *11:03) are in linkage disequilibrium (LD) with DQB1*03:01 (TRAELDT positive) and HLA-DRB1*15:01 alleles (FLEDR positive) in LD with HLA-DQB1*06:02 (TRAELDT positive). Therefore one could argue that the TRAELDT association observed is only due to LD with FLEDR.

However TRAELDT alone is statistically associated with dcSSc and ATA positive individuals as the frequency of individuals negative for FLEDR (0 dose) but positive (1 or 2 doses) for TRAELDT is statistically higher in patients with dcSSc ($\chi^2 = 12.9$, $p = 0.0003$) and in patients with ATA ($\chi^2 = 19.4$, $p = 0.000013$) compared to controls (**Tables S5 and S6**). This last result indicates TRAELDT has its own contribution to disease susceptibility and autoantibody specificity.

Increased mRNAs of Beta Chains with FLEDR Motif in Patients and Controls

The HLA-DRB1*1104/DRB1*1501 genotype was present in 7 out of 19 patients with ATA positive dcSSc with double dose FLEDR but rarely seen in healthy controls with double dose FLEDR (3/42, 2 tailed Fisher's test, $p<0.007$, data not shown).

The FLEDR motif is expressed on the ß1 chain in some HLA-DR haplotypes (i.e. DR11), and on the ß5 chain on other HLA-DR haplotypes (i.e. DR15, see **Table 1**). Ratios of HLA-DRB5*01 ($FLEDR^{pos}$)/HLA-DRB1*15 ($FLEDR^{neg}$) mRNA expression were compared in 14 patients with SSc and 6 healthy controls (**Figure 1**). Levels of ß5 mRNA ($FLEDR^{pos}$) were systematically higher than levels of ß1 mRNA with a mean ratio of 5.6. This difference was observed in patients and controls.

As a control of our experiments and validation of our quantitative comparisons, we checked whether levels of ß1 mRNA were higher than ß3 mRNA in HLA-DR11 haplotypes as previously described. Indeed, analyses on 5 subjects (2 patients

Table 5 Prevalence of FLEDR and TRAELDT in patients with dcSSc divided by antibody status.

		DcSSc ATA pos			DcSSc ATA neg			Healthy controls		
		N = 52			N = 33			N = 468		
Motif	**Doses**	#	%	Adj.Std Resd[a]	#	%	Adj.Std Resd[a]	#	%	Adj.Std Resd[a]
FLEDR	**2**	16	30.8	**4.8**	3	9.1	−0.4	42	9.0	*−3.6*
	1	28	53.8	1.9	18	54.5	1.6	184	39.3	*−2.5*
	0	8	15.4	*−4.9*	12	36.4	−1.3	242	51.7	**4.8**
TRAELDT	**2**	27	51.9	**2.6**	13	39.4	0.5	155	33.1	*−2.5*
	1	23	44.2	−1.1	16	48.5	−0.3	244	52.1	1.1
	0	2	3.8	*−2.1*	4	12.1	−0.2	69	14.7	1.9

[a]adjusted standardized residual >1.96 indicates that the number of cases in that cell is significantly larger than would be expected if the null hypothesis were true (represented in bold), with a significance level of.05. An adjusted residual < −2.0 indicates that the number of cases in that cell is significantly smaller than would be expected if the null hypothesis were true (represented in italic).

[b]when comparing different subgroups for FLEDR association: dcSSc ATA pos/dcSSc ATA neg/healthy controls: Fisher's exact test $p<10^{-6}$; dcSSc ATA pos/healthy controls: $\chi^2 = 35.2$, $p<10^{-7}$; dcSSc ATA neg/healthy controls: not significant.

[c]when comparing different subgroups for TRAELDT association: dcSSc ATA pos/dcSSc ATA neg/healthy controls: Fisher's exact test $p = 0.043$; dcSSc ATA pos/healthy controls: $\chi^2 = 9.4$, $p = 0.009$; dcSSc ATA neg/healthy controls: not significant.

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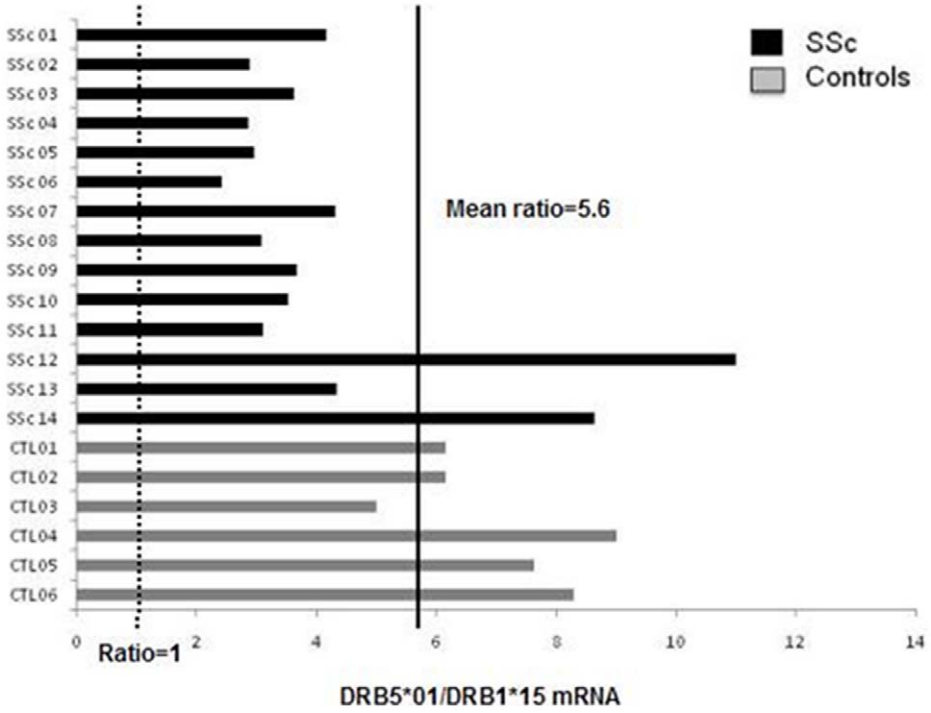

Figure 1. mRNA expression of DRB5*01 and DRB1*15 in patients with SSc and controls (CTL).
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with SSc and 3 controls) confirmed an increased quantity of ß1 mRNA ($FLEDR^{pos}$), with a mean ratio of 4.5, compared to ß3 mRNA (data not shown).

Discussion

The most important genetic factors for scleroderma, as for many autoimmune diseases, are in the HLA locus. Indeed, results from worldwide cohorts of patients with SSc, going from early HLA allele frequency analyses to more recent Genome Wide Association studies, all show HLA association with SSc. Most studies have used the classification of diffuse or limited disease, while others, more recently, have analysed patients subsets classified according to their autoantibody status. Overall, the consensus is that HLA-DRB1*11:04 is a risk factor in numerous Caucasian populations for diffuse SSc and presence of ATA [3,5] and HLA-DRB1*15:02 and DRB1*0802 in Asian populations [7]. Sequence identity on SSc-associated HLA-DRß chains conducted to a model of FLEDR shared epitope at amino acid positions 67 to 71 [16]. Similarly, SSc-associated HLA-DQß1 chains have a common ^{71}TRAELDT^{77} sequence, which has been associated with patients with dcSSc and ATA. Although many studies have analysed HLA allele frequencies in several ethnic groups including North American Caucasians, Japanese and Choctaw Indians [2], no study has evaluated the strength of FLEDR motif compared to TRAELDT motif. Moreover, to our knowledge, this is the first study analysing HLA class II shared epitopes in French Caucasian patients with systemic sclerosis (SSc) stratified by clinical subsets *and* autoantibodies. We confirmed two previous findings in Japanese and Korean patients [16] and showed that FLEDR is the most prevalent shared epitope for the most severe type of SSc (dcSSc ATA+) in French Caucasians. We further showed that the association is linked to autoantibody production rather than clinical subtype. Finally by using standardized adjusted residual method we showed that having 2 doses of FLEDR is the higher risk to develop dcSSc with ATA.

Since TRAELDT association is weaker compared with FLEDR association, and since TRAELDT is often in linkage disequilibrium (LD) with FLEDR, one could think TRAELDT association is solely due to LD. However we showed that TRAELDT has its own contribution to disease susceptibility and autoantibody specificity. A tyrosine at position 30 (^{30}Y), previously shown to reinforce the strength of the TRAELDT association with patients with ATA [9], added significance to the association but still remained lower than the FLEDR association. Similarly, on the DRß chain, by a novel approach which consists in subdividing into biologically relevant smaller sequence features and their variant types, Karp et al. showed that additional residual amino acids played a role in the risk to develop SSc [17]. Risk alleles had the sequence ^{26}F-^{28}D_^{30}Y_^{37}Y_^{67}F/I_^{70}D_^{71}R_^{86}V. However this additional effect of residual amino acids on DRß chain was not as obvious in our cohort as most double dose FLEDR shared epitopes were HLA-DRB1*11:04/*15:01 and HLA-DRB1*15:01 does carry FLEDR but not the whole risk sequence described above (ie: ^{86}V).

A parallel can be made between shared epitope in Rheumatoid Arthritis (RA) and in SSc. In patients with RA, the shared epitope ^{70}QK/RRAA^{74} has a strong effect on the risk to develop Anti-Citrullinated Peptide Antibodies (ACPA) positive RA, whereas this association, although significant, is weaker in patients without ACPA [18]. This observation argues the admitted hypothesis that a particular HLA shared epitope presents particular auto-antigenic peptides triggering to a T cell helper response, which itself conducts to a particular auto-antibody production.

The FLEDR motif, by its position in the peptide binding groove, is determinant for efficient presentation of antigenic peptides to T cells. Interestingly, we showed that this motif would be overexpressed when carried by DRß5 chains in HLA-DR15

molecules, as well as when carried by DRß1 in HLA-DR11 molecules. Indeed, our results, very similar to Prat et al. recently found in patients with multiple sclerosis and controls [14], showed a 5 fold increase of ß5 chain at mRNA level. Prat et al. further showed that this mRNA increase correlated with a two-fold increase at protein level. ß5 chains might be then sufficiently expressed at cell surface to combine with the DRß chain to form additional DR molecules on the cell surface and be involved in antigen presentation [14]. These "accessory chains" serve to extend and complement the peptide repertoire of DRB1 in antigen presentation [19].

In the current study, not only we confirmed that some HLA-DRB1 and DQB1 alleles are highly associated with the production of ATA, but for the first time we statistically evaluated the strength of each HLA allele common motives. FLEDR is the main presenting motif involved in ATA production. Knowing better motives involved in peptide and autoantibody production could allow developing blocking therapies to prevent ATA production, a hallmark of higher risk for severe organ involvement, for internal malignancies and for reduced survival. Indeed, in a recent publication, by using an *in silico* molecular docking program to screen a large "druglike" chemical library, Michels et al. were able to find small molecules capable of occupying the pockets along the I-A^{g7} binding groove in the NOD mouse model of spontaneous autoimmune diabetes [20].

The focus of this paper has been the amino-acid sequence from position 67 to 71 encoded by *HLA-DRB* and the amino-acid sequence from position 71 to 77 encoded by *HLA-DQB1*, but classification of *HLA-DRB1* genotypes according to their risk should provide diagnostic markers for SSc. Indeed we found that HLA-DRB1*1104/DRB1*1501 was the most common FLEDR double dose genotype among patients with ATA and was rarely seen in healthy controls. This highlights a synergistic effect of different alleles from each haplotype. Double dose of shared epitope but also compound heterozygosity, may confer a higher risk to disease as it has been shown in rheumatoid arthritis, type 1 diabetes, celiac disease and systemic lupus erythematosus suggesting a common autoimmune pathway [19,21,22,23,24].

Future larger studies should also focus on classification by HLA genotypes at risk for SSc to provide help in clinical practice for a disease still difficult to diagnose.

Supporting Information

Table S1 HLA-DRB1 allele frequencies in patients with SSc divided by clinical subtypes and compared with healthy controls. [a] Odds ratios (OR) and confidence intervals [CI] are given only for HLA-DRB1 allele frequencies statistically higher (susceptibility alleles) or statistically lower (protective alleles) in patients compared with controls. [b] Otherwise statistics are noted as non-significant (ns). [c] $p<0.05$ after correction for multiple comparisons.
(DOCX)

Table S2 HLA-DQB1 allele's frequencies in patients with SSc divided by clinical subtypes and compared with healthy controls. [a] Odds ratios (OR) and confidence intervals [CI] are given only for HLA-DRB1 allele frequencies statistically higher (susceptibility alleles) or statistically lower (protective alleles) in patients compared with controls. [b] Otherwise statistics are noted as non-significant (ns). [c] $p<0.05$ after correction for multiple comparisons.
(DOCX)

Table S3 HLA-DRB1 allele frequencies in patients with SSc divided by autoantibodies status and compared with healthy controls. [a] Odds ratios (OR) and confidence intervals [CI] are given only for HLA-DRB1 allele frequencies statistically higher (susceptibility alleles) or statistically lower (protective alleles) in patients compared with controls. [b] Otherwise statistics are noted as non-significant (ns). [c] $p<0.05$ after correction for multiple comparisons.
(DOCX)

Table S4 HLA-DQB1 allele's frequencies in patients with SSc divided by autoantibodies status and compared with healthy controls. [a] Odds ratios (OR) and confidence intervals [CI] are given only for HLA-DRB1 allele frequencies statistically higher (susceptibility alleles) or statistically lower (protective alleles) in patients compared with controls. [b] Otherwise statistics are noted as non-significant (ns). [c] $p<0.05$ after correction for multiple comparisons.
(DOCX)

Table S5 FLEDR and TRAELDT haplotype analyses in patients with SSc classified by clinical subgroups. Haplotypes $FLEDR^0$ -$TRAELDT^{1/2}$ in dcSSc/Haplotypes $FLEDR^0$–$TRAELD^{1/2}$ in healthy: $\chi^2 = 12.9$, $p = 0.0003$
(DOCX)

Table S6 FLEDR and TRAELDT haplotype analyses in patients with SSc classified by autoantibody status. Haplotypes $FLEDR^0$ -$TRAELDT^{1/2}$ in SSc ATApos/Haplotypes FLEDR0 -$TRAELDT^{1/2}$ in healthy: $\chi^2 = 19.4$, $p = 0.000013$
(DOCX)

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Author Contributions

Conceived and designed the experiments: DFA JMR NCL. Performed the experiments: DFA JMR MM SBK PPP. Analyzed the data: DFA JMR JR NCL. Contributed reagents/materials/analysis tools: IF YA KT DFB EH JRH RD BG JC JR. Wrote the paper: NCL DFA.

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17: Do men with Scleroderma or men with Rheumatoid Arthritis have a greater magnitude of maternal and/or fraternal microchimerism than women with similar diseases?

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Keywords: gender, systemic sclerosis, rheumatoid arthritis, microchimerism, HLA.

Background. Fetal microchimerism (Mc), naturally arising from pregnancy, could contribute to the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) and systemic sclerosis (SSc) in women.[1,2] Men are less affected by autoimmune diseases and have also fewer possibilities than gravid women to acquire natural Mc. Nevertheless, as anybody, they can acquire from their in utero passage, maternal Mc and/or cells from a twin.[3] The aim of this study is to investigate whether men with SSc or men with RA acquire these 2 sources at a greater magnitude than women with similar diseases.

Methods. We studied 31 subjects with SSc and 26 healthy individuals for maternal Mc in their whole blood and/or peripheral blood mononuclear cells (PBMC), using HLA-specific real-time PCR as previously described. HLA typing for A, B, DRB1 and DQB1 genes was obtained for probands and their mothers. Cell sorting for granulocytes, T lymphocytes, B lymphocytes and monocytes has been performed on a Robosep (Stem Cell) from whole blood samples.

Results. Among the 31 subjects with SSc tested for Mc in their PBMCs, 8 were men and 23 women. Preliminary results show that 50% of men were positive for maternal Mc, whereas 22% of women were positive for maternal Mc. A similar frequency (23%) of healthy women (n = 26) was positive for maternal Mc, but at lower quantities than patients with SSc (range [0–29.5 genome equivalent/million of host cells] vs. [0–321 gEq/M]).

Conclusion and perspectives. Although these results are very preliminary and far from reaching statistical power, they seem to confirm higher maternal Mc levels in women with SSc compared with healthy women. Furthermore they may indicate that men with SSc have more often maternal Mc than women with similar disease. A larger number of subjects with SSc are currently being recruited in each group to confirm our hypothesis. In parallel, 26 men with RA are under investigation, with family members being recruited in order to develop suitable maternal HLA-specific Q-PCR. We will furthermore define the phenotype of Mc cells by testing all the different cell subsets already obtained.

Investigating whether the impact of different natural sources of Mc varies between men and women with autoimmune diseases will give new insights to the yet unclear risk factors in sex disparities in autoimmunity.

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infliximab treatment. In reviewing the literature we find that anti TNF paradoxically brings about an immediate response in erythema nodosum patients, however provokes erythema nodosum and others skin manifestations in patients with either rheumatic pathology or inflammatory bowel disease. [1, 2]

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7. Genetics and epigenetics of rheumatic diseases

A7.1 A GENETIC VARIANT IN THE REGION OF *MMP-9* IS ASSOCIATED WITH SERUM LEVELS AND PROGRESSION OF JOINT DAMAGE IN RHEUMATOID ARTHRITIS

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Background and Objectives The severity of joint destruction is highly variable between Rheumatoid Arthritis (RA) patients. We aimed to identify new genetic risk factors by studying genetic susceptibility loci of several auto-immune diseases.

Patients and Methods In phase-1, 646 Dutch RA-patients with yearly X-rays of hands and feet over 7 years follow-up were genotyped for 148,880 SNPs by Immunochip which contains 186 loci previously associated with autoimmune diseases. Association of SNPs with MAF > 0.01 (130,841 SNPS) with joint destruction was analysed using a marginal regression model. Correction for multiple testing was done by Bonferroni correction for the number of uncorrelated SNPs (threshold $p < 1.1 \times 10^{-6}$). In phase-2, 686 North American RA-patients with repeated hands X-rays over 15 years follow-up, for which Immunochip genotyping data were also available, were studied. SNPs that were significantly associated in phase-1 were selected and evaluated. All X-rays were scored by Sharp van der Heijde score (ICC 0.91 and 0.98 for phase-1 and 2 respectively). MMP-9 levels were measured in baseline serum by ELISA (Ebioscience) in 120 RA-patients that were selected on the Rs11908352-genotype.

Results In phase-1, 109 SNPs were significantly associated with joint destruction ($p < 1.1 \times 10^{-6}$). Of these, 76 variants were on the HLA region. The 33 non-HLA genetic variants, though several were in high LD, were studied in the North-American RA-patients. Here, after correction for the number of uncorrelated SNPs (threshold $p < 0.0036$), two variants were associated with the severity of joint destruction: Rs451066 on chromosome 14 ($p_{uncorrected} = 0.002$, MAF = 0.20) and Rs11908352 on chromosome 20 ($p_{uncorrected} = 0.002$, MAF = 0.21). The region of Rs451066 on chromosome 14 has previously been linked to type-1 diabetes susceptibility. Presence of a risk allele was associated with a 3.7% higher rate of joint destruction per year; this equaled 29% over 7-years. Rs11908352 is located at the *MMP-9* region on chromosome 20. Patients with a risk allele had a 2.7% higher radiological progression rate per year, which equaled 20% more joint destruction over a 7-years period. Furthermore, the minor genotype was associated with significantly higher levels of MMP-9 compared to the common genotype ($p = 0.007$).

Conclusions Two new risk loci for progressive joint destruction in RA were identified (Rs451066 and Rs1190852). The risk allele in Rs11908352 also associated with higher serum MMP-9 levels, indicating to a role for MMP-9 in progression of joint destruction in RA.

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A7.2 ALLOGRAFT INFLAMMATORY FACTOR 1 (AIF1) POLYMORPHISMS IN FRENCH CAUCASIANS WITH RHEUMATOID ARTHRITIS

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Background Allograft inflammatory factor 1 (AIF1) is a cytoplasmic inflammatory protein encoded within the HLA class III genomic region on chromosome 6 (6p21.3). Although several risk loci for Rheumatoid Arthritis (RA) have been identified by Genome Wide Association Studies (GWAS), none of them involved AIF1 polymorphisms. However, two studies on small cohorts have shown that AIF1 single nucleotide polymorphism (SNP) Rs2269475 (C/T), causing a non-synonymous change of amino acid, is associated with RA (Harney, SM *et al*, 2008; Pawlik A *et al*, 2008). Moreover, AIF1 overexpression in inflammatory synovial tissues and macrophages isolated from synovial fluids of patients with RA, confirms its potential role in RA.

Objective We propose to examine the association of the seven most described AIF1 SNPs in our French RA patients.

Methods We have tested 99 Anti-Citrullinated Protein Antibody (ACPA) positive Caucasian RA patients who fulfilled ACR/EULAR criteria and 104 healthy Caucasians. We designed AIF1 primers to specifically amplify the AIF1 gene region containing the 7 SNPs: Rs2844475, Rs4711274, Rs2736182, Rs2736181, Rs2259571, Rs2269475 and Rs13195276. PCR products were sequenced (Cogenics Beckman Coulter) and chromatogram results analysed for the 7 SNPs positions in patients and controls. Patients and controls were genotyped for HLA-DRB1.

Results Two SNPs out of the 7 were associated with RA: Rs4711274 (G/A) and Rs2269475 (C/T). Regarding Rs4711274, G/A and A/A genotypes were increased when compared with controls ($p = 0.0005$). The minor A allele was strongly associated with RA ($p = 0.0005$). Regarding Rs22699475, in linkage disequilibrium with the former, we found a similar pattern with increased T/T and C/T genotypes ($p = 0.0009$) and increased minor T allele frequency ($p = 0.0008$) in patients with RA. Interestingly, patients carrying the minor associated AIF1 allele expressed HLA-DRB*04 more often than the patient's group carrying the C/C or G/G genotype (63.8% versus 44.4%), although the difference was marginal ($p = 0.06$).

Conclusions In this study of French Caucasians with RA, we confirmed Rs2269475 association already described in British and Polish Caucasians. Additionally, we find an association with Rs4711274 in linkage disequilibrium with Rs2269475. Intriguingly, such associations have never been found in GWAS.

A7.3 ASSOCIATION OF CIRCULATING MIR-223 AND MIR-16 WITH DISEASE ACTIVITY IN PATIENTS WITH EARLY RHEUMATOID ARTHRITIS

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19 HLA-G A PUTATIVE SUSCEPTIBILITY GENE IN SCLERODERMA, BUT ONLY IN WOMEN

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Background Systemic sclerosis or Scleroderma (SSc) is an autoimmune disease affecting mostly women with a higher incidence between 40 and 60 years old. Several HLA class II genes have been associated with SSc depending on the clinical subset of the disease, limited cutaneous SSc (lc-SSc) or diffuse cutaneous SSc (dc-SSc) or on specific autoantibodies, anticentromere antibodies (ACA) or antitopoisomerase (ATA) antibodies, respectively hallmarks of the above clinical subsets. HLA-G is a non classical HLA molecule with tolerance functions that may play a role in inflammatory diseases. A 14 bp sequence insertion/deletion polymorphism (rs16375) in the 3′-untranslated region of the HLA-G gene has been associated to levels of soluble HLA-G (sHLA-G). The insertion is associated with lower levels.

Objectives In the current study, the authors propose to analyse this polymorphism in patients with SSc compared to healthy individuals. The authors furthermore stratified patients according to sex, clinical subsets and autoantibody status.

Patients and methods The authors genotyped 145 patients with SSc and 95 healthy controls by a previously developed two step multiplex SNaPshot method several HLA-G Single Nucleotide polymorphisms and the -14bp Ins/Del polymorphism.

Results Patients with SSc tend to have higher frequency of ins/ins (29/145, 20.0%) genotypes compared with healthy controls (11/95, 11.6%, p=0.008). The frequency of Ins alleles compared to Del alleles is significantly increased among patients with SSc (p=0.024). This association reveals interesting patterns when patients are divided by sex (women, N=110 and men, N=35). Men had similar frequencies of Del/Del, Del/Ins, Ins/Ins genotypes than healthy controls (respectively, 45.7%, 42.9%, 11.4% and 50.5%, 37.9%, 11.6%), whereas women had different proportions of genotypes with Del/Del: 36.4%, Del/Ins: 40.9% and Ins/Ins: 22.7%. The difference in genotype repartition was statistically significant in women with SSc compared with healthy controls (p=0.047) and the difference in allele frequency even stronger (Ins: 43.1% compared with 30.5%, p=0.008).

Apart from the gender association an association with dcSSc with the Ins allele (p=0.004), and a marginal association with lcSSc (p=0.08)were also observed. Interestingly both autoantibody subgroups, ATA and ACA, were associated with higher Ins allele frequencies (p=0.018 and p=0.03, respectively).

Conclusion Women with SSc but not men have a genotype of low sHLA-G secretor. Further studies need to be conducted to better understand this gender dichotomy and its meaning in tolerance in a disease affecting mostly women in their childbearing years.

A206 SKEWED X CHROMOSOME INACTIVATION IN RHEUMATOID ARTHRITIS WOMEN

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Background and objectives It has long been recognised that women have a greater prevalence of autoimmune diseases. Rheumatoid arthritis (RA) does not escape this rule with a women:men ratio of 3:1.

X chromosome inactivation (XCI) is a dosage compensation mechanism used by mammals to ensure that XX females and XY males equalise X chromosome gene expression. As a consequence, females are a mosaic of two cell lines, one expressing maternal X-linked and the other expressing paternal X-linked genes with a ratio close to 50:50 when XCI is random.

However skewing, defined as a deviation from the 50:50 ratio has been described in females with autoimmune thyroid diseases, scleroderma and juvenile idiopathic arthritis (for review[1]). The aim of the current study is to test whether women with RA also have a skewed XCI.

Methods The highly polymorphic CAG repeat on the first exon of the androgen receptor gene was genotyped, as described elsewhere[2] to determine XCI bias in 84 women with RA and 100 healthy women.

Results A total of 54 patients and 69 controls were informative for androgen receptor polymorphism. Among them 31.5% of women with RA (17/54) had a skewed XCI (≥80:20) compared to only 17.4% of healthy women (12/69). Only extreme skewing was statistically significant with 18.5% of patients following this pattern and 2.9% of controls (p=0.004).

Conclusions Our preliminary data indicate that skewed XCI may be a risk factor for the occurrence of RA in women. Further studies need to be done to analyse whether women who have a skewed pattern have less genetic susceptibilities (shared epitope) or less specific autoantibodies (anti-CCP) as their risk factor is X chromosome linked.

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X-LINKED GENETIC FACTORS BEHIND GENDER BIAS IN RHEUMATOID ARTHRITIS

***Discipline*:** Immunology

***Key words*:** Female predominace – Rheumatoid arthritis – Scleroderma – X chromosome inactivation – Copy number variations – Microchimerism – *TLR7* and *TLR8*

Cover abstract:

As in many autoimmune diseases, a female predominance is observed in rheumatoid arthritis (RA). The X chromosome, present in 2 copies in females, is of particular interest as it contains many genes with immune functions. In this work, we show an increase with age in copy number of some X-linked genes in peripheral blood cells of men, healthy or with RA. Importantly, this increase is not observed in women. On the other hand, when in fact females generally randomly inactivate (50:50) either the paternally-derived or the maternally-derived X chromosome, we show a skewed inactivation (≥ 80:20) in women with RA. Moreover this skewing correlates preferentially with women carrying disease susceptibility genes. Altogether, our findings highlight the importance of this fascinating chromosome in the development of autoimmunity in a step forward to better understand female predilection to autoimmune diseases.

FACTEURS DE RISQUE LIES AU CHROMOSOME X A L'ORIGINE DE LA PREDOMINANCE DES FEMMES DANS LA POLYARTHRITE RHUMATOÏDE

Discipline : Immunologie

Mots clés : Prédominance féminine – Polyarthrite rhumatoïde – Sclérodermie systémique – Inactivation du chromosome X – Variation du nombre de copies – Microchimérisme – *TLR7* et *TLR8*

Abstract de couverture :

Comme dans la plupart des maladies auto-immunes une prédominance féminine est observée dans la polyarthrite rhumatoïde (PR). Le chromosome X, présent en 2 exemplaires chez la femme, est intéressant puisque beaucoup de gènes à fonctions immunitaires y sont localisés. Dans ce travail, nous montrons que certains de ces gènes peuvent augmenter leur nombre de copies quand l'individu vieillit. En outre, cette variation est spécifique au sexe avec une augmentation chez les hommes et l'inverse chez les femmes. D'autre part, alors que généralement les femmes inactivent aléatoirement (50:50) le chromosome X d'origine maternel ou X d'origine paternel, nous montrons un biais d'inactivation (≥ 80:20) chez les femmes atteintes de PR. De plus ce biais est préférentiellement associé à celles qui portent les gènes de susceptibilité à la maladie. Ces résultats soulignent l'importance du chromosome X dans le développement de l'auto-immunité et aident à la compréhension du biais féminin dans ces maladies.