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Ro52 Antibodies and Susceptibility Genes in Congenital Heart Block

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To my husband
with love

ABSTRACT

Congenital heart block (CHB) develops in fetuses of Ro/SSA and La/SSB positive women. During pregnancy, the autoantibodies cross the placenta and affect the fetus in which a potentially lethal atrioventricular (AV) block may develop. This thesis is aimed at identifying clinically useful maternal serologic markers predictive of risk for CHB, and to define genes linked to susceptibility in the child. Ro52-p200 antibodies, binding amino acid 200-239 of the Ro52 protein, was recently suggested by our group as a marker for high risk pregnancies. Performing a multinational study we now show that p200 antibodies are highly relevant as a second step analysis in Ro52-positive pregnancies, and increase the positive predictive value for fetal cardiac involvement.

The incidence of CHB in Ro/La positive women is 1-2%. This risk is only increased to 20% in subsequent pregnancies despite persisting antibodies, indicating that there are other factors involved in disease susceptibility than antibody specificity alone. Ro/La antibody levels and Ro52 subclass profiles were investigated longitudinally through pregnancies and revealed no significant differences between affected and healthy pregnancy outcomes. There were no significant decreases or peaks in antibody levels corresponding to or preceding the time point when CHB is usually detected. We therefore investigated differences in fetal susceptibility to CHB. Fetal genetic factors in susceptibility to CHB have been suggested, but not previously investigated experimentally. To investigate MHC and non-MHC associations of the disease, an immunization model of CHB was established in rat. Analysis of MHC and non-MHC genetic influences using congenic rat strains and an F2 cross revealed significant associations with MHC encoded genes. Maternal generation of pathogenic antibody specificity was linked to a specific MHC haplotype, whereas fetal susceptibility to development of CHB was linked to a separate MHC haplotype in the fetus. Patterns of inheritance also indicated a possible epigenetic influence in susceptibility to CHB. Our data suggest complex genetic prerequisites for susceptibility, and explain why simple associations with MHC genes have not been observed in human studies of CHB.

The cellular function of the Ro52 autoantigen was also investigated. We show that Ro52 is an E3 ubiquitin ligase and using a panel of Ro52 monoclonal antibodies which was generated, we show that R52 locates predominantly to the cytoplasm. Stimulation of cells with the systemic autoimmune-related cytokine IFN-alpha induced translocation of Ro52 from the cytoplasm to the nucleus, which preceded apoptosis of the cells.

In summary, we identify Ro52-p200 antibodies as a clinically useful marker for risk of CHB and show that fetal susceptibility to these pathogenic autoantibodies depend on fetal MHC-encoded genes. We also demonstrate that Ro52 is an E3 ligase, and that cytokines involved in systemic autoimmunity regulate the cellular localization of the Ro52 autoantigen.

LIST OF PUBLICATIONS

I. *Strandberg L, *Winqvist O, Sonesson S-E, Mohseni S, Salomonsson S, Bremme K, Buyon JP, Julkunen H, Wahren-Herlenius M. **Antibodies to amino acid 200-239 (p200) of Ro52: Candidate serologic marker for the risk of developing congenital heart block.** Submitted manuscript.

II. Strandberg L, Salomonsson S, Bremme K, Sonesson S, Wahren-Herlenius M. **Ro52, Ro60 and La IgG autoantibody levels and Ro52 IgG subclass profiles longitudinally throughout pregnancy in congenital heart block risk pregnancies.** Lupus. 2006;15(6):346-53.

III. Feist E, Keitzer R, Gerhold K, Horvath L, Wahren-Herlenius M, Dörner T. **Development of systemic lupus erythematosus in a patient with congenital heart block.** Arthritis & Rheumatism 2003; 48(9): 2697-8.

IV. Strandberg L, Ambrosie A, Jagodic M, Åden U, Klauninger R, Salomonsson S, Olsson T, Wahren-Herlenius M. **Maternal MHC regulates generation of pathogenic antibodies and fetal MHC-encoded genes determine susceptibility in congenital heart block.** Submitted manuscript.

V. Espinosa A, Zhou W, Ek M, Hedlund M, Brauner S, Popovic K, Horvath L, Wallerskog T, Oukka M, Nyberg F, Kuchroo VK, Wahren-Herlenius M. **The Sjögren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death.** J Immunol. 2006 May 15;176(10):6277-85.

VI. *Strandberg L, *Ambrosi A, Zhou W, Ottosson L, Eloranta M-L, Espinosa A, Elfving Å, Edward Greenfield, Vijay K. Kuchroo and Marie Wahren-Herlenius. **Interferon-α induces up-regulation and nuclear translocation of the Ro52 autoantigen as detected by a panel of novel Ro52-specific monoclonal antibodies.** Submitted manuscript.

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CONTENTS

LIST OF ABBREVIATIONS

ANA	anti-nuclear antibodies
APS	Antiphospholipid syndrome
ATD	Autoimmune thyroid disease
AVB	atrioventricular block
CFA	complete Freund's adjuvant
CHB	Congenital heart block
CREST	Calcinosis, Raynaud, Esophagus, Sclerodactylia, Telangiectasia
dsDNA	double-stranded deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
HLA	human leukocyte antigen
IFA	incomplete Freund's adjuvant
IgG	immunoglobulin G
i.p.	intraperitoneal
IRF-8	Interferon regulating factor-8
ISGs	interferon-stimulated genes
i.v.	intravenous
Lyp	Lymphoid tyrosine phosphatase
MCTD	Mixed connective tissue disease
MHC	major histocompatibility complex
MS	Multiple sclerosis
NC	normal conduction
NHR	normal heart rate
NLE	Neonatal lupus erythematosus
pAPS	primary Antiphospholipid syndrome
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pSS	primary Sjögren's syndrome
RA	Rheumatoid arthritis
RF	rheumtoid factor
RFP	Ring finger protein
RBCC	RING/B-box/coiled-coil
SA node	sino-atrial node
sAPS	secondary antiphospholipid syndrome
s.c.	subcutaneous
SS	Sjögren's syndrome
sSS	secondary Sjögren's syndrome
SCLE	Subacute cutaneous lupus
SLE	Systemic lupus erythematosus
T1D	Type 1 diabetes
TRIM	Tripartite motif
U1nRNP	anti-nuclear ribonucleoprotein

1 INTRODUCTION

Congenital heart block is a passively transferred autoimmune disease where maternal antibodies cross the placenta and are, potentially, initiators in an inflammatory reaction, which can lead to fibrosis and calcification of the fetal conduction system. This may lead to an incomplete or complete atrioventricular block, as well as more general functional effects on the whole heart. Research has been focused on the involvement of these antibodies for decades, but the mechanisms of antibody injury and susceptibility in these fetuses has not been elucidated.

The main objective of this thesis is to better understand the antibody specificity and induction of congenital heart block and elucidate the genetic involvement in fetal susceptibility to the disease. In order to understand the mechanism of Ro52 antibody production and its immunopathogenesis in the disease, it is also important to understand the function and cellular localization of the Ro52 protein. The function of Ro52 may give us clues as to whether or not the Ro52 antibodies involved in CHB induction are recognizing the Ro52 protein or cross-reacting with another protein on the fetal heart. This thesis includes studies investigating the involvement of Ro52 autoantibodies in congenital heart block, both clinical human studies and animal models, as well as more basic studies with *in vitro* investigations of Ro52 function.

PART I involves studies characterizing Ro52 antibodies, their involvement in congenital heart block pregnancies, as well as in experimental models of CHB (**paper I, II, IV, VI**). **PART II** encompasses human studies and animal models describing a genetic influence in congenital heart block (**paper III, IV**). Finally in **PART III**, we describe studies involving the cellular function and localization of the Ro52 autoantigen (**paper V, VI**).

In summary this thesis aims to characterize the Ro52 antibodies involved in the induction of CHB and to, for the first time, describe a fetal genetic susceptibility factor which could explain the low incidence of disease in pregnancies where the pathogenic antibodies are present. I will also present data on the function of the Ro52 protein and its cellular localization, and discuss whether or not this has implications for CHB pathogenesis.

2 BACKGROUND

In order to discuss the results of my thesis, it is necessary to first give a brief background of related topics and present the current results and literature in the field. An overview of autoimmune rheumatic diseases, with particular attention to systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), as well as cytokines involved in autoimmunity will be presented. Autoantibodies commonly found in autoimmune diseases, as well as antibodies hypothesized to be involved in congenital heart block will be summarized. New findings regarding the Ro52 autoantigen function will be discussed and finally, aspects of the disease congenital heart block, as well as *in vitro* and *in vivo* models will be described.

2.1 AUTOIMMUNE DISEASES

Autoimmune diseases are conditions in which damage to organs results from the presence of autoantibodies or autoreactive cells (Griesmacher 2001). These diseases comprise a heterogeneous group of disorders which are poorly understood (Davidson 2001, Marrack 2001). Autoimmune diseases can be divided into organ-specific or cell-specific and systemic disease (Table 1). But, there are diseases which can have characteristics of both organ specific and systemic autoimmune diseases.

Cell-specific	**Organ specific**	**Systemic**
Autoimmune hemolytic anemia	Diabetes mellitus type 1 (pancreas)	Anti-phospholipid-syndrome
Autoimmune thrombopenia	Hashimoto thyroiditis (thyroid)	Chronic polyarthritis
Goodpasture's syndrome	Addison's disease (adrenal)	CREST-syndrome
Myasthenia gravis	Basedow's disease (thyroid)	Mixed connective tissue disease
	Pernicious anemia (stomach)	Polymyositis/ dermatomyositis
	Autoimmune hepatitis	Rheumatoid arthritis
	Primary biliary cirrhosis	Sjögren's syndrome
		Systemic lupus erythematosus
		Systemic scleroderma
		Wegener's granulomatosis

Table 1. Cell-specific, organ-specific and systemic autoimmune diseases.

There are a number of autoimmune rheumatic diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), anti-phospholipid syndrome (APS), polymyalgia rheumatica, systemic sclerosis, Sjögren's syndrome (SS), polymyositis and dermatomyositis, necrotising arteritis, myasthenia gravis, sarcoidosis, and a spectrum of related syndromes. I will limit my discussions to SLE and SS since they have been the patients of focus in my studies.

2.1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, characterized by inflammation of multiple organs through the deposition of autoantibodies and immune complexes (Klippel 1997). SLE is the most diverse of the autoimmune diseases because it may affect any organ of the body and display a broad spectrum of clinical and immunological manifestations. It is thought that SLE is caused by interactions between susceptibility genes and environmental factors, which results in abnormal immune responses. It is characterized by hyperreactivity of T and B lymphocytes as well as impaired removal of apoptotic cells and immune complexes.

Non-erosive arthritis and photosensitive rashes occur in approximately 75% of cases, whereas serositis, nervous system disease and renal involvement occur in about 50% of cases. Lymphopenia occurs in a majority of SLE cases, and is most often present during active disease. Hemolytic anemia and thrombocytopenia are present in about 50% of cases (Rose NR 1998).

Since the 1950's, the studies of SLE have been dominated by the study of autoantibodies. This interest has largely been focused on antinuclear antibodies, anticytoplasmic antibodies as well as antibodies reactive with cell membranes. The overall prevalence of SLE is estimated to approximately one per 2000, varying by ethnicity, gender, and ages (Tsao 2003, Wakeland 2001). SLE is more prevalent in black, Hispanic, and Asian populations, suggesting a genetic component in the development for the disease. SLE predominantly affects women in their child-bearing years (female vs male ratio 9:1), suggestive of a hormonal factor predisposing to the disease (Petri 2002).

2.1.2 Sjögren's syndrome

Sjögren's syndrome is a chronic autoimmune disease characterized by destruction and dysfunction of the exocrine glands (Jonsson 2000). The salivary and lacrimal glands are affected leading to dryness of the mouth and eyes. In the exocrine glands there are dense lymphocytic infiltrates and the disease is characterized by B cell hyperactivity. Extraglandular manifestations of Sjögren's syndrome include fatigue, general malaise, low grade fever, as well as myalgias and arthralgias.

Sjögren's syndrome may be found alone, primary Sjögren's syndrome (pSS), or in association with one of several other autoimmune diseases, and is then denoted secondary Sjögren's syndrome (SS). A small but significant number of patients may even develop lymphoid neoplasia (Theander 2006). Sjögren's syndrome is a disease which affects primarily women, and can develop at any time but usually at 30-50 years of age (Jonsson 2000). The prevalence of SS is 0.1-3% in the general population depending on what classification criteria is used (Fox 1986, Jacobsson 1989, Dafni 1997, Bjerrum 1997).

Sjögren's syndrome patients often have a number of antibodies in their sera, such as rheumatoid factor (RF), Ro/SSA and La/SSB. Autoantibodies to Ro/SSA and La/SSB in Sjögren's syndrome are associated with earlier disease onset, longer disease duration, salivary gland enlargement, severity of lymphocytic infiltration in minor salivary glands and certain extraglandular manifestations (Moutsopoulos 2006).

Recently, alpha-fodrin has been implicated as a new autoantigen in Sjögren's syndrome. The prevalence of alpha-fodrin autoantibodies in SS has been

investigated, but is difficult to determine and has varied from 33%-88% (IgA) and 21-95% (IgG) in different studies, depending on classification criteria used, as well as patient selection methods (Witte 2005).

2.1.3 Cytokines in autoimmunity

Cytokines have essential roles in immune cell development, immunoregulation as well as immune effector functions. Cytokines have been extensively studied with respect to their role in autoimmune disease, but in fact not much is understood of their role in autoimmunity. It was once thought that cytokines were either pro-inflammatory and induced autoimmune disease, or they were anti-inflammatory, and suppressed autoimmunity. It turns out that it is a much more complicated role the cytokines play with combinations of cytokines exerting variable effects during the evolution of an autoimmune disease. One good example of pleiotropic effects of a cytokine is the complex effects of the interferons (IFNs) on autoimmunity (O'Shea 2002)(see Table 2).

Cytokine	**IFN-α/β**	**IFN-γ**
Immunostimulatory	Antiviral ↑antigen presentation TH_1 differentiation in human cells Induction of IL-15 (promotes NK and memory T-cell development and differentiation ↑ DC maturation	Activation of macrphages ↑antigen presentation ↑expression of MHC class II Promotion of TH_1 differentiation
Immunosuppressive	↑ Fas ↑ IL-10 ↓ IL-12 Inhibition of IL-12 signalling Anti-proliferative effects Differentiation of T-reg cells	Upregulation of SOCS1 Anti-proliferative effects on myeloid and lymphoid cells

Table 2. Complex effects of interferons on immune functions.

2.1.3.1 IFN-α in SLE and SS

There are a number of cytokines involved in the development of Sjögren's syndrome and SLE, but numerous studies in the past decades have concentrated on the role of IFN-α in the pathogenesis of these two diseases.

Increased serum levels of IFN-α have been found in patients with SLE (Bengtsson 2000). The significance of IFN-α in SLE is also substantiated by the expression of interferon-stimulated genes (ISGs), often referred to as an IFN signature, in peripheral blood mononuclear cells (PBMCs) of SLE patients (Bennett 2003, Baechler 2003).

Studies of Sjögren's syndrome pathogenesis have also implicated IFN-α, where IFN-α producing cells are detected in the salivary glands (Båve 2005).

Microarray analysis of biopsies from affected glands in Sjögren's syndrome patients also revealed an activation of IFN pathways (Gottenberg 2006).

2.1.3.2 IFN therapy

Autoimmune complications are well recognized in IFN-α therapy, which is widely used for treatment of specific types of cancers and chronic viral infections, particularly chronic hepatitis C virus infection (Selmi 2006). IFN-α treatment can cause new autoantibody production, but also induce disease symptoms in people with existing autoantibodies. Autoimmune disorders occurring during IFN-α treatment include diseases such as SLE (Niewold 2005), autoimmune thyroid disorders (Huang 2006), type I diabetes (Okanoue 1996), myasthenia gravis (Oishi 2005), celiac disease (Cammarota 2000), autoimmune hepatitis (Sezaki 2003), immune mediated dermatological disease (Guillot 2004), as well as immune-mediated haematological complications (Lambotte 2005).

A recent study of IFN-α treatment in chronic hepatitis C patients demonstrated that the rheumatological symptoms of these patients were 30% improved, 44% worsened, and 26% were unchanged after treatment. Fifteen percent of the patients developed rheumatologic symptoms on beginning IFN-α treatment which disappeared when therapy was discontinued (Nissen 2005).

SLE symptoms have been observed during IFN-α treatment of several different conditions (Niewold 2005), and these SLE symptoms generally resolve on discontinuation of therapy. Neonatal lupus following maternal treatment for essential thrombocytopenia with IFN-α has also been described, with thrombocytopenia, rash, photosensitivity, positive ANA and anti-Ro antibodies. The mother also developed high titer ANA, but was asymptomatic (Fritz 2005).

2.2 AUTOANTIBODIES

Autoantibodies are antibodies towards self antigens and found in autoimmune rheumatic diseases. It is not certain whether all autoantibodies are pathogenic but many have been confirmed to be involved in the development of autoimmune rheumatic diseases (Bizzaro 2007). It has been suggested that autoimmune diseases are multifactorial and are comprised of a number of phases (Vanderpump 1995, Betterle 1997, Arbuckle 2003), see Figure 1. In the initial phase, patients are asymptomatic and have no autoantibodies but are carriers of particular genes which predispose them to an autoimmune disease. At the next stage, specific autoantibodies appear in the serum yet the patient is still asymptomatic. This phase before symptoms appear may be short or last for a number of years, depending on the specific disease. The third phase corresponds to the appearance of disease symptoms and is thought to be triggered by an environmental factor.

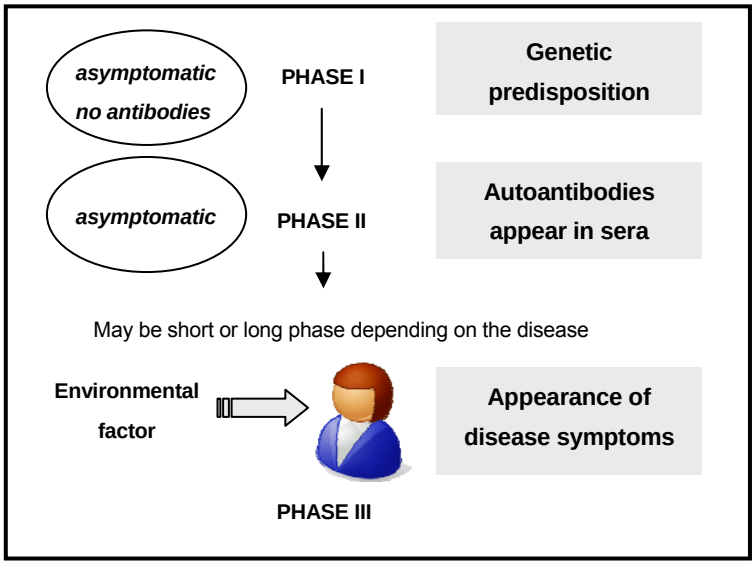

Figure 1. Phases of autoimmunity induction. Progression from asymptomatic genetic predisposition to appearance of antibodies and the induction of disease perhaps initiated by an environmental factor.

2.2.1 Autoantibodies in rheumatic diseases

Several groups of autoantibodies have been described in autoimmune diseases, the grouping depending on the chemical structure of the corresponding antigens (Griesmacher 2001), summarized in Table 2. Some autoantibodies are included in the diagnosis and the classification criteria for diseases, such as anti-Sm-antibodies and anti-double-stranded DNA (dsDNA) antibodies in SLE, anti-nuclear-ribonucleoprotein (U1nRNP) in mixed connective tissue disease (MCTD), and antibodies against Ro/SSA and La/SSB in Sjögren's syndrome.

Antibodies
Rheumatoid Factor
Antinuclear antibodies
Anti-neutrophil cytoplasmic antibodies
Anti-phospholipid antibodies
Anti-endothelial antibodies
Antibodies to blood cells
Anti-neuronal antibodies
Antibodies to stress proteins
Antibodies to hormones
Antibodies to microsomes

Table 2. Groups of antibodies. Most autoantibodies are not specific for a clinical syndrome. They can be detected in patients with different clinical features as well in some healthy individuals.

Antibodies which bind to the Fc region of IgG are called rheumatoid factor (RF) and are present in the sera of 70-90% patients with rheumatoid arthritis (Griesmacher 2001). RF may also be found in several other diseases, such as, for example Sjögren's syndrome (75-95% incidence), SLE (15-35%), MCTD (50-60%), among others.

Antinuclear antibodies (ANA) are autoantibodies which bind to different cellular nuclear antigens. Currently the determination of ANA is widely used as screening for autoimmune diseases such as SLE, MCTD, Sjögren's syndrome, RA, polymyositis, scleroderma, dermatomyositis and CREST-syndrome (Calcinosis, Raynaud, Esophagus, Sclerodactylia, Telangiectasia). Anti-dsDNA are antinuclear antibodies which are of great importance in SLE and nephritis. The presence of anti-dsDNA have also been correlated with SLE disease activity (Schur 1968). The presence of ANA is evidence of some underlying autoimmune diseases, however, it is not itself considered diagnostic. Prevalence of some common antinuclear antibodies are presented in Table 3 (Griesmacher 2001).

Antinuclear antibodies	**Disease**	**Incidence (%)**
Anti-ds-DNA	SLE	50-90
Anti-ss-DNA	SLE	70-95
	MCTD	20-50
	Polymyositis/dermatomyositis	40-50
	SS, RA, systemic scleroderma	8-15
Anti-Sm	SLE	25-75
Anti-SS-A/Ro (Ro52/Ro60)	SS	40-95
	SLE	20-60
	NLE	-100
Anti-SS-B/La	SS	40-95
	SLE	10-20
anti-U_1RNP	MCTD	95-100
	SLE	30-40
Anti-histone	Drug-induced LE	20-40
Anti-Scl-70	Progressive systemic sclerosis	40-70
Anti-centromere	CREST-syndrome	80-90

Table 3. Antinuclear antibodies and incidence of disease.

The anti-phospholipid syndrome (APS) is characterized by severe thrombotic events, recurrent fetal loss or thrombocytopenia and detection of anti-phospholipid antibodies (APA). This syndrome can be associated with other autoimmune diseases, particularly SLE (sAPS), or may be unrelated to another disease (pAPS). Disease activity in SLE is reported to be associated to levels of antiphospholipid antibodies (Buttgereit 1997).

2.2.2 Immunoglobulin isotypes and subclasses

Immunoglobulins, or antibodies, are made up of four polypeptide units: two light chains and two heavy chains (see Figure 2). The light chains are bound to the heavy chains by disulfide bridges and noncovalent interactions. The two heavy chains are then joined together by covalent disulfide bridges as well as by noncovalent hydrophilic and hydrophobic interactions. The heavy chains determine the class of the antibody. There are two different light chains denoted κ and λ. Each antibody can have only one light chain, not both (Janeway CA 2001). The Fc region of the immunoglobulin is what interacts with effector molecules and cells, and the Fab fragments contain the antigen-binding activity (indicated in figure 2).

There are five classes of antibodies, IgA, IgD, IgE, IgG and IgM classes which contain α, δ, ε, γ, and μ heavy chains, respectively. The human IgG class of antibodies is further divided into four IgG subclasses; IgG_1, IgG_2, IgG_3, and IgG_4, having γ_1, γ_2, γ_3, γ_4 heavy chains respectively. IgG is the main antibody found in the blood after antigen stimulation, and it also has the ability to cross the placenta. IgA mainly functions in body secretions such as saliva and breast milk, for example. The role of IgD is unknown. IgD and surface bound IgM are the two isotypes which constitute the B cell receptor. Soluble IgM is a pentamer that binds to invading microorganisms and activates complement killing and phagocytosis of the cells. IgE provides immunity against some parasites but is also responsible for the clinical symptoms of allergic reactions (Janeway CA 2001).

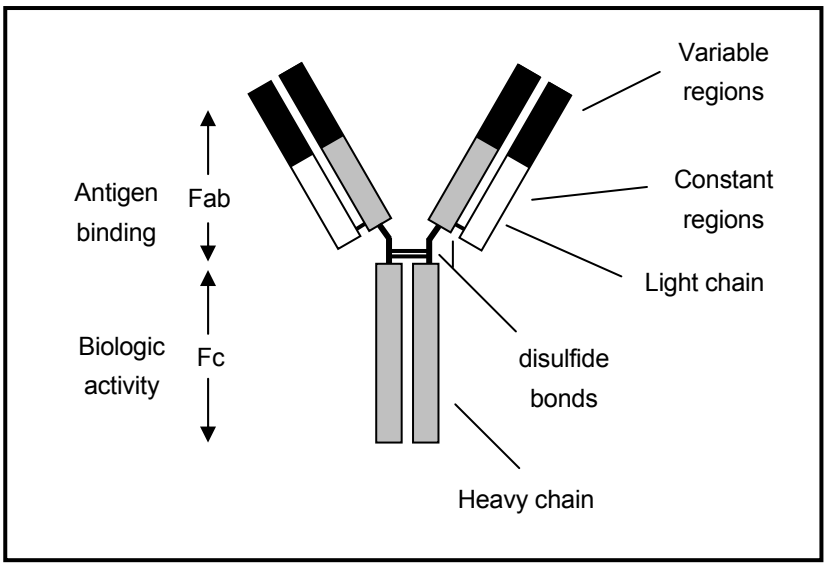

Figure 2. Antibody structure. All antibodies have the same basic unit made up of two light chains and two heavy chains.

During pregnancy, maternal immunoglobulins are actively transported to the fetus via Fc receptors (Jenkinson 1976). Maternal IgG concentrations in fetal blood increase from early in the second trimester through term, most antibodies passing to the child in the third trimester. It has been demonstrated that IgG_1 is the most efficiently transported subclass, then IgG_3 and IgG_4, and IgG_2 is the least, across the human placenta (Simister 2003, Hashira 2000, Costa-Carvalho 1996).

Protein antigens usually induce IgG_1 and IgG_3 responses and these isotypes are able to activate all types of Fc receptors and the C1 component of complement. The IgG_4 subclass may be characteristic of chronic antigen stimulation, as in autoimmune disease. IgG_4 has restricted Fc receptor activating abilities and does not activate C1q. The IgG_2 subclass is predominant in responses to carbohydrate antigens. IgG_2 also has restricted Fc receptor and C1 activating abilities (Janeway CA 2001, Ravetch 2001, van de Winkel 1993, Woof 2004).

2.3 Ro52 AUTOANTIGEN

Of the Ro/SSA autoantigens, Ro52 is a member of the tripartite motif (TRIM) protein family (Reymond 2001), also referred to as the RING/B-box/coiled-coil (RBCC) family (Reddy 1992, Borden 1998). Several studies have demonstrated that RING-finger proteins (many which are in the TRIM family) act as E3 ubiquitin ligases (Jackson 2000, Liu 2004). Before discussing the function of Ro52, I will give a brief background on TRIM proteins and ubiquitination. A discussion of cellular localization of Ro52 will be presented in the *Results and Discussion* section.

2.3.1 TRIM proteins

These proteins consist of a RING finger, one or two b-box motifs and a coiled-coil region (Figure 3). These TRIM/RBCC proteins are involved in a broad range of biological processes such as apoptosis, cell cycle regulation and viral response. This is a family of proteins sharing overall domain structure and 40-50% identity at the amino acid level. Ro52 has a RING finger, one b-box, a coiled-coil region and also has a B30.2 (sometimes referred to as SPRY) domain at the C-terminus. Other members in the TRIM/RBCC family of proteins with high homology to Ro52 include RFP, Staf-50, and RPT1 (Reymond 2001).

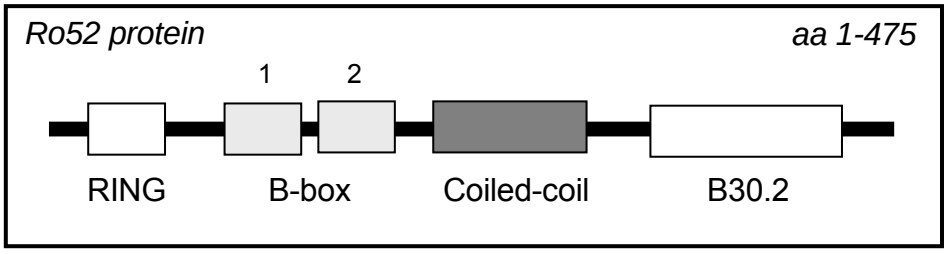

Figure 3. The common structure of the TRIM/RBCC proteins. These proteins consist of a RING finger, one or two b-box motifs and a coiled-coil region.

2.3.2 Ubiquitination and E3 ligases

Ubiquitination is a post-translational modification mechanism used by eukaryotic cells mainly to control protein levels through proteosome-mediated proteolysis, but may also modify protein trafficking, or lysosomal degredation (d'Azzo 2005). Protein ubiquitination involves three classes of enzymes and it is a multi-step process. The first enzyme (E1) is the ubiquitin activating enzyme which activates ubiquitin by forming an E1-ubiquitin thioester intermediate. This activated ubiquitin is then transferred to the second enzyme, a ubiquitin-conjugating enzyme (E2). The third enzyme, which is a ubiquitin ligase enzyme (E3) interacts directly with this E2 enzyme and its substrate, and mediates the transfer of ubiquitin to a lysine residue of the target protein. In the proteosome, polyubiquitinated proteins are degraded.

2.3.3 Ro52 function

The cellular function of the Ro52 autoantigen has been unknown until recently. It is impossible to discuss the function of Ro52 and not mention the Ro60 and La autoantigens. There has been so much debate about their relationship, since most often antibodies to these antigens are found together; it has also been shown in immunized mice that epitope spreading between these antigens can occur (Topfer 1995). Furthermore, it has been debated whether they all associate with the Y RNA complex.

2.3.3.1 Ro52

The human Ro52 protein was cloned in 1991 (Chan 1991), Itoh 1991), and the homologous Ro52 protein in mice (Keech 1996) and cattle (Shusta 2003) were cloned in 1996 and 2003 respectively. Human Ro52 is a 475 amino acid protein and sequence similarity between the murine and human protein is 70% on the amino acid level, and even higher in the predicted functional domains (Keech 1996). The Ro52 gene has been mapped to chromosome 11 in the region 11p15.5 (Bepler 1999).

Ro52 function has been investigated for years, but earlier studies have not elucidated the function of the protein. It has previously been proposed that the function is associated with transcription processes, since the Ro52 protein shares homology with gene-regulating proteins and has been suggested to bind to nucleic acids (Frank 1995, Frank 1999). It was early on suggested that the RING finger motif was a strictly DNA-binding motif, but more recent studies have demonstrated it to be associated with ubiquitination, and especially as a functional domain in E3 ubiquitin ligases (Trockenbacher 2001, Urano 2002, Linares 2003), as well as in protein-protein interactions (Saurin 1996). Ro52 in later years has also been demonstrated to induce CD28-mediated IL-2 production in Jurkat T cells (Ishii 2003), and to interact with IgG (Rhodes 2006), Skp2 and Cul1 (Sabile 2006).

There has also been an alternatively spliced Ro52 mRNA expressed in a variety of tissues including the fetal heart (Chan 1995), which has also been suggested to be an E3 ubiquitin ligase, but the significance is unclear since a Ro52-β protein has never been demonstrated. The mRNA expression of Ro52-α (full-length protein) and Ro52-β was assessed in Sjögren's syndrome patients' salivary glands and controls and there was no difference in expression found (Bolstad 2003).

2.3.3.2 Ro60 and La

Ro60 is complexed with small noncoding RNAs of unknown function called Y RNAs (cytoplasmic RNAs). The La protein is also found in a fraction of these Ro60/Y RNA complexes, and approximately half of patients with anti-Ro60 antibodies also have antibodies against La. It is hypothesized that the ribonucleoprotein particle is the immunogen (Hardin 1985, Tan 1989). Most patients with anti-Ro60 proteins also produce antibodies against the structurally unrelated Ro52 protein (Ben-Chetrit 1988), but studies have failed to demonstrate an association of Ro52 with this complex (Kelekar 1994, Boire 1995).

Although the function of Ro60 has been investigated for many years, it was not until recently that it has been demonstrated that the protein binds misfolded, defective, noncoding RNAs that are eventually degraded (O'Brien 1994, Labbe 1999,

Chen 2003). Ro60 is therefore proposed to function in a quality control pathway in which incorrectly folded and otherwise imperfect noncoding RNAs are targeted for decay (Chen 2004, Wolin 2006). La is a nuclear phosphoprotein that binds many newly transcribed noncoding RNAs, including Y RNAs (Wolin 2002). There is accumulating evidence that La is necessary for correct folding of many small RNAs, suggesting a general RNA chaperone function for this protein (Wolin 2002).

2.4 NEONATAL LUPUS ERYTHEMATOSUS

Congenital heart block is the most serious symptom of a syndrome called neonatal lupus erythematosus (McCauliffe 1995). The most common manifestation of NLE are skin rash and CHB, but other symptoms can occur, such as cytopenias and hepatitis (Buyon 1998a). Nearly half of NLE infants develop skin lesions (Petri 1989), and about 50% develop CHB. In about 10% of cases, skin rash and CHB symptoms occur in the same infant (Lee 1990). Skin rash may be present at birth and this cutaneous NLE may be precipitated or exacerbated by UV light exposure (McCauliffe 1995). The NLE symptoms, except CHB, are transient, and disappear when the maternal antibody levels have decreased in the neonate system at approximately 6-8 months (Lee 1994).

It has been suggested that HLA haplotypes, autoantibodies and isotype specificity of antibodies may be important factors to determine what NLE symptoms the infant will develop (Kim 2001). It has been demonstrated that Ro52 antibodies are more prevalent in mothers with children with CHB and that Ro60 antibodies are more prevalent in mothers with these transient symptoms (Lee 1994). It has also been speculated that maternal diagnosis in NLE with skin manifestations differs from that of CHB (Lawrence 2000). It was observed that mothers of children with cutaneous NLE frequently had SLE or another autoimmune disorder, whereas mothers of children with CHB were more often asymptomatic. This however may be due to study inclusion criteria's where healthy mothers with children having only transient neonatal lupus rash may not be included as frequently. A recent study has suggested that women with Ro/SSA and La/SSB antibodies and hypothyroidism have an increased risk for CHB pregnancies (Spence 2006).

NLE has been reported to occur in approximately 1-2% of children born to mothers with SLE, and in 15-20% of children born to mothers with SLE and Ro/SSA antibodies (Cimaz 2003b).

2.4.1 Congenital heart block

CHB occurs in fetuses of Ro/SSA and La/SSB positive women with rheumatic diseases such as SLE and SS but also of asymptomatic women. The antibodies cross the placenta to the fetus and have been demonstrated to be associated with atrioventricular block (AVB), as well as other general pathological effects on the fetal heart which may be associated with AV block (Jaeggi 2005, Eronen 2001a). This condition can lead to a life-long pacemaker dependence or even death. Fetal heart block occurs in 1/15 000 to 1/20 000 live births (Michaelsson 1972).

Congenital heart block is usually detected between 18-24 weeks of gestation, and the incidence of CHB in Ro/SSA and La/SSB positive women is approximately 2% (Brucato 2001). The recurrence of CHB in subsequent pregnancies

is 10-16% (Buyon 1998b, Eronen 2000, Brucato 1995), despite persisting antibodies (Strandberg 2006), indicating that the antibodies are necessary, but that other fetal factors may be involved in the susceptibility to CHB.

2.4.1.1 Ro/SSA and La/SSB is associated with CHB

Autoantibodies towards the Ro and La ribonucleoprotein particles are commonly found in the sera of patients with SS, SLE, subacute cutaneous lupus (SCLE), as well as mothers giving birth to children with neonatal lupus erythematosus (McCauliffe 1995, Harley 1992). Ro/SSA in fact refers to two unrelated proteins which have been identified and are encoded by two distinct genes, Ro52 and Ro60 (Itoh 1991). Cross-reactivity of these antibodies has been suggested but has not been observed consistently (Ben-Chetrit 1988, McCauliffe 1994) and though initial reports claimed an interaction between Ro52 and Ro60 (Cheng 1996), subsequent analysis has failed to confirm these observations (Kelekar 1994, Boire 1995).

The specificity of the immune response to the Ro52, Ro60 and La proteins has been extensively studied and dominant epitopes have been described for all three proteins (Wahren-Herlenius 1999, Scofield 1999). Several groups have identified an epitope in the central part of the Ro52 protein corresponding to the predicted leucine zipper (Blange 1994, Buyon 1994, Frank 1994, McCauliffe 1994, Kato 1995, Dörner 1996). There have also been two conformation-dependent epitopes identified in the zinc-finger region, in the N-terminal of the protein, which are detectable under reducing conditions (Pourmand 1998b).

Several studies show a close correlation between passively aquired Ro/SSA and La/SSB antibodies and neonatal lupus syndrome in infants born to mothers with connective tissue disease (Michaelsson 1972, Scott 1983, Manthorpe 1992, Ramsey-Goldman 1986, Buyon 1998b, Brucato 2001, Waltuck 1994, Gordon 2004). Most recent studies confirm that Ro52 antibodies seem to be more associated with congenital heart block than Ro60 or La (Julkunen 2004, Salomonsson 2002, Fritsch 2005), however the presence of La antibodies has been suggested to increase the risk for CHB (Gordon 2004). There is also the clinical observation that congenital heart block is more often associated with Sjögren's syndrome than with SLE, and these patients often have Ro52 antibodies (Brucato 2001, Julkunen 2001, Julkunen 2004). The association of Ro/SSA and La/SSB antibody levels and subclass antibodies will be discussed more in the *Results and Discussion* section.

It has been demonstrated that these Ro/La autoantibodies are associated with a number of cardiac abnormalities. Complete atrioventricular block (AV block) has been associated with these antibodies for a number of years, but recently, there are reports of effects on the function of the whole heart including first- and second-degree AV block, myocardial inflammation, endocardial fibro-elastosis and dilated cardiomyopathy (Jaeggi 2002, Jaeggi 2005, Eronen 2001a, Taylor-Albert 1997, Nield 2002a, Nield 2002b). Effects on the sinoatrial node (SA node) such as bradycardia, have also been demonstrated (Brucato 2001) as well as prolongation of the QTc interval (Cimaz 2000, Gordon 2001a, Lazzerini 2004). Previously it was believed that these cardiac effects of the Ro/La antibodies only affect the fetus, but there are some studies which report Ro/La antibody effects on the maternal heart as well (Dörner 1993, Lazzerini 2004), but not all studies support these findings (Costedoat-Chalumeau

2005a, Gordon 2001b). It is generally believed that the maternal hearts are more resistant than the fetal hearts to the autoimmune insult.

Although these maternal autoantibodies are presumed to cross the placenta and induce damage on the fetal heart, direct evidence of this is still lacking. The mechanism of fetal injury is still not well understood and there are a number of hypotheses.

2.4.1.2 Specific antibodies involved in heart block development

There are many theories and speculations to the initiating factors and mechanism of pathology in CHB. Serum screens have revealed that Ro52 antibodies seem to be more associated with CHB than Ro60 antibodies (Julkunen 2004, Fritsch 2005, Julkunen 1998, Buyon 1993, Salomonsson 2002). La antibodies have more recently been demonstrated to possibly add to the risk of CHB (Gordon 2004).

A. Ro52 antibodies and congenital heart block:

An antibody response to amino acids 200-239 (peptide p200) of the Ro52 protein have been associated with CHB (Salomonsson 2002). Higher levels of Ro52-p200 antibodies were also correlated to longer atrioventricular time intervals in a prospective study in which Doppler echocardiography was performed weekly during susceptibility weeks 18-24 in Ro52 positive women (Salomonsson 2005).

Monoclonal Ro52-p200 antibodies were produced with the intent of making antibodies for use in *in vitro* and *in vivo* studies. ScFv antibody fragments were cloned from B-cell libraries derived from lupus patients, which generated 2 antibodies, S3A8 and M4H1 (Salomonsson 2004). The two antibodies were both p200 specific antibodies, but mapping with mutated peptides revealed that they had different epitope binding profiles (Ottosson 2005). The antibody called S3A8 represented the p200 specificity found in CHB sera. In *in vitro* experiments, S3A8 antibody bound to the cell surface of cultured cardiomyocytes, causing dysregulation of intracellular calcium levels and leading to cell death (Salomonsson 2005). These effects were not observed with the control antibody M4H1, under the same conditions, indicating that it is a defined p200 specificity which can bind cardiomyocytes and cause calcium dysregulation and death (Salomonsson 2005). The target of these Ro52 p200-specific cross-reacting antibodies is till unclear and will be an important step in identifying the mechanism of CHB pathogenesis.

Since Ro52 is an intracellular protein it is possible, and probable, that the Ro52 antibodies are cross-reacting with another protein on the cell surface and not directly binding to Ro52 to cause the pathology of CHB. There are four proteins which have been suggested as cross-reactive targets of Ro52 antibodies and have been given quite some attention. The 5-HT_4 serotoninergic receptor (Fritsch 2005, Eftekhari 2000), and the α_{1C} and α_{1D} subunits of the L-type calcium channel (Qu 2005) and the T-type calcium channel (Hu 2004). Antibodies to Ro52 peptide 365–382 have been shown previously to cross-react with residues 165–185 of the heart 5-HT_4 serotoninergic receptor, and have been suggested to be important for the pathology in congenital heart block. In some studies it has been demonstrated that the sera from mothers with CHB children have antibodies which recognize the 5-HT_4 serotoninergic receptor (Eftekhari 2000, Kamel 2005) but other studies did not confirm these results (Buyon

2002). Cross-reactivity of Ro52 antibodies with the α_{1C} subunit of the L-type calcium channel has been supported by interactions between Ro/La positive IgG and the α_{1C} subunit, detected by immunoblotting (Xiao 2001). The α_{1D} Ca channel has been demonstrated to be expressed in the human fetal heart (Qu 2005), and α_{1D} $I_{Ca\text{-}L}$ α_{1C} $I_{Ca\text{-}L}$, and $I_{Ca\text{-}T}$ have been demonstrated to be inhibited by Ro/La positive IgG (Qu 2005, Qu 2001, Xiao 2001). The authors suggest that blockade of these calcium channel subunits in the human fetal heart may contribute to the symptom of sinus bradycardia in CHB.

B. Other antibodies associated with congenital heart block:

There are other antibodies identified which may be involved in CHB, or in some cases at least be markers for CHB, but the studies on most of these are small and few. In one study, antibodies to (ERV3) have been found to be associated more with CHB mothers than in mothers with healthy children (Li 1996). Calreticulin antibodies have also been found more frequently in CHB mothers compared to women with healthy pregnancies in one study (Orth 1996). Sachiko and colleagues propose that maternal antibodies to the 120 kDa protein may be an additional marker for the risk of NLE in Ro/SSA and La/SSB positive women (Miyagawa 1998).

Borda and colleagues have described that the frequency of antibodies to the second extra cellular loop of the M_1 muscarinic acetylcholine receptor are associated with CHB children more than with healthy children (Borda 2001), and that these antibodies interact with the neonatal myocardium by activating these receptors (Borda 1999). This same group has also demonstrated antibodies against this muscarinic acetylcholine receptor in the sera of Sjögren's syndrome patients and claim that these antibodies alter receptor activation as well as nitric oxide synthase (NOS) activity (Reina 2004). The significance of these antibodies in the pathology of CHB and Sjögren's syndrome is however still unclear.

Some investigators believe that La autoantibodies play a key role in the pathogenesis of CHB (Tran 2002) and there are several studies describing that La antibodies cross-react with Laminin (Li 1995, Horsfall 1996, Chang 1998). Although few studies have pointed to La antibodies as the initiating factor responsible for CHB, the majority of sera with Ro52 and Ro60 antibodies do contain La antibodies as well, and they may add to the risk of CHB (Gordon 2004). In one study by Tran and colleagues it has been demonstrated that La IgG alone can cross the placenta and bind to apoptotic cells in the fetus (Tran 2002). Fetal AVB has been induced in a mouse model of CHB by immunizing with calriticulin, Ro60 as well as La proteins (Suzuki 2005) demonstrating that Ro52 may not be the only antigen involved in the pathogenesis of CHB. Miranda-Carus *et al* has also induced AV block in BALB/c mice upon immunization with Ro52α, Ro52-β, Ro60, La and mouse Ro52 (Miranda-Carus 1998a).

2.4.1.3 Physiology of the heart and the electrocardiogram

In order to explain the pathology of CHB, I will first introduce heart physiology with an explanation of conduction through the heart. The heart consists of the atrium and ventricles, with a sino-atrial node (SA node), an atrioventricular node (AV node), the His bundle and branches, as well as the Purkinje fibers (see Figure 4).

In the right atrium, the SA node is the hearts pacemaker. It paces the heart by emitting action potentials at distinct intervals. The two divisions of the autonomic nervous system control the rate of sinus pacing. The sympathetic system stimulates the release of norepinephrine and epinephrine (adrenaline) and increases the pacing rate. The parasympathetic nervous system on the other hand inhibits the pacing rate of the SA node. Conduction branches from the SA node innervate and depolarize the left and right atrium, meeting in the AV node. Atrial depolarization causes atrial contraction.

Conduction of depolarization through the AV node is quite slow to allow for complete ventricular filling. Once depolarization has passed the AV node and reaches the His bundle, conduction passes rapidly through the ventricular conduction system, which consists of rapidly conducting bundles of Purkinje fibers. The conduction divides into left and right bundle branches which terminate in fine terminal purkinje fibers. Ventricular depolarization begins at the endocardial lining (inside the heart) and moves outwards through the myocardium (the cardiac muscle) and out to the epicardium (outer layer). After depolarization, there is ventricular repolarization which allows the myocytes to regain their intracellular charge so they can be depolarized again (Dubin 2003).

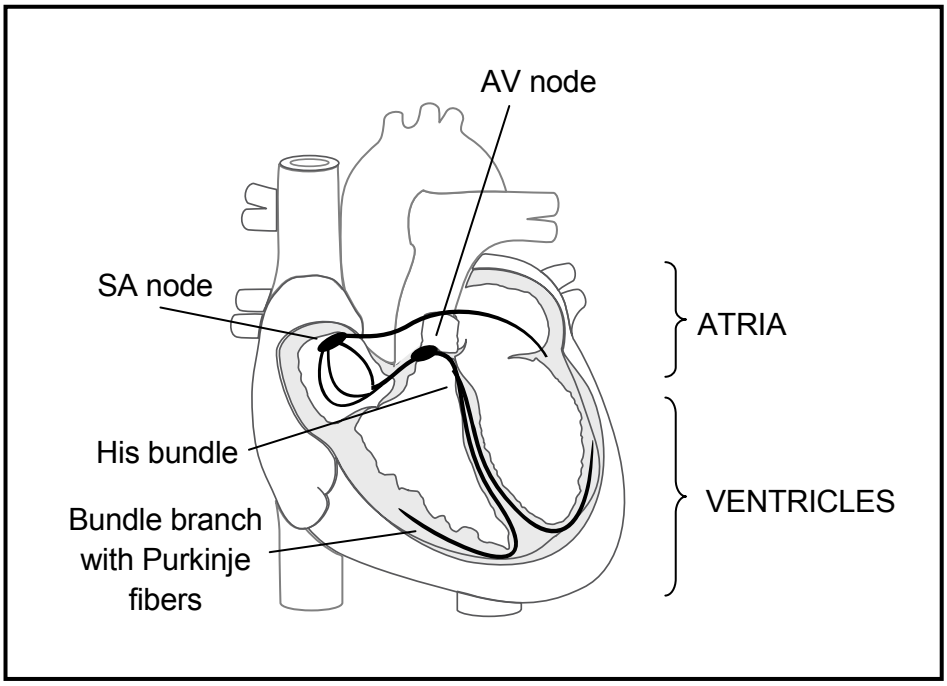

Figure 4. Human heart with SA node, AV node and conduction through the heart. The AV node is the only electrical connection between the atria and the ventricles.

An electrocardiogram (Figure 5) consists of a P wave, a PR interval, a QRS wave, ST segment, T wave, QT interval, and possibly a U wave. The P wave of an electrocardiogram represents the atrial activation. The QRS complex corresponds to the ventricular depolarization and the T wave (as well as the U) to the repolarization. The PR interval is measured from the beginning of the P wave to the onset of the QRS complex (see Figure 5). A first-degree AV block is defined as a prolongation of the PR interval. A second degree block is characterized by some impulses not being conducted through the AV node, and thus disappearance of some QRS complexes. In a third-degree block no impulses are conducted through the AV node at all (Dubin 2003).

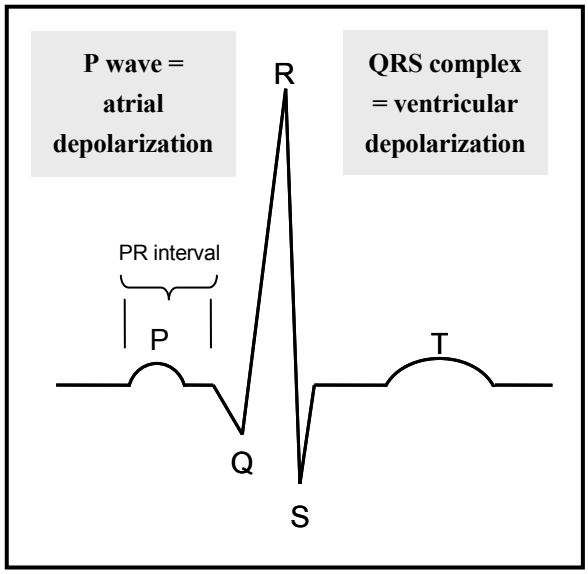

Figure 5. Electrocardiogram which shows the P, Q, R, S and T waves. The P wave corresponds to the atrial depolarization and the QRS complex to the ventricular depolarization. Prolongation of the PR interval and observation of missing QRS complexes in an ECG are used for detection of AV block.

2.4.1.4 Atrioventricular block

CHB is a conduction abnormality which affects the atrioventricular node (AV node) of the fetal heart (John 2004). An AV block is a block or delay in the signal conduction through the heart (John 2004). A first-degree AV block is characterized by a prolonged AV conduction time, measured by PR interval in an ECG (Figure 5), but all impulses are conducted (Jaeggi 2005). Second-degree AV block refers to a failure to conduct some of the atrial impulses to the ventricles. There are several types of second-degree AV block. Type I (Wenckeback) second-degree AV block is characterized by a lengthening of the AV conduction time until conduction is blocked. Type II (Mobitz II) second-degree AV block includes a sudden or occasional block without prior lengthening of the AV conduction time. There is also a 2:1 AV block where every second impulse is conducted through the AV node. In third-degree AV block, there are no impulses conducted through the AV node.

Significant bradycardia is seen with high-degree second- or third-degree AV block. When this occurs, the fetal heart begins to compensate the heart block by increasing its stroke volume. If the fetal heart does not manage to adapt, fetal hydrops can develop which is associated with a high risk of fetal or neonatal death (Groves 1996, Jaeggi 2002, Buyon 1998b).

It is hypothesized that CHB progresses gradually from a first-degree AV block to a second-degree AV block and finally this could progress to the irreversible third-degree AV block (Sonesson 2004). Fetuses of anti-Ro52 positive women frequently have signs of first-degree AV block, but the majority of these blocks revert spontaneously (Gerosa 2007, Motta 2007, Sonesson 2004). But since some do progress to a more severe form of heart block it is important to follow these high risk pregnancies. AV time interval surveillance with Doppler echocardiography has been suggested as a method for early detection of these fetuses which are at risk for CHB (Glickstein 2000, Andelfinger 2001, Van Bergen 2004, Bergman 2006).

2.4.1.5 Fetal echocardiographic screening

Congenital heart block is believed to be a gradually progressing disease, and therefore a method to diagnose a first-degree AV block early in pregnancy before it progresses to a complete block is necessary. Transmaternal fetal electrocardiography is still difficult to use as a surveillance technique due to low voltage signals transmitted and fetal P waves being difficult to separate from noise and maternal signals. The PR interval with this technique is constructed by a summation of several heart beats, and is therefore not regarded as suitable for the individual complex analysis needed when diagnosing higher degrees of AV block (Andelfinger 2001).

Magnetocardiography has also been used to evaluate developmental changes in fetal cardiac time intervals, however, this requires expensive equipment and is not suitable for routine surveillance of fetuses (Horigome 2000). Ultrasonography which is the routine method widely used to diagnose fetal arrhythmias can also be used to estimate PR intervals.

By using standard fetal echocardiographic techniques atrial and ventricular depolarizations can be identified indirectly by their mechanical (m-mode, tissue Doppler) or hemodynamic (blood flow Doppler; *i. e.* pulse-wave Doppler) consequenses and allow detection of first-degree AV block (Glickstein 2000, Andelfinger 2001, Van Bergen 2004, Sonesson 2004, Bergman 2006, Nii 2006). Reference values obtained by using these different Doppler techniques have been established and reported in several studies (Glickstein 2000, Andelfinger 2001, Van Bergen 2004, Nii 2006). Recent experimental (Dancea 2000) and clinical (Fouron 2000) studies have demonstrated the superiority of the pulsed Doppler technique compared with the M-mode approach for measuring fetal AV time intervals and thus for diagnosing first-degree AV block.

It has been shown that AV time intervals are positively correlated with increasing gestational age (Nii 2006, Andelfinger 2001, Van Bergen 2004), which is thought to be related to the increase in cardiac size and in parasympathetic tone with advancing gestation (Wheeler 1978). This emphasizes the importance of standard reference values for detection of first-degree AV block.

In the study by Sonesson *et al* in 2004 it was demonstrated that indirect signs of first-degree AV block was actually present in one third of a group of Ro52 positive women followed by using Doppler echocardiography (Sonesson 2004). The limits set for first-degree AV block, based on 284 normal pregnancies (Sonesson 2004), have been challenged by other authors (Buyon 2005, Rein 2005) based on reference values in only 60 normal pregnancies in an older study (Glickstein 2000). The reference values for prolonged PR intervals in the study by Sonesson *et al* were recently confirmed in a large independent study of 110 cases in 2006 (Nii 2006).

The presence of first-degree AVB in approximately 30% of Ro52 positive pregnancies indicates that the spectrum of heart abnormalities and effects may be wider than previously thought. Since these first-degree AV block fetuses have not been detected in the majority of previous studies, they have thus been included in the control groups. Thus, pregnancies with early signs of CHB in the fetus are included in the control group, thus making correlations with antibody specificity and levels as well as genetic associations difficult to achieve. This suggests that caution should be exercised in interpreting studies performed where fetuses are not followed *in utero*.

2.4.1.6 Pathogenesis of heart block

These pathogenic Ro/SSA and La/SSB antibodies enter the fetal system in pregnancy and in susceptible fetuses and are believed to be associated with progressive destruction of the AV node (Jaeggi 2002, Jaeggi 2005). Histological studies of CHB cases have demonstrated antibodies in cardiac tissue (Litsey 1985, Lee 1987). Deposition of complement, lymphocytic infiltrates, calcification and fibrosis have also been found in fetuses dying from CHB (Litsey 1985, Ho 1986, Lee 1987, Clancy 2004). Histological studies have confirmed antibody deposition in CHB fetal hearts (Litsey 1985), and fetal heart eluates from a CHB case were found to contain Ro52 and Ro60 antibodies (Reichlin 1994).

Also, the observations of antibody deposition, calcification and fibrosis have been reported in the entire myocardium and not just in the AV node of affected fetuses (Litsey 1985, Lee 1987, Meckler 1998, Piercecchi-Marti 2003). More general effects on the whole heart caused by these antibodies include myocardial inflammation, endocardial fibro-elastosis and dilated cardiomyopathy (Jaeggi 2005, Eronen 2001b, Taylor-Albert 1997, Nield 2002a, Nield 2002b). Prolongation of the Qt interval have also been described (Cimaz 2000, Gordon 2001a, Cimaz 2003a). Sinus bradycardia has also been suggested to be a symptom of CHB, and an effect of maternal Ro/SSA and La/SSB antibodies (Cimaz 1997) (Brucato 2000, Brucato 2001, Menon 1998). This has been demonstrated in a number of CHB murine models (Mazel 1999, Suzuki 2005, Eftekhari 2001, Xiao 2001, Boutjdir 1997) and in infants born to anti-Ro positive mothers (Cimaz 2000), as well as in perfusion studies of rat hearts (Restivo 2001).

Some studies do not confirm the prolongation of the QT interval and the sinus bradycardia effects which other studies have demonstrated in children to Ro/SSA positive mothers, and it has been speculated that the effects seen by other authors may occur independently of these antibodies (Costedoat-Chalumeau 2004). The majority of studies however support the notion that transplacental passage of maternal Ro/SSA and La/SSB antibodies should be considered as a cause of other conduction abnormalities, other than AV block.

2.4.1.7 Treatment

Current treatments of CHB include *in utero* administration of steroids (Saleeb 1999), plasmaphoresis (Buyon 1987), intravenous immunoglobulin therapy (IVIG) (Buyon 2001), sympathomimetics (Groves 1995) and cardiac pacing (Carpenter 1986). Although there is no cure for CHB, a reliable marker for CHB pregnancies may allow early treatment which may have a better effect if initiated before an inflammatory reaction has begun in the fetal heart.

In order to prevent the inflammatory damage which may occur in the fetal hearts of Ro/SSA and La/SSB positive pregnancies, maternal treatment with steroids such as prednisone, betamethasone and dexamethasone has been employed. The effect of prednisone however has been shown to be less efficient since only a small percentage of the drug reaches the fetus in its active form due to placental degradation and metabolism into its non-active form (Blanford 1977, Beitins 1972). Maternally administered betamethasone and dexamethasone are however poorly metabolized in the placenta and readily cross into the fetal circulation (Kream 1983). Once a complete AV block has developed, maternal administration of steroids is not useful for affecting the

block itself, since complete block has persisted in all treated cases and seems to be permanent (Breur 2004, Saleeb 1999, Shinohara 1999). The effect of steroids in the treatment of fetal hydrops however, has been demonstrated to be effective (Brackley 2000, Tseng 2000).

Breur and colleagues in 2004 summarized the literature and described 93 CHB cases treated with fluorinated steroids (Breur 2004). One significant observation was that complete CHB was always irreversible. Only three of 13 cases of incomplete heart block improved, and multiple side effects of the steroids were reported. Other studies have suggested that dexamethasone treatment combined with β-adrenergic stimulation for signs of bradycardia constitutes an effective therapy program for CHB (Jaeggi 2004). The overall result in this study was that untreated fetuses had a 1-year survival rate of 46%, whereas dexamethasone treated fetuses had a 90% 1-year survival rate, and this increased to 95% in those cases also treated with β-adrenergic stimulation.

Incomplete second-degree AV block has been shown to revert upon therapy with high dose fluorinated steroids (Carreira 1993, Buyon 1995, Rosenthal 1998, Saleeb 1999, Theander E 2001), but a majority of cases continue on to a complete AV block (Breur 2004), indicating the need to identify these pregnancies early and start therapy before the inflammatory reaction has begun. Treatment of choice for many physicians for an incomplete AV block has been dexamethasone and betamethasone therapy, but alarming side effects of dexamethasone therapy such as neurodevelopment impairment (Barrington 2001), diminished birth weight (Lazzerini 2004), as well as adverse obstetric events (Costedoat-Chalumeau 2003) have been reported by some investigators, and they are questioning the positive effects of dexamethasone on CHB treatment compared to the negative effects of the drug on the fetus (Breur 2004). However, a recent study by Brucato and colleagues has demonstrated that in a small study with 11 CHB children exposed to high-doses of dexamethasone, no negative effects on the neuropsychological development was detected (Brucato 2006). This data together however may indicate that betamethasone is a better choice for treatment than dexamethasone.

One report has suggested the prophylactic treatment of pregnancies at risk for CHB pregnancies with maternal prednisone (Shinohara 1999), but reports of CHB developing in women who were taking prednisone through pregnancy indicate that this may have little effect (Waltuck 1994), possibly due to placental inactivation. Prophylactic treatment with fluorinated steroids however is not recommended by most researchers and clinicians at this time because the risk for CHB is very low in these Ro/SSA and La/SSB positive women, only 2% (Brucato 2001). The negative risks of treatment must be weighed against the benefit, and possibly in a mother with a previously affected pregnancy, it may be an option.

Treatment with intravenous immunoglobulin (IVIG) prophylactically has also been suggested (Hughes 2004), but clinical success with this method to date is limited to a few cases (Wong 2001, Tsai 2001, Kaaja 2003). Plasmaphoresis has been unsuccessful in treating complete blocks (Herreman 1985, Buyon 1987, Arroyave 1995) but its prophylactic use has been successful in several cases (van der Leij 1994, Feist 1996). Complete block requires lifelong pacing in 64% of cases (Buyon 2003), and despite adequate pacing, about 10% develop life-threatening cardiomyopathies. Most deaths occur *in utero* or in the first 3 months of life approaching a mortality of 20% (Buyon 2003).

2.5 EXPERIMENTAL MODELS OF CONGENITAL HEART BLOCK

To date there is no perfect model to study CHB pathogenesis, but many groups are developing *in vivo* and *in vitro* models which will enable us to elucidate the cause of CHB. Since this disease involves unborn fetuses it makes it difficult to do experiments on humans and thus an adequate experimental model is important in understanding the mechanisms of the disease and to develop future therapies. *In vivo* animal models will also be of significant help in understanding the possible fetal genetic factors of CHB. Since CHB disease incidence is low, it makes large genetic studies difficult. However, an inbred large animal population may help to give us an indication of what genes may be important in the human disease.

There are a number of *in vitro* and *in vivo* models designed to better understand how CHB develops and to explain the mechanisms of the pathology. The *in vitro* studies demonstrate CHB effects by adding Ro/La positive IgG, or affinity purified Ro/La IgG to perfused hearts, commonly rabbit or human from aborted fetuses. Most groups induce *in vivo* effects of CHB by immunizing rats, mice or rabbits with Ro52, Ro60 and La proteins or peptides or by transferring IgG from mothers of CHB children to mice.

This indicates that the antibodies are an important factor and researchers are in agreement about their involvement. However, the specificity of the antibody and what the target of these antibodies is, is however a topic of debate. I will give an overview of the different models and their success in inducing CHB symptoms.

2.5.1 Ro52, Ro60 and La induce AV block in animal models

A number of animal models are available to help understand the induction of the disease CHB. Summarizing CHB models, it is evident that Ro52 is most associated with the induction of AVB block (Table 4). Higher degrees of AV block are also only induced in models where mice and rabbits are immunized with the Ro52 protein. However, only 5/197 (2.5%) pups (mice and rabbits) developed AV block II-III in three studies where Ro52 immunization was used (Boutjdir 1997, Miranda-Carus 1998b, Xiao 2001), so this indicates that second- and third-degree AV block is uncommon in animal models. The incidence of first-degree AV block in these same 3 models taken together is 21% (41/196 mouse and rabbit pups), which is not far from the human situation, where the incidence of first-degree Av block in Ro52 positive women is close to 30% (Sonesson 2004).

Inducing factor	Species (strain)	Total AVB (%)	2º/3º AVB	Reference
Ro52	Mouse (BALB/c)	**25**	2/20 pups	Boutjdir 1997
	Mouse (BALB/c)	**9**	2/56	Miranda-Carus 1998
	Rabbit (New Zealand)	**12**	1/121	Xiao 2001
Ro52-β	Mouse (BALB/c)	**12**	5/86	Miranda-Carus 1998
Ro52 (mouse)	Mouse (BALB/c)	**9**	0	Miranda-Carus 1998
Ro52-peptide (aa 200-239)	Rat (DA)	**19**	0	Salomonsson 2005
Ro52-peptide (aa 365-382)	Mouse (BALB/c)	**0**	0	Eftekhari 2001
Ro52-peptide (aa 366-379)	Mouse (BALB/c)	**0**	0	Eftekhari 2001
Ro60	Mouse (BALB/c)	**19**	0	Miranda-Carus 1998
	Mouse (C3H/HEJ)	**14**	0	Suzuki 2005
La	Mouse (BALB/c)	**7**	0	Miranda-Carus 1998
	Mouse (C3H/HEJ)	**7**	0	Suzuki 2005

Table 4. A comparison of AV block induced in animal models with Ro52, Ro60 and La protein and peptides. Ro52 appears to be most associated with AV block induction and higher degrees of AV block are only demonstrated in Ro52 models.

Three different Ro52 peptides were used to try and induce AV block in rats and mice. One is the Ro52-p200 peptide, antibodies to which have been shown to be associated with a higher risk for CHB pregnancy in humans (Salomonsson 2002), and also demonstrated to bind to fetal rat cardiomyocytes and cause dysregulation of calcium levels and cell death (Salomonsson 2005). This peptide induced first-degree AV block in 19% (10/52) of DA rat pups (Salomonsson 2005).

The other two Ro52 peptides (aa 365-382, and aa 366-386) have been described to cross-react with the 5-HT_4 receptor. It is suggested that these Ro52 antibodies antagonize the serotonin receptor and could explain the symptoms of CHB (Eftekhari 2001). Eftekhari and colleagues were able to demonstrate that pups from mice immunized with the 5-HT_4 peptides displayed bradychardia, AV block, as well as skin rash and neuro motoric problems (Eftekhari 2001). However as evident by their data, these Ro52 peptides which cross react with the 5-HT_4 receptor are not immunogenic in an *in vivo* system. More recent studies have demonstrated that the 5-HT_4 receptor is important for the cardiovascular and central nervous system development in BALB/c mice and that anti-receptor antibodies result in anatomical abnormalities which would help to explain the effect they are seeing in these mice (Kamel 2007).

Immunization of BALB/c mice with the Ro52-β protein (aa 169-245 of Ro52) induced AV block in 12% (10/86) in total, where half of the affected pups (5/86) had complete AV block (Miranda-Carus 1998b). This is the highest incidence of third-degree AV block in any model to date. Immunization with mouse Ro52 in this same

study induced only 9% (2/22) first-degree AV block in pups (Miranda-Carus 1998b). One group has also induced AV block in 33% of C3H/HeJ mice immunized with Calreticulin (Suzuki 2005). But these results, along with the Ro52-peptide immunizations have not been repeated.

Ro60 and La induce first-degree AV block in total looking at the results of 2 studies, with an incidence of 17% (16/97 mouse pups) for Ro60 and and 7% (4/55) for La (Miranda-Carus 1998b, Suzuki 2005). As suggested in human studies, Ro60 and La demonstrate to be less associated with CHB induction (Julkunen 2004, Salomonsson 2002, Fritsch 2005).

In conclusion, immunization with Ro52 protein in mice was observed to be the best way to induce higher degrees of AV block, and also a higher incidence of first-degree AV block than Ro60 or La immunization. Ro52 p200-peptide also induced first-degree AV block better than Ro60 and La immunization, but no second- or third-degree AV block was observed there indicating that immunization with a peptide may be more difficult, since the conformation of the peptide is important for its immunogenicity.

2.5.2 Induction of AV block with Ro/La positive serum

Other models include studies where Ro/La sera and affinity purified sera are used to induce block in mice and in *in vitro* models with perfused rat and human hearts. There is only one IgG transfer model described, where affinity purified IgG from 2 mothers with CHB pregnancies were used to induce AV block in BALB/c mice in 88% (14/16), 90% (9/10) and 47% (14/30) of pups injected 8, 11 or 16 days gestation, respectively (Mazel 1999). Bradycardia was also demonstrated in this study in 44% (7/16), 70% (7/10) and 33% (10/30) of the pups injected 8, 11, or 16 days gestation. Transferring the correct antibody specificity (IgG from mothers with CHB pregnancy) induced a higher incidence of first-degree AV block in these pups than the Ro52-immunization models did, however, second- and third-degree AV block was not observed. This study did however reveal that the induction of AV block by injection of purified sera in the animal model is time dependent (Mazel 1999), which corresponds with the human situation where AV block is usually detected at 18-24 weeks gestation.

Boutjdir and colleagues also demonstrated that IgG from mothers with CHB children, or affinity purified Ro52 sera, cause AV block in a Langendorff beating rat heart model (Boutjdir 1998), as well as in a human perfused heart (Boutjdir 1997). Also, a number of studies have employed a model using Langendorff-perfused rabbit hearts to demonstrate the effects of Ro/SSA and La/SSB positive IgG on cardiac conduction. AV block and sinus bradycardia were seen in different degrees (Restivo 2001, Viana 1998, Garcia 1994, Hamilton 1998). Qu and colleagues have adopted a model where they add Ro/SSA and La/SSB positive IgG to human and rat fetal cardiomyocytes and demonstrate inhibition of L- (Xiao 2001, Qu 2001, Qu 2005) and T-type calcium channel currents (Xiao 2001). They believe the pathogenic effects of the antibodies are due to a cross-reaction with calcium channel subunits.

Creating a model of CHB using purified IgG from mothers which have had CHB pregnancies seems to be an effective way to induce first-degree AV block in mice. However, complete AV block was not induced in this model even though the incidence of first-degree block was high. It is possible that the concentration of the heart block inducing antibodies was not high enough in IgG transferred to the mice.

The use of Ro/La serum to induce AV block in hearts does not give us information about the antibody specificity and is only useful as a first step in developing a model of CHB.

2.6 GENETIC INFLUENCES IN AUTOIMMMUNE DISEASE

Autoimmmune diseases are chronic conditions initiated by loss of immunology tolerance to self antigens. Autoimmune disease share a number of characteristics that suggest common etiologic pathways or mechanisms, including reactivity to self antigens by the humoral and/or cellular immune system, as well as genetic associations with human leukocyte antigen genes and with non-HLA genes (Ghodke 2005, Serrano 2006). Generally autoimmune disease has a higher incidence in females compared to males (Lockshin 2006).

2.6.1 MHC and non-MHC influences

Genetic predisposition to complex autoimmune disease was first identified by analysis of concordance rates in monozygotic twins. The monozygotic disease concordance rate varies between 15% for rheumatoid arthritis (RA), 20% for multiple sclerosis (MS) and 40% for SLE, but the disease concordance rate for dizygotic twins is lower suggesting that multiple genes shared by each pair of twins greatly influence their susceptibility to disease (Wandstrat 2001). This model infers that an individual will develop disease when their susceptibility to disease exceeds a certain threshold. This threshold is determined by the cumulative content of disease susceptibility alleles in that individual. It has been demonstrated that genetic predisposition is a major factor in disease susceptibility. MHC influence is the most powerful genetic influence in most autoimmune diseases (Ghodke 2005), but linkage analysis in a number of affected families has established a strong relationship between non-MHC chromosomal susceptibility regions and autoimmune disease (Serrano 2006).

2.6.1.1 MHC genes

The major histocompatibility complex (MHC) is a gene-dense region of the human genome on Chromosome 6p21.31. The complex covers > 120 expressed genes (1999). The MHC is divided into three major regions: class I, class II and class III. The region is associated with more diseases than any other region of the genome, especially the autoimmune diseases (1999).

2.6.1.2 MHC region in rat and mouse

Since my work involves genetic studies investigating the MHC and non-MHC influence in CHB using a rat model, I will give an overview of the organization of the rat MHC region, called the RT1 region in rats, compared to the human HLA and the mouse H-2 region (Figure 6). The human HLA is located on chromosome 6 (*HAS 6*), H-2 on mouse chromosome 17 (*MMU 17*) and RT1 on rat chromosome 20 (*RNO 20*). The MHC in humans (HLA), mice (H-2), and rats (RT1) was originally identified by its prominent role in transplant reactions and then in the immune response to various

immunogens and infections (McDevitt 1969, Benacerraf 1972, Zinkernagel 1979). Genes of the MHC contribute to the susceptibility of several autoimmune diseases and their animal models (Vyse 1996, McDevitt 1998). These MHC associations are thought to arise through the binding and presentation of antigenic peptide fragments of potential autoantigens to MHC class I and/or class II molecules leading to activation of specific T cells and leading to activation of immune cells in a cascade which eventually causes tissue damage and inflammation. There are however some proteins encoded in the MHC region which have non-immune-related functions (Klein 1986).

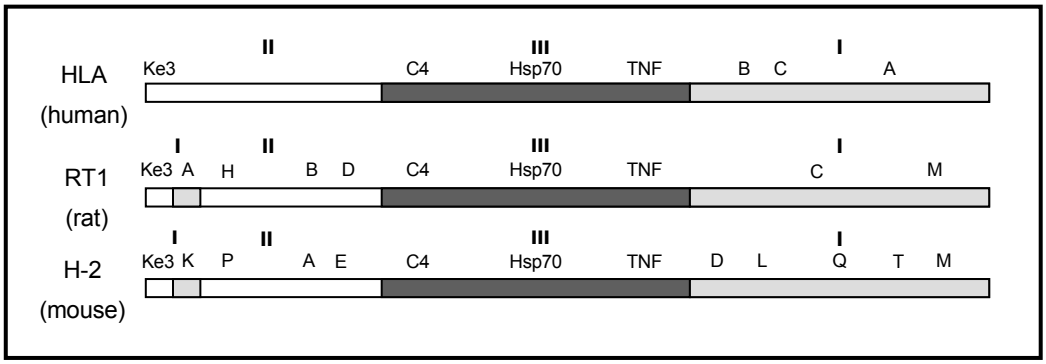

Figure 6. The organization of MHC in human, rat and mouse (Weisert 1999).

2.6.1.3 Non-MHC genes

Non-HLA genes have been associated with the development of rheumatoid arthritis (RA), autoimmune thyroid diseases (ATD), Type 1 diabetes (T1D), multiple sclerosis (MS), and systemic lupus erythematosus (SLE). These diseases represent 50% of all autoimmmune disease (Serrano 2006). Besides HLA, there are 3 non-HLA genes which have been convincingly associated with several autoimmune diseases. They include, *Cytotoxic T lymphocyte associated antigen 4 (CTLA4), Protein tyrosine Phosphatase (PTPN22) and Tumor necrosis factor-α (TNF)*.

CTLA-4 is a negative regulator of T cells, and polymorphisms within the gene have been associated with a variety of T-cell associated diseases (Serrano 2006). *PTPN22* encodes a lymphoid tyrosine phosphatase (Lyp) involved in inhibition of T-cell activation. Lyp is an important molecule in the regulation of T-cell antigen receptor signaling in memory/effector T cells. Genetic variations of this gene have been shown to be important in a number of autoimmune diseases such as T1D, RA, SLE, ATD and primary Sjögren's syndrome (pSS), but not in MS (Criswell 2005). In the inflammatory response cytokines play an important role as immune cellular response regulators. It is common in autoimmune disease to find high levels of cytokines and their receptors. An important member of this proinflammatory cytokine group is TNF-α. The *TNF* gene, is a non-HLA gene, but it is located in the MHC region, and it has been implicated as a susceptibility factor in a number of studies and diseases such as RA, SLE and pSS (Correa 2005).

3 AIMS OF THE THESIS

For simplification this thesis has been divided into 3 parts, where **PART I** describes studies characterizing Ro/SSA and La/SSB antibodies and their involvement in CHB (**papers I, II, IV, VI**); **PART II** describes two genetic studies of CHB with a patient case study and an animal model (**paper III, IV**), and **PART III** which encompasses the Ro52 protein and its function (**paper V, VI**).

Part I: Characterization of Ro52 antibodies in CHB

The aim of these studies was to investigate the significance of Ro52-p200 antibodies as a marker for congenital heart block pregnancies, characterize the Ro/SSA and La/SSB antibodies in mothers with congenital heart block, to develop an experimental model of CHB and to produce monoclonal Ro52 antibodies for use in *in vitro* and *in vivo* studies. Specific questions asked were:

- Are Ro52-p200 antibodies a good marker for CHB pregnancies?
- What other factors related to the Ro/La autoantibodies are important for CHB development?
- Can the heart block inducing capabilities of Ro52 antibodies be verified in an experimental model?

Part II: Genetic influence in CHB

It has been demonstrated that specific human genotypes are associated with Ro/SSA and La/SSB antibody positivity, but a genetic association with CHB in the children has not been shown or an association of congenital heart block with development of autoimmune disease later in life. This part aims to describe a genetic influence in a case study of a child with congenital heart block and to dissect genetic influence in a rat model of heart block. Since the Ro52 antibodies are necessary but not sufficient to cause disease in congenital heart block, we speculate that a fetal factor such as genetics is involved in the development of disease. Specific questions asked were:

- Is CHB associated with autoimmune disease later in life?
- Are maternal or fetal genes involved in congenital heart block development?

Part III: Function and localization of Ro52

Although antibodies to Ro52 are present in a number of rheumatologic diseases such as SS and SLE, the function of the protein was not known. Cellular localization of the Ro52 protein has also been unclear due to conflicting results over the years. The aim of these studies was to understand the function and cellular localization of Ro52. Specific questions asked were:

- What is the function of the Ro52 protein?
- Is the cellular localization of Ro52 cytoplasmic or nuclear?

4 RESULTS AND DISCUSSION

4.1 PART I. CHARACTERIZATION OF RO52 ANTIBODIES IN CHB

In order to understand the mechanism of fetal heart tissue injury in CHB, it is first important to characterize the antibodies responsible for the pathology. Many studies have demonstrated the significance of Ro52 antibodies in CHB development (Julkunen 2004, Salomonsson 2002, Fritsch 2005), but there is still some uncertainty of the role Ro60 and La antibodies may play in the disease pathogenesis.

I have aimed at investigating other factors which may be involved in initiation of CHB, such as Ro/SSA and La/SSB antibody levels, the influence of different Ro52 IgG subclasses, as well as profiles of the antibodies transferred to the child. It has also been important to ascertain whether or not an assay for Ro52-p200 antibody levels can be useful as a marker in a second-step analysis for CHB-risk pregnancies in the Swedish population, as well as in other populations.

In order to understand the *in vivo* effects of these antibodies studied, it was important to (1) develop an experimental model of CHB, and to (2) produce Ro52 monoclonal antibodies with different specificities in order to investigate their pathogenecity in *in vitro* and *in vivo* models.

4.1.1 Ro52-p200 peptide antibodies as a marker of CHB-risk

In previous studies it has been shown that antibodies towards this specific Ro52 peptide p200 (corresponding to aa 200-239 of Ro52, encompassing the leucine zipper motif) are more common in mothers with fetuses affected by congenital heart block (Salomonsson 2002). In order to confirm these results in a large international group of patients (**Paper I**), 515 sera were collected in total, from congenital heart block pregnancies and control sera, from Finland, Sweden and the United States.

4.1.1.1 Detection of Ro52-p200 antibodies

In order to improve the assay for detection of Ro52-p200 antibodies a new protocol was developed at the Clinical Immunology Department at Karolinska University Hospital. To allow free folding of the alpha helical p200 sequence and to give a set orientation to the peptide during assay performance, biotin was conjugated at the N-terminal end during synthesis before coating to streptavidin-plates and subsequent ELISA. This was of importance for the Ro52-derived p200 peptide, as the epitope formation and antigenicity of aa 200-239 of Ro52 is dependent on correct folding and structure (Ottosson 2005). Intra- and inter-assay variability for the ELISA were established at 3% and 3.8%, respectively (**Paper I**). Analysis of all 3 national cohorts together (Finnish, Swedish and American), including 515 samples, demonstrated that the mothers of fetuses with second- and third-degree AV block had significantly higher Ro52-p200 levels than mothers with healthy children **(Paper I)** ($p<0.001$).

Currently, the method of screening for CHB-risk pregnancies is often by determining presence of Ro/SSA and La/SSB antibodies, and specifically presence of antibodies to Ro52. We therefore performed an analysis of Ro52-p200 antibody levels exclusively in Ro52 positive sera to determine if anti-p200 levels are a better marker

than the current method of detection. It is not practically possible to follow all Ro52-positive pregnancies with ECG, and not economically justifiable considering the low incidence of disease. It is therefore important to find a more specific marker for these high risk pregnancies.

Because of substantial differences in p200 levels between the three populations, as well as differences in inclusion criteria and diagnostic procedures, we found it necessary to analyze the national cohorts separately for more detailed analyses. Finnish and American sera generally had lower Ro52-p200 antibody levels than the Swedish sera. This could possibly be due to factors such as the time of sampling, as they were often taken many years after affected pregnancies, and storage of samples, since the Finnish and American cohorts have been collected for decades, they have certainly been thawed and frozen a number of times. It is also possible that the difference is due to ethnic or genetic differences in the populations. We found significantly higher levels of Ro52-p200 antibody levels in sera from mothers of fetuses with second- and third-degree AV block compared to mothers of fetuses with NHR in the Swedish and American cohorts, but not in the Finnish patients **(Paper I)**.

Recent studies by Clancy and colleagues analyzing sera from the Research Registry for Neonatal Lupus did not confirm p200-binding antibodies as specific for children with complete congenital heart block, although the antibodies were present in the majority of sera (Clancy 2005). The discrepancy in results, as the difference was significant when we analyzed the same samples, could be due to differences in the assay. Peptide coating was performed differently in the assay by Clancy, where the peptide was attached directly to the surface of the ELISA plate. In our study the peptide was conjugated to biotin at the N-terminal end and attached to streptavidin-coated plates allowing for proper folding and orientation. It has been demonstrated that the antigenicity of the p200 peptide is dependent on correct folding and structure (Ottosson 2005). Other differences such as serum dilutions and incubation times exist, as well as the fact that Clancy detected several Ig-isotypes in one combined signal, whereas we have detected only IgG, as these are the immunoglobulins which cross the placenta during pregnancy (**Paper I**).

There are most likely a number of undiagnosed first-degree AV block fetuses in the normal heart rate group both in the American and the Finnish cohort, as these pregnancies were not monitored by fetal Doppler echocardiography (**Paper I**). This could explain the lack of significance in the Ro52-p200 antibody levels between the mothers with fetuses which had second- and third-degree AV block compared to those having children with normal heart rate in the Finnish cohort. Mother's with Sjögren's syndrome in the Finnish cohort had higher Ro52-p200 antibody levels, which is in agreement with previous studies demonstrating that higher Ro and La antibody levels have been observed in these patients (Harley 1986). Many of the controls in the Finnish cohort had Sjögren's syndrome which could also contribute to higher Ro52-p200 antibody levels in the controls.

4.1.1.2 Significance of detecting first-degree AV block

There is controversy surrounding the finding of first-degree AV block *in utero* (Sonesson 2004), since there is a discrepancy in the reference values for a prolonged PR interval (Glickstein 2000, Andelfinger 2001, Sonesson 2004, Nii 2006). Several authors have demonstrated that AV time intervals are positively correlated with

increasing gestational age (Nii 2006, Andelfinger 2001, Glickstein 2000), which emphasizes the importance of well established reference values. A study by Sonesson *et al* demonstrated an incidence of 33% (8/24) of first-degree AV block in fetuses diagnosed *in utero* in a Ro/SSA positive group of women (Sonesson 2004). Although the high incidence of AV block has been questioned (Buyon 2005, Rein 2005), the reference values in this study were confirmed in an independent study (Nii 2006).

It is thought that a first-degree AV block is a first stage in CHB development and that this may develop into higher degrees of block. There may not be a direct clinical significance in detecting first-degree AV block *in utero* since most revert spontaneously (Sonesson 2004), and do not seem to have obvious clinical effects on these children at birth. However, there may be an effect on these children which is not recognized as of yet since these children have not usually been detected and represented in large studies of CHB and they may give important clues to the pathogenesis of CHB. Cases of first-degree AV block *in utero* may represent the fetuses which have some resistance to the antibody insult which is apparent in the second- and third-degree AV block cases. The long term effects on these children have not been determined and it is important from a biological stand-point to identify these children as they may give us more insight into the pathology of CHB and especially clues as to why not all fetuses are equally affected.

In **paper I** in the Swedish cohort, a distinction between fetuses with normal conduction, first-degree AV block, second-degree AV block as well as third-degree AV block was possible. An analysis of Ro52-p200 levels revealed no difference between mothers with fetuses having signs of first-degree AV block and the group of mothers with fetuses having second- and third-degree AV block. There was however a significant difference between both these groups with mothers giving birth to children with normal conduction ($p<0.001$). This indicates that first-degree AV block pregnancies do not have different antibody levels from the second- and third-degree AV block pregnancies, but both these groups differ significantly in Ro52-p200 antibody levels from Ro52 positive pregnancies with unaffected children. They are not a separate entity in initiation of disease, but they probably are able to handle the immunological insult to the heart better than the fetuses with complete block. This is also supported by case studies of mothers having effects on their cardiac conduction systems (Dörner 1993), possibly indicating that they too are affected but are better equipped to handle this inflammatory reaction which probably occurs in all Ro52-p200 positive pregnancies.

4.1.1.3 Ro52-p200 antibody screening and surveillance of CHB

In **paper I** we show that in the Swedish population, p200 antibody levels are an effective marker of incomplete and complete atrioventricular block. As discussed in the paper, the American and Finnish populations were not well characterized for first-degree AV block and this can also account for p200 antibodies not being as good a marker in these populations. Another possibility is assay procedures and performance.

As a result of this p200 antibody study in **paper I,** as well as studies by Salomonsson *et al* which first identified the association of p200-specificity with CHB pregnancies (Salomonsson 2002), a study by Salomonsson *et al* correlating p200 antibody levels with PR intervals, as well as *in vivo* and *in vitro* studies demonstrating the pathogenicity of these antibodies (Salomonsson 2005), a p200 assay has been set up

at the Clinical Immunology Department (Karolinska University Hospital) for further screening of Ro52-positive women in order to help in selecting high risk pregnancies for Doppler echocardiographic surveillance during susceptibility weeks (see Figure 7).

A program is in the process of being established at the Karolinska University Hospital today which involves the following: Patients which are positive for antinuclear antibodies (ANA), or suspected of SLE or SS are screened for Ro/SSA and La/SSB antibodies. If Ro/SSA antibodies are identified, a further analysis of Ro52 antibodies is undertaken, especially if a pregnancy is identified, or planned. Once the patient is found to be Ro52 positive, an ELISA identifying p200 antibody levels is run in a highly reproducible assay at the Department of Clinical Immunology, at the Karolinska University Hospital. A p200 index value is an optical density (OD) value normalized to a high titer patient serum. A p200 index < 30 has been evaluated to equate to a low risk for a CHB pregnancy, whereas a p200 index > 30 is associated with a medium to high risk for developing AV block in the fetus (Paper I).

High risk pregnancies can be monitored with weekly echocardiograms during susceptibility weeks (18-24 weeks gestation) and with ECG at birth. Upon identification of a first-degree AV block, more frequent echocardiograms may be initiated depending on the level of prolongation of the PR interval. Identification of a progressing first-degree or second-degree AV block however leads to an initiation of treatment with betamethasone, which in some cases does help to revert the block or at least stop the block from progressing to a complete third-degree AV block.

Recommendations for treatment upon discovery of a complete third-degree AV block are not established in Sweden, and is evaluated for every individual case. However, treatment of third-degree AV block is practiced in some countries (Friedman 2002), but views on this differ between clinicians. However, betamethasone should be considered to treat complications such as fetal hyrops, low ventricular rate (< 55 bpm), fibroelastosis, as well as myocarditis (Costedoat-Chalumeau 2005b). The occurrence of complete fetal AV block usually leads to fetal demise or pacing at birth.

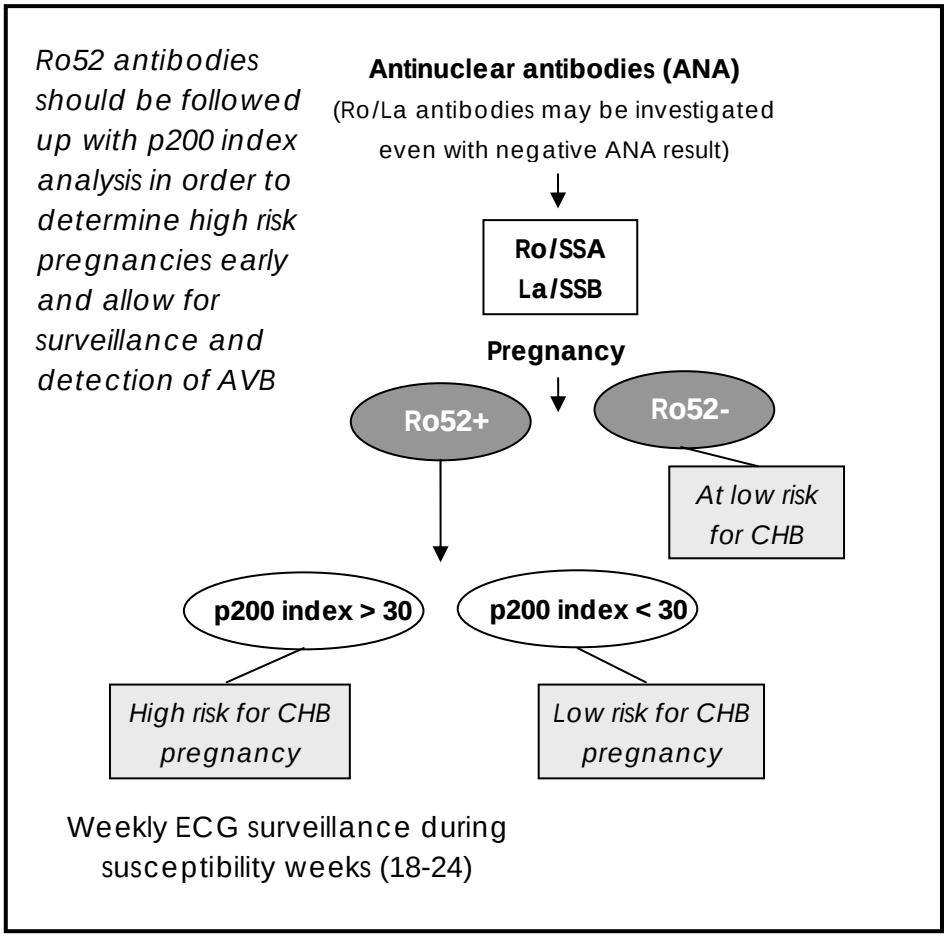

Figure 7. Future screening of CHB risk pregnancies with the p200 antibody assay and ECG surveillance during susceptibility weeks.

4.1.2 Ro/La antibody levels and subclasses in pregnancy

Mothers with Ro/SSA and La/SSB antibodies may have a healthy child even after giving birth to an affected child which indicates that factors other than Ro/SSA and La/SSB specificity play a role in the penetrance of the disease. There have been some small studies investigating the significance of levels of antibodies (Derksen 1992, Dörner 1995), as well as different antibody subclasses (Tseng 1996), but on a limited number of patients without conclusive results. The study by Buyon and colleagues (Tseng 1996) investigated subclasses of the Ro/La antibodies in mothers with neonatal lupus, but since control mothers had also had previously affected pregnancies this made the results difficult to interpret.

Longitudinal studies of Ro/SSA and La/SSB autoantibody levels in rheumatic patients have indicated that levels remain stable in patients with Sjögren's syndrome over long periods of time, but that levels in young or recently diagnosed SLE patients may fluctuate (Wahren 1998, St Clair 1990, Meilof 1997, Praprotnik 1999).

4.1.2.1 Ro52, Ro60 and La antibody levels in pregnancy

We investigated Ro52, Ro60 and La antibody levels throughout pregnancy in 10 Ro/SSA and La/SSB positive women, including 11 pregnancies. We demonstrate that Ro52 and Ro60 antibody levels significantly decrease from early to late pregnancy, and that this trend can also be seen in La antibody levels (**Paper II**). The antibody levels fluctuated over time, but did not change enough to make an anti-Ro/SSA and anti-La/SSB positive mother negative or an anti-Ro/SSA and La/SSB negative mother positive. There were also no obvious dips or peaks in the pregnancies affected by atrioventricular block compared to the healthy pregnancies.

Paper II demonstrated that there was a decrease of Ro52, Ro60 and La IgG antibody levels from early to late pregnancy, possibly due to expanded plasma volumes affecting IgG levels (Ailus 1994). However, other studies demonstrate that hemodilution can not fully explain the drop in immunoglobulin concentration and that other factors such as transfer of antibodies to the child may play a role (Marolis 1971). Immune suppression as a physiological part of pregnancy has also been suggested (Thellin 2003).

Ro52 antibodies were present in all mothers with affected pregnancies (4 mothers/5 pregnancies), and only one of these mothers had antibodies to Ro60. Three out of the four mothers with affected pregnancies did have La antibodies. These results indicate that Ro52 may be more associated with CHB pregnancies than Ro60 and La, which has been demonstrated in previous studies (Julkunen 2004, Salomonsson 2002, Fritsch 2005), but our study was not large enough to demonstrate this association.

Since Ro52 antibody levels seem to be associated more with CHB and our studies have demonstrated an association with a Ro52 p200-peptide antibody (recognizing the Ro52 peptide corresponding to aa 200-239), we also focused our investigations on p200 antibody levels in these 10 Ro/SSA and La/SSB pregnancies.

4.1.2.2 Longitudinal levels of Ro52-p200 antibodies in pregnancy

Ro52-p200 antibody levels were also investigated longitudinally throughout pregnancy in mothers with pregnancies affected by AV block and mothers with healthy pregnancies (Strandberg unpublished data). The purpose of investigating Ro52-p200 antibodies through pregnancy was to determine if there was a peak or dip in antibody levels which possibly coincided, or proceeded, the gestational time AV block usually is detected.

These are the same patients which were included in the study investigating Ro52, Ro60 and La antibody levels (Table 5, see **Paper II** for more patient details), but p200 antibody levels were not explored in that study. Six Ro/SSA and La/SSB negative patients were also analyzed as controls for Ro52-p200 antibody levels and had OD values < 0.1 in all samples.

SSA/SSB positive patients	No of months followed	Maternal diagnosis	Child diagnosis
1	2	pSS	AVB III
2	15	SLE	AVB III
3	48	pSS	1. Sinus bradycardia, anemia, skin rash 2. Fetal and neonatal AVB I
4	10	SLE	AVB I, skin rash
5	84	SLE	Healthy
6	15	pSS	Healthy
7	27	pSS	Healthy
8	36	SLE	Healthy
9	66	UCTD	Healthy
10	78	SLE	Healthy

Table 5. Characterization of patients analyzed for Ro52-p200 antibodies through pregnancy. Brief description of mothers and children included in the longitudinal study in **Paper I** and analyzed for Ro52-p200 antibodies (Strandberg, unpublished data).

Four mothers, five pregnancies, affected by CHB (Figure 8 A) and six mothers with healthy pregnancies (Figure 8 B) were analyzed for p200 levels throughout pregnancy. Results indicate low levels of p200 antibodies in mothers with healthy pregnancies, except for mothers 6, 8 and 10 which have high levels of p200 antibodies (Figure 8 B). However, on observation, there is not as much of a dip of antibody levels at delivery as there is in the graph (A) depicting the p200 levels in mothers with affected pregnancies.

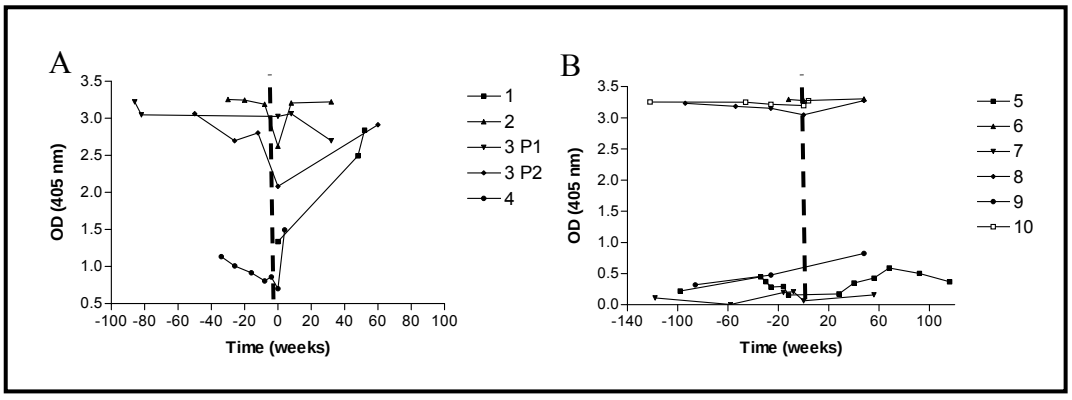

Figure 8. p200 antibody levels throughout pregnancy in Ro/SSA and La/SSB mothers affected by CHB pregnancies (A) and mothers with healthy pregnancies (B). The dotted vertical lines indicate 0 weeks which coincides with delivery. P1 and P2 indicate two pregnancies by the same mother.

4.1.2.3 Ro52 subclass antibodies

Since different IgG subclasses have different biological properties with respect to complement fixation, and binding of Fc receptors, immune responses to antigens may give different profiles of subclass antibodies. There have been few studies investigating

the Ro52, Ro60 and La IgG subclass antibodies and their involvement in CHB. One study investigated Ro52 subclass antibodies in patients with primary Sjögren's syndrome and demonstrated high levels of specific Ro52 IgG_1, as well as moderately high IgG_3 and IgG_4, and extremely low, undetectable levels of Ro52 IgG_2 (Lindström 1994).

In 1990 it was demonstrated in a study by Bennion *et al* that Ro/SSA antibodies are predominantly IgG_1 in SCLE and NLE sera (Bennion 1990). Tseng and colleagues investigated the subclass distribution in pregnancies affected and unaffected by development of CHB (Tseng 1996). There was no association of CHB with any Ro52, Ro60 or La subclass demonstrated, but a major drawback of the study was that the control group consisted mostly of mothers which had children with NLE in a previous pregnancy. It would be desirable to have mothers which have given birth to only healthy children as the control mothers.

Both Ro/SSA and La/SSB IgG and IgA antibodies have been demonstrated in breast milk, yet their implication in NLE seems minor since the IgG antibodies are already present in the infant circulation from passage of IgG across the placenta (Askanase 2002).

In **paper II** we investigated the antibody subclass distribution of Ro52-specific antibodies in the 10 Ro/SSA and La/SSB positive mothers. Ro52 IgG subclass antibody profiles were also compared at birth in 9 of the same mother/child pairs, and two additional mother/child pairs where samples at birth were available.

IgG_1 was the predominant Ro52 antibody subclass in all the patients, whereas Ro52 specific IgG_2, IgG_3 and IgG_4 levels were low in most patients (**Paper II**). This is in agreement with previous studies of Ro/La antibody subclass distribution (Bennion 1990, Tseng 1996).

This antibody profile did not change significantly throughout pregnancy, but IgG1 and IgG4 antibody levels decreased significantly from early to late pregnancy. There was no obvious difference in the longitudinal Ro52 subclass levels or profile between a mother with a healthy child, and a mother giving birth to an affected child.

Ro52 IgG_1, IgG_2, IgG_3 and IgG_4 profiles in the children at birth were similar to that of the mother, but the levels were lower. In summary, there were no specific Ro52 IgG subclass profiles which correlated with disease and the autoantibodies tended to decrease, rather than to increase, during pregnancy.

Several reports have indicated that Ro/SSA and La/SSB antibodies are dominated by an IgG_1 subclass profile (Lieu 1988, Tseng 1996, Wahren 1994, Pearce 1986, Lindström 1994). This is in agreement with our observations of all maternal Ro52 profiles being dominated by the IgG_1 subclass.

4.1.3 Induction of AV block in an experimental model of CHB

In order to study the pathogenic effects of Ro52 and the p200 antibodies, an animal model of heart block was developed. Colleagues had previously immunized DA rats with Ro52-p200 peptide and pups born to these rats demonstrated prolonged PR intervals (Salomonsson 2005).

To study the disease CHB, I immunized different rat strains (DA, PVG.AV1, Lew.AV1 and Lew rats) with Ro52 protein and used the fusion partner MaBP as control protein (**Paper IV**). See figure 9 for a schematic of the Ro52-immunization model.

Prolonged PR intervals were observed in approximately 45% of three of the rat strains and 10% in one strain. This calculation was made by taking the mean of the control group PR and adding 2 x the standard deviation, as a threshold for PR prolongation, or first-degree AV block. This was done separately for each rat strain.

Forty-five percent first-degree AV block is quite a high incidence of first-degree AV block compared with previous immunization models by other researchers (Boutjdir 1997, Miranda-Carus 1998b, Eftekhari 2001, Xiao 2001, Suzuki 2005, Salomonsson 2005). A comparison of experimental models of CHB in mice and rats where authors have immunized with different proteins or peptides to induce AV block is summarized in Table 6. All studies defined the first-degree AV block cut-off in the same way (average +2 SD of control litter), except for Eftekhari and colleagues (Eftekhari 2001) which defined a parameter as abnormal when they were over the upper limit of the highest value in the control group. Two studies did not define their AV block determination (Xiao 2001, Boutjdir 1997).

It is evident that different antigens immunized into different strains of mice and rats, as well as rabbits give a different incidence of AV block. Eftekhari had a low incidence of heart block in Ro52-peptide immunized mice (Eftekhari 2001), but this I believe is explained by the Ro52 peptide chosen for immunization, which does not prove to be immunogenic in an *in vivo* model even though it cross-reacts with the 5-HT_4 receptor.

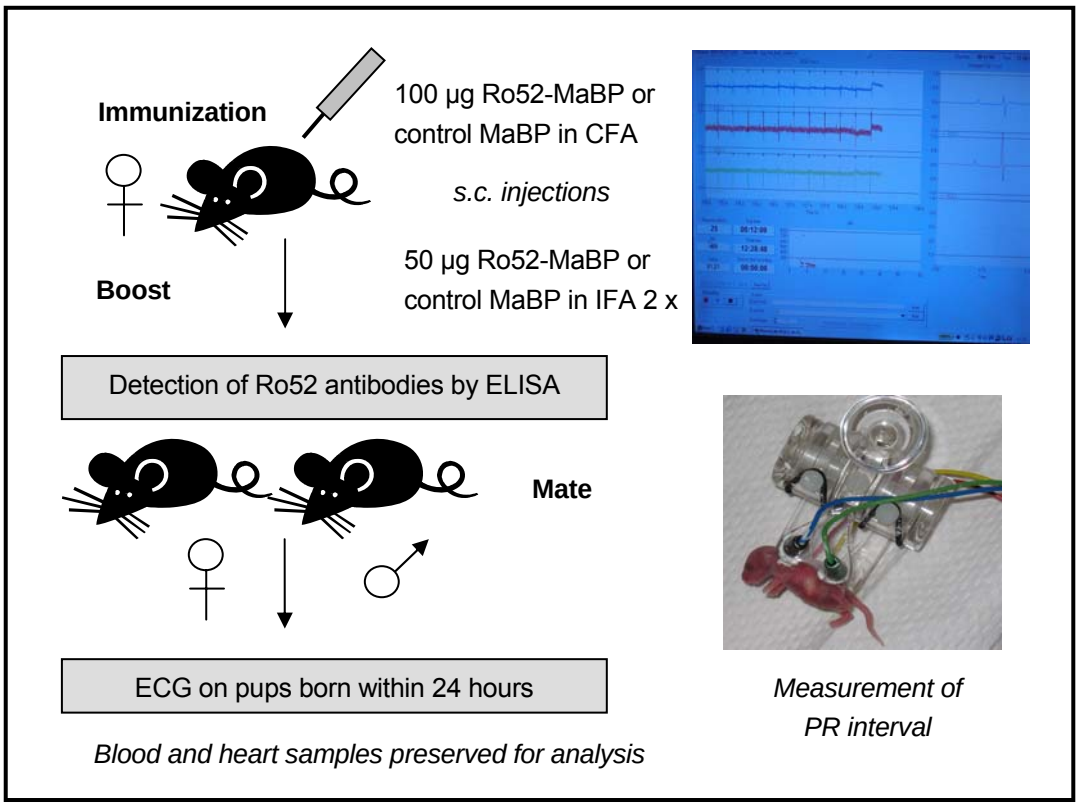

Figure 9. Ro52 immunization model of heart block. Rats were immunized and boosted with Ro52 or control protein, after Ro52 antibody titers were high, the rats were mated and ECG was performed on pups born within 24 hours of birth.

The lower incidence of heart block when immunizing DA rats with Ro52-p200 peptide (Salomonsson 2005) compared to immunizing DA rats with the full-length Ro52 protein (**Paper IV**) could be due to protein antigens allowing for a higher number of linear and conformational epitopes (Van Regenmortel 2001).

It is difficult to compare incidence of AV block with the incidence in the study by Suzuki *et al* because the group sizes vary between 6, 28 and 43 (Suzuki 2005). The protein Calreticulin induced 33% block in C3H/HEJ mice, but the group was very small with only 6 mice (Suzuki 2005). In the study by Miranda-Carus, Ro60 has induced more first-degree AV block in BALB/c mice than Ro52, but second- and third-degree AV block was induced in the Ro52 immunized mice only. The protein La seems to consistently induce less AV block than both Ro52 or Ro60 (Miranda-Carus 1998b, Suzuki 2005).

Unfortunately, no second-degree or third-degree AV block was observed in our rat immunization model, but many litters and individual pups have not been surviving long enough to perform ECG analysis. This may relate to higher degree of block, but it is difficult to ascertain the cause of death since the rats often eat their young upon poor health of the pup, or as a stress-related behavior of the mother. In the study by Xiao and colleagues, 20% of rabbit pups born died after birth (Xiao 2001), and they speculate that this is possibly due to third-degree AV block.

I believe our Ro52-immunization model is a good model of first-degree AV block, but it is uncertain if second and third-degree AV block is not ascertainable in these rats, or if once they are severely affected they simply do not make it to term. Hopefully, ultrasound observations of the pups during pregnancy in the future will help to establish how many pups are in each group in total and how many die *in utero*. Suzuki and colleagues have demonstrated an embryonic Doppler technique, but it is still not possible to detect dead pups, and the percentage of affected pups in embryonic Doppler versus fetal echocardiography suggested that some blocks may revert before birth and/or some pups are not surviving until birth (Suzuki 2005).

Other experimental models of CHB include *in vivo* models with transfer of Ro/La positive IgG to mice (Mazel 1999), as well as *in vitro* studies with perfused rabbit hearts (Garcia 1994, Viana 1998, Hamilton 1998, Restivo 2001), and human hearts perfused with affinity-purified Ro52 serum (Boutjdir 1997). We have in our studies been able to induced first-degree AV block in preliminary studies transferring Ro52-p200 monoclonal antibodies to DA rats (Dzikaite, preliminary data in Appendix A) and both first- and second-degree AV block transferring Ro52-p200 monoclonal antibodies to DBA1 mice (Dzikaite, unpublished data).

Antigen	Species (Strain)	Pups born	AVB (%)	AVB I	AVB II	AVB III	Reference
Ro52	Mouse (BALB/c)	20	**25**	3	0	2	Boutjdir 1997
Ro52-α	Mouse (BALB/c)	56	**9**	3	1	1	Miranda-Carus 1998
Ro52-β		86	**12**	5	0	5	
Ro60		54	**19**	10	0	0	
La		27	**7**	2	0	0	
Mouse Ro52		22	**9**	2	0	0	
G21V (5-HT_4 peptide)	Mouse (BALB/c)	29	**3**	2	1	0	Eftekhari 2001
C15Q (5-HT_4 peptide)		24	**3**	0	3	0	
R18L (Ro52 peptides)		20	**0**	0	0	0	
C15T (Ro52 peptides)		19	**0**	0	0	0	
Ro52	Rabbit (New Zealand)	121	**12**	13	1	0	Xiao 2001
Ro52 (p200 peptide)	Rat (DA)	52	**19**	10	0	0	Salomonsson 2005
Ro60	Mouse (C3H/HEJ)	43	**14**	6	0	0	*Suzuki 2005
La		28	**7**	2	0	0	
Calreticulin		6	**33**	2	0	0	
Ro52	Rat (DA)	49	**45**	22	0	0	Paper IV
	Rat (PVG.AV1)	48	**44**	21	0	0	
	Rat (LEW.AV1)	51	**47**	24	0	0	
	Rat (LEW)	49	**10**	5	0	0	

* Sukuki and colleagues also demonstrated non-advanced second-degree AV block in 3-9%, 16-17% and 8-13% of Ro60, La and Calreticulin immunized mice, respectively upon embryonic Doppler technique (tested at <18 days as well as ≥18 days).

Table 6. Summary of CHB immunization models in mice, rats and rabbit.

4.1.4 Monoclonal Ro52 antibodies for *in vitro* and *in vivo* studies

Monoclonal antibodies were developed for use in a number of studies investigating Ro52. *In vitro* studies included FACS staining of mouse and rat neonatal cardiomyocytes (Dzikaite, preliminary data, see Appendix A), fluo-4 measurement of calcium flux in cardiomyocytes after addition of different Ro52 monoclonal antibodies (Park, preliminary data), as well as immunofluorescence staining of cells to identify the

cellular localization of Ro52 under different conditions (**Paper V, VI**). *In vivo* studies include transfer of different Ro52 monoclonal antibodies to mice and rats to induce AV block as a model of CHB (Dzikaite, preliminary data, see Appendix A).

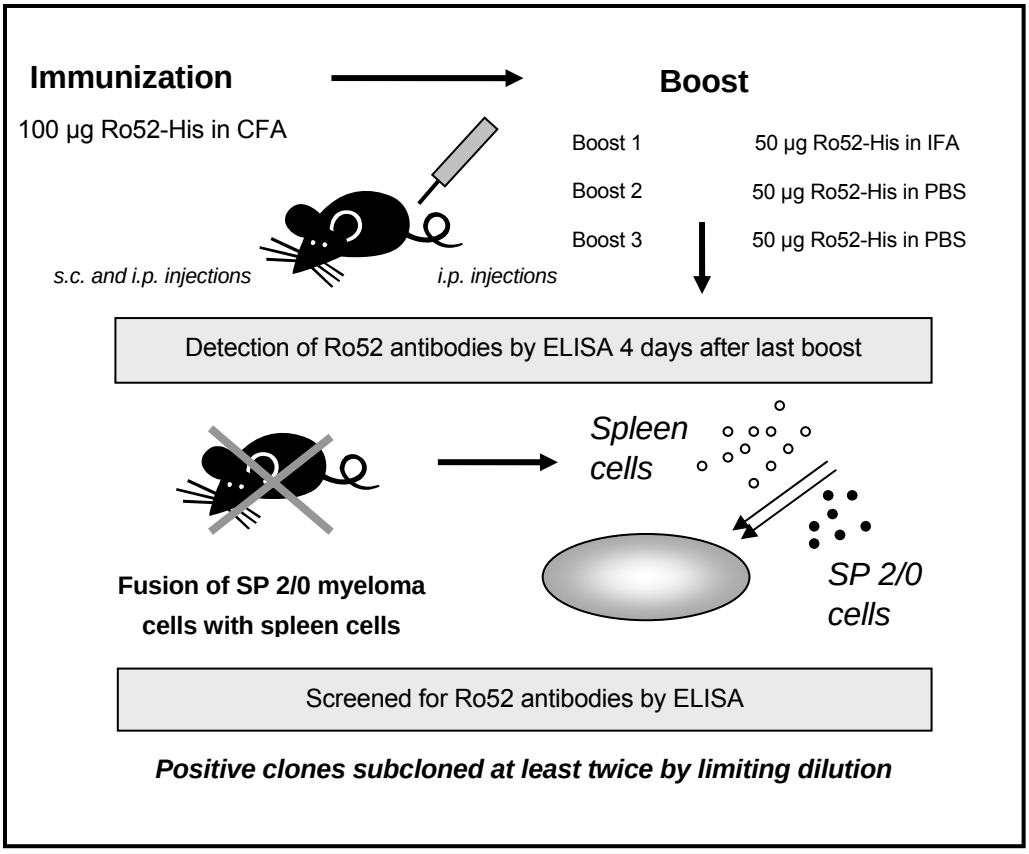

Figure 10. Schematic representation of hybridoma monoclonal antibody production. Four BALB/c mice were immunized and boosted with Ro52, and spleen cells were fused with SP 2/0 cells to make antibody producing hybridomas.

4.1.4.1 Characterization of Ro52 monoclonal antibodies

For generation of Ro52 monoclonal antibodies, four BALB/c mice were immunized with 100 µg Ro52-His protein in complete Freund's adjuvant (CFA), subcutaneous (s.c.) and intraperitoneal (i.p.) injections, and boosted three times i.p. with 50 µg Ro52-His, the first time in IFA, and the last two in PBS (Figure 10). Four days after the last boost, Ro52 antibody levels were confirmed in the mice and hybridomas were created by fusing the mouse spleen cells with SP 2/0 myeloma cells. After two weeks, hybridoma supernatants were screened for Ro52 IgG antibodies in ELISA and all clones of interest were subcloned at least 2-3 times. Antibodies with different specificities for Ro52 were produced. The most common antibody produced was antibodies against the coiled-coil region and specifically against the p200 peptide. This indicates that even in mice this is a common antibody epitope. Thirty-eight hybridomas were found to be positive after several screens (Figure 11), and a selection of these were sub-cloned 2-3 times each for further use.

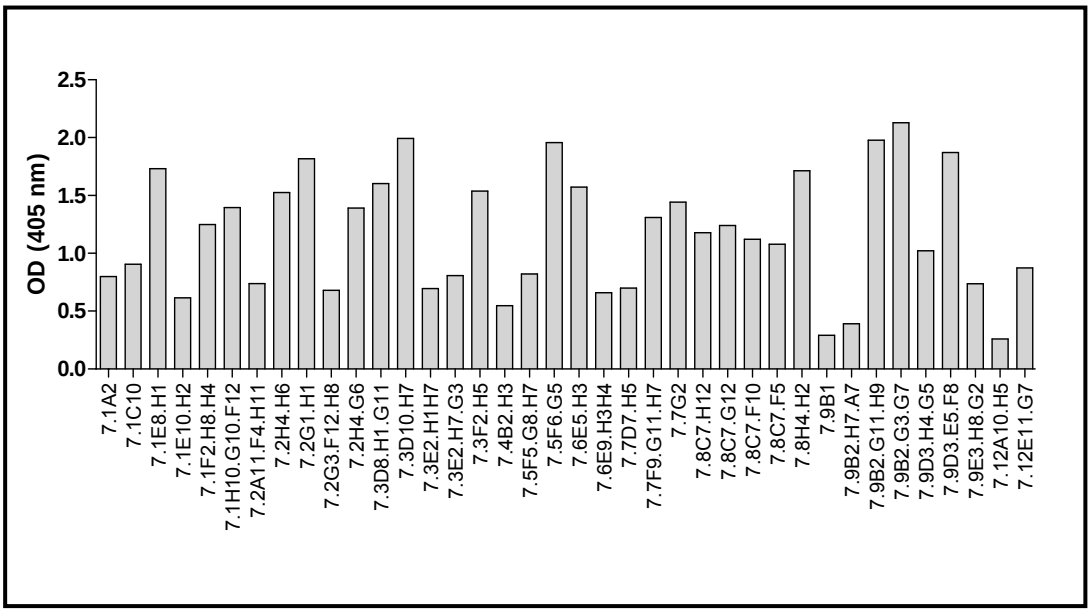

Figure 11. A panel of Ro52-positive monoclonal antibodies detected by ELISA.

Many of these hybridomas were further characterized with ELISA, western blot, immunoprecipitation as well as immunofluorescence (**Paper VI**). Of the eleven hybridomas selected for *in vitro* and *in vivo* studies one was specific for the RING finger motif, six were specific for the coiled-coil region and four were specific for the B30.2 region of Ro52 (Figure 12).

These eleven monoclonal antibodies were selected for further characterization and use in studies because of their mono-specific reactivity in all assays. They were highly specific for the different regions of Ro52. Antibodies specific for different regions of Ro52 would be critical for understanding what Ro52 specificity induces CHB. They would also be beneficial for *in vitro* studies of Ro52 function and localization and understanding the importance of different functional regions of the protein in biological processes.

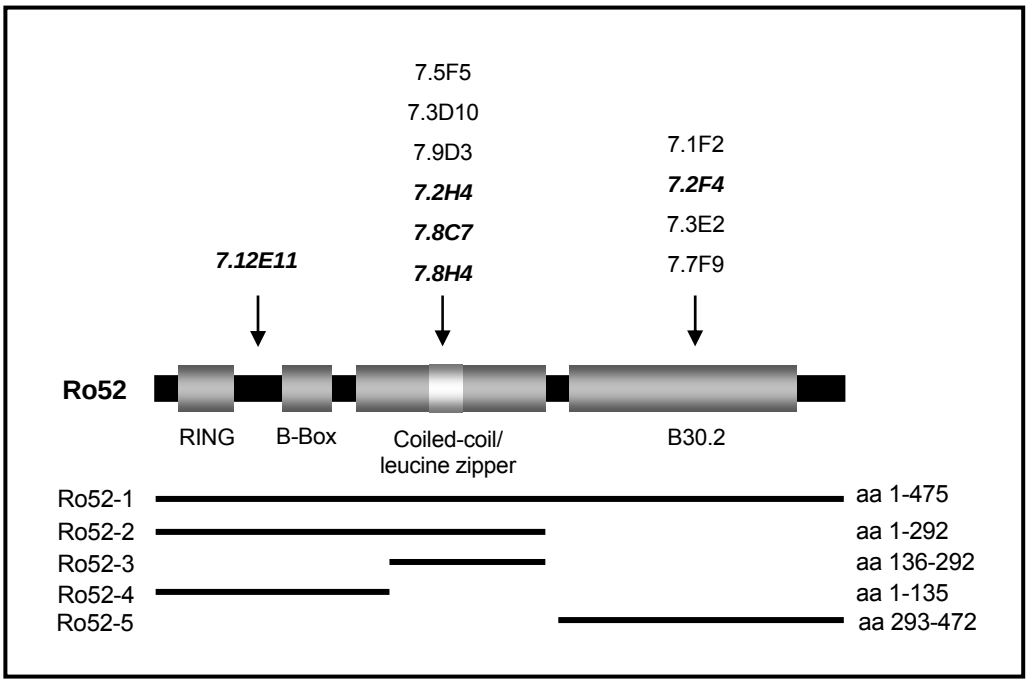

Figure 12. **A selection of Ro52 monoclonal antibodies available for *in vitro* and *in vivo* studies.** Ro52 protein and peptides (1-5) aid in illustratation of epitope specificity of monoclonal antibodies. Bold and italicized antibodies have been used in Ro52 localization studies (**paper VI**) as well as in preliminary transfer studies (Dzikaite preliminary data, Appendix A).

4.1.5 Summary Part I:

Taken together these studies indicate that Ro52-p200 antibodies can be a useful marker for detection of CHB-risk pregnancies. Furthermore, Ro/La antibody levels and Ro52 subclass profiles were not shown to be associated with CHB affected pregnancies, indicating that antibody specificity may be more important for the fetal outcome. It has been demonstrated that Ro52 p200-specific antibodies may play an important role both as a marker for congenital heart block risk, but also having a role in the pathogenesis of congenital heart block as demonstrated by our *in vitro* studies and animal models.

4.2 PART II. GENETIC INFLUENCE IN CHB

Both the fact that recurrence rates are low, and that twins can be discordant for disease, indicate that a genetic fetal factor is most likely responsible for susceptibility in CHB. Due to the low incidence of disease, it has been difficult to demonstrate any HLA associations in humans with CHB. Other genetic susceptibility factors in these children include their susceptibility to autoimmune disease later in life. Associations of CHB with autoimmune disease later in life are infrequent, but case reports indicate the need to follow up these children and to document their outcome.

4.2.1 Autoimmune disease risk in CHB

It has been demonstrated that specific genotypes are associated with Ro/SSA and La/SSB antibody positivity but an association of congenital heart block with development of autoimmune disease later in life has not been shown. **Paper III** is a case study which describes a young girl with congenital heart block, who at puberty develops SLE. The girl's mother developed SLE 7 years after her pregnancy and the maternal grandmother also had SLE.

The girl had a genetic background normally associated with Ro/SSA La/SSB antibodies, her HLA type was A1,25(10); B8(Bw6),62(15); Cw3,7; DR3,12(5),52; DQ2,7. Her SLE symptoms included butterfly rash, photosensitivity, skin rashes, polyarthritis, and severe headaches. Her laboratory analysis revealed that she was ANA positive, she had IgM antiphospholipid antibodies and was anti-DNA negative.

The mother was diagnosed 7 years after the child's birth with SLE and secondary Sjögren's syndrome. She had photosensitivity, skin lesions, polyarthritis, myalgias, involvement of the nervous system, and sicca symptoms. She was positive for ANA but negative for anti-DNA antibodies.

ELISA analysis of sera from the mother and child revealed that the mother was positive for Ro52, Ro60 and La with highest levels of Ro52 (Figure 13 A), whereas the daughter was negative for all of these antibodies (Figure 13 B), including the Ro52 peptides (Figure 13 D). Analysis of Ro52 peptides demonstrated that the mothers profile was dominated by antibodies to the p200 peptide of Ro52, but showed little or no reactivity to overlapping or neighboring peptides (Figure 13 C).

We have suggested that having certain factors predisposes a child with CHB to developing autoimmune disease in later life (**Paper III**). These factors include being female and not male, maternal diagnosis of SLE and not Sjögren's syndrome, as well as a genetic background for anti-Ro antibodies (Figure 14). This is substantiated by reports such as the girl described in our study, as well as 2 other reports of girls with CHB which were both 15-years of age when they developed autoimmune disease (Esscher 1979, Hubscher 1997). One 15-year old was described to develop an undefined connective tissue disease and she was positive for Ro/SSA and U1 RNP antibodies (Hubscher 1997). The other 15-year-old developed SLE at 15-years of age and had a mother with arthritis symptoms for many years (Esscher 1979). Because of the strong genetic predisposition to SLE, it is possible that CHB children to these mothers have an increased risk to SLE. But it is not certain if the risk is increased if the child displays symptoms of CHB or not.

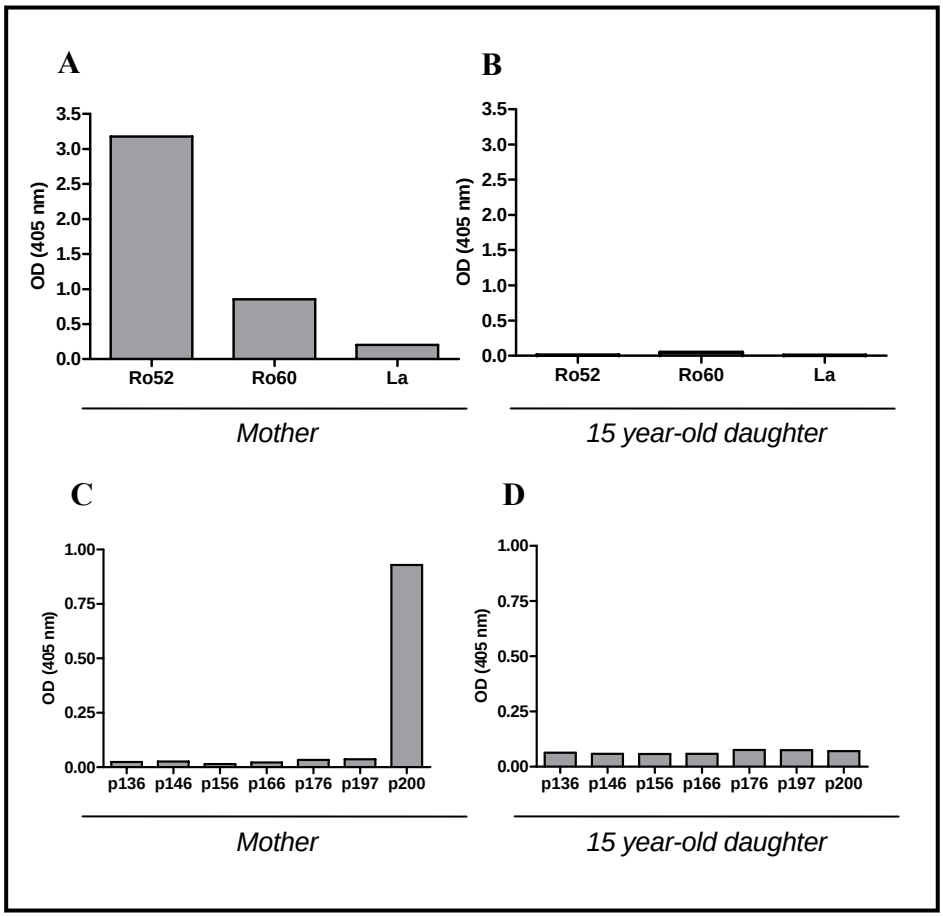

Figure 13. Ro52, Ro60 and La antibody levels in (A) mother and (B) daughter (Paper III, data not shown), as well as Ro52 peptide antibodies in (C) mother and (D) daughter.

In a long-term follow-up of 49 children with neonatal lupus (30 having CHB) by Martin *et al*, it was described that 2 females with CHB developed autoimmune disease (Martin 2002). One female developed psoriasis and iritis at 13-years of age, and the other female developed type 1 diabetes mellitus at 7-years of age. This may demonstrate that children with CHB are at risk for susceptibility to all kinds of autoimmune disease later in life, but that the risk is low. Three children in the study by Martin *et al* with cutaneous neonatal lupus rash also developed autoimmune disease in their first 10 years of life. Two developed jeuvenile rheumatoid arthritis and one Hashimoto thyroiditis. There have been other descriptions of children with neonatal lupus without CHB developing SLE later in life at the ages of 15 and 19 years old (Jackson 1979, Fox 1979).

However, other studies investigating the long-term outcome of children with CHB have not demonstrated development of rheumatic disease in 105 infants from an American study in 1998 (Buyon 1998b), or in 192 infants with CHB in a Finnish study (Eronen 2000). This paper describes no manifestations of autoimmune disease in the follow-up of 23 of 36 infants with CHB followed at a clinic in Germany since 1984 (**Paper III**). A smaller Italian study with 16 patients did not find any specific HLA related to CHB affected children nor describe any rheumatic disease in follow-up of children with CHB (Brucato 1995).

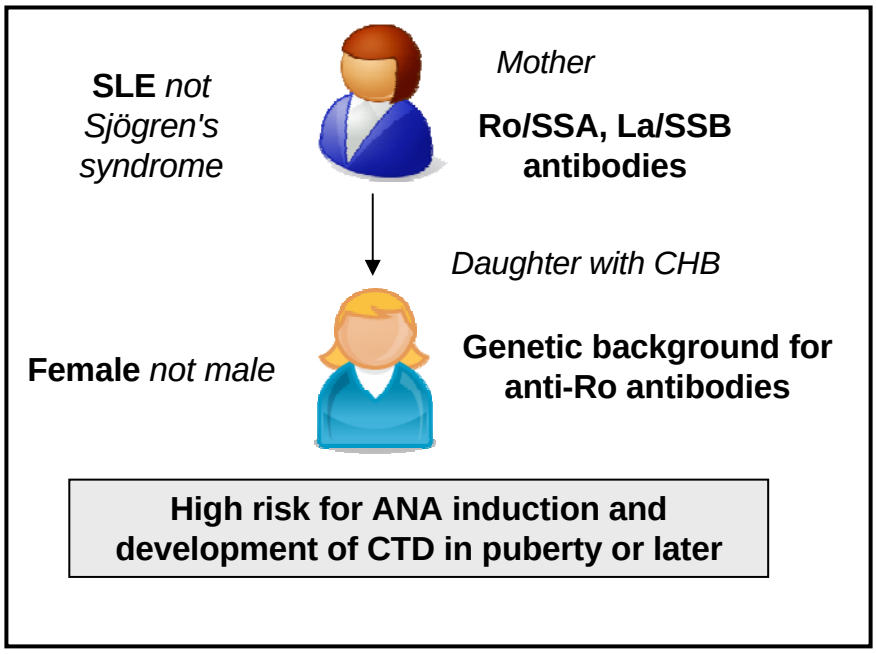

Figure 14. Proposed risk factors for CHB children developing rheumatic disease later in life.

Unfortunately, the number of cases which are necessary to determine if there is an increased risk for autoimmune disease later in life in CHB children is extremely numerous, since the incidence of CHB is low.

4.2.2 MHC and non-MHC associations with CHB

The Ro/SSA and La/SSB antibodies have for decades been associated with congenital heart block, and the risk is estimated to be 1-2% in mothers with these antibodies (Brucato 2001, Cimaz 2003b). Since the risk for a subsequent affected pregnancy is only 20% (Solomon 2003), this indicates that there are further factors involved in susceptibility to disease other than antibody specificity.

There are a number of reports of dizygotic twins discordant for disease, which also indicates that there may be a genetic fetal factor involved in susceptibility to disease (Watson 1994, Eronen 2000, Callen 1985, Harley 1985, Lee 1987, Solomon 2003, Fesslova 2003, Buyon 1998b). However, there are also reports of monozygotic twins discordant for disease (Cooley 1997), indicating that not only fetal genetics are responsible for induction of disease or not. This infers that there may be multiple genes involved in susceptibility and that the sum of these susceptibility genes in each individual determines the outcome of disease. A certain threshold of susceptibility must be exceeded for disease to be expressed in each individual. There are no data on what the discordance rates for CHB are for monozygotic and dizygotic twins at this time because the numbers of cases are too few.

Associations of mothers with CHB and MHC genes have been described, particularly associations with haplotype B8/DR3 (Arnaiz-Villena 1989, Watson 1992, Julkunen 1995, Brucato 1995, Colombo 1999, Lee 1983, Watson 1984, Buyon 1990, Kassan 1998, Siren 1999b), but these are believed to be related to the presence of Ro/SSA and La/SSB antibodies, as well as with the disease pSS and not to CHB. This association of Ro/SSA and La/SSB antibodies and pSS with the B8/DR3 haplotype is

well documented (Nakken 2001, Gottenberg 2003). Of particular interest is that this B8/DR3 association found in Caucasians is not found in the Japanese population (Miyagawa 1997c, Miyagawa 1997b, Miyagawa 1999). The association with B8/DR3 was however confirmed in the Italian population (Brucato 1995) and the Finnish population (Julkunen 1995).

These same genetic associations are not found in the children born with CHB (Siren 1999a). There have been some suggested HLA class I and II alleles which predispose a child to CHB, but the studies are too small and few to draw any conclusions (Siren 1999a Lupus- children). There is however a small study which indicates that the MHC class II associations, in at least the Japanese population, may not be the same in mothers with NLE and CHB (Miyagawa 1997a).

Non-HLA genes have also been suggested to play a role in CHB predisposition and susceptibility to disease. Clancy and colleagues demonstrated an association of CHB with cytokine polymorphisms as there was an increased frequency of TGF-β and TNF-α cytokine polymorphism in 40 CHB children compared to a large number of healthy controls (Clancy 2003). In fetal CHB hearts, protein expression of TGF-β, and not TNF-α, was demonstrated in septal regions. An association of TNF-α has however been proposed with cutaneous neonatal lupus (Clancy 2004). A more recent study with twin and triplet pregnancies corroborated the association of TGF-β and CHB in the twin pair but not in the family with triplets (Clancy 2006).

Since the Ro52 antibodies are necessary but not sufficient to cause disease in congenital heart block, it has been speculated that a fetal factor such as genetics is involved in the development of disease. In a pilot study, two strains, DA ($RT1^{av1}$) and LEW ($RT1^{l}$), differing in both MHC and non-MHC genes were investigated. The strains have previously been shown to differ in their susceptibility to other induced autoimmune diseases such as EAE (Lorentzen 1997) and collagen-induced arthritis (Lorentzen 1996, Griffiths 1993). Heart block developed three times more often in pups of the DA strain than in LEW pups (Salomonsson, data not shown), and a study was therefore designed to dissect the influence of MHC and non-MHC genes by use of several inbred strains and MHC congenic rats (Table 1).

In **paper IV** we present an experimental model which helped to elucidate a genetic influence in heart block development and susceptibility.

4.2.2.1 MHC and non-MHC associations of CHB in an experimental model

In order to investigate the influence of MHC and non-MHC genes in the disease CHB, an immunization model with four different strains was developed (Figure 15). Three shared the same MHC haplotype $RT1^{av1}$(DA, PVG.AV1 and LEW.AV1), and the fourth strain (LEW) shared the background genes with the congenic LEW.AVI strain, but differed in the MHC genes, as the original RT1 locus of the LEW strain is $RT1^{l}$.

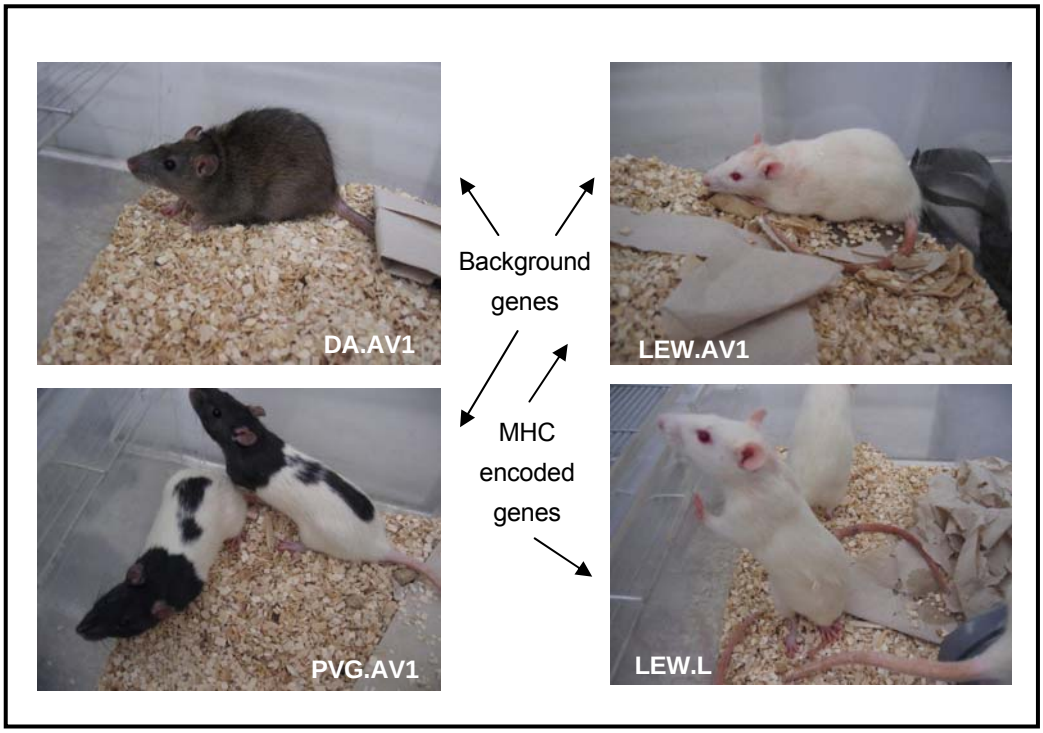

Figure 15. Dissection of MHC and non-MHC genes in an immunization model of CHB. A number of rat strains were used for comparison of PR levels in pups after immunization with Ro52 or control protein.

After Ro52-immunization of rat females, they were mated, and PR intervals were analyzed in the pups by ECG measurement within 24 hours of birth. First-degree AV block developed in 45% (22/49) of the DA ($RT1^{av1}$) pups, 44% (21/48) of the PVG.AV1 and 47% (24/51) of the LEW.AV1 pups. However, only 10% (5/49) of pups in the LEW ($RT1^{l}$) group developed AV block (**Paper IV**). This calculation was made by taking the mean of the control group PR (for each strain) and adding 2 x the standard deviation, as a threshold for PR prolongation, or first-degree AV block. The pups of Ro52 immunized animals in the 3 rat strains sharing the $RT1^{av1}$ haplotype (DA, PVG.AV1, LEW.AV1) had significantly longer PR intervals than did the LEW ($RT1^{l}$) pups ($p<0.001$), while there was no significant difference in PR-intervals between the three $RT1^{av1}$ carrying strains (**Paper IV**).

Bradycardia was also detected, and calculated by taking the mean of the control group heart rate and adding 2 x SD for a cut-off. Bradycardia was detected in 19% (9/49) of the DA ($RT1^{av1}$) pups, 40% (19/48) of the PVG.AV1, 43% (22/51) of the LEW.AV1 pups, and only 10% (5/49) of pups in the LEW ($RT1^{l}$) group (unpublished data).

Bradychardia has been demonstrated in other immunization models as well and in an immunization model of CHB by Suzuki *et al* they found immunization with Ro60, La and Calreticulin induced 29%, 39%, 33% bradycardia respectively in mice (Suzuki 2005). In the study by Eftekhari investigating 5-HT_4 peptide and Ro52-peptide immunization of mice, only 17% of the R18L Ro52-peptide immunized mice showed signs of bradychardia (Eftekhari 2001). Bradycardia was also demonstrated in 9% (12/121) rabbit pups born to Ro52-immunized rabbits (Xiao 2001). Boutjdir *et al* also reported 20% (4/20) mice with bradycardia in a Ro52-immunization model of

CHB (Boutjdir 1997). Sinus bradycardia was not observed in any of the groups in the large mouse immunization study by Miranda-Carus with immunization of mice with Ro52-α, Ro52-β, Ro60, La and murine Ro52 (Miranda-Carus 1998b). Signs of bradycardia in the study by Salomonsson *et al* was not investigated (Salomonsson 2005). In human studies bradycardia has been reported in a number of children affected by CHB (Brucato 2000).

It was demonstrated that the maternal antibodies in the $RT1^{av1}$ (DA, PVG.AV1, LEW.AV1) rat strains had a different Ro52-p200 antibody binding profile than the $RT1^{l}$ (LEW) rats did. Using a set of mutated peptides it was demonstrated that binding to the pZIP and pOUT peptides differed in these strains and thus the antibody specificity could possibly explain the difference in AV block incidence. However, it was still uncertain whether this association with MHC-encoding genes in the RT1 region was with maternal genes defining antibody specificity or with fetal genes inferring susceptibility.

4.2.2.2 Maternal antibody specificity and fetal susceptibility to AV block

In order to determine whether the MHC-region association with AV block from the immunization study was with maternal or fetal genes an F2 cross was performed between the susceptible LEW.AV1 rat strain and the resistant LEW rat strain. The LEW.AV1 and LEW rats were mated, and the F1 heterozygous females ($RT1^{av1/l}$) were immunized with Ro52. These F1 females were then mated with heterozygous F1 males ($RT1^{av1/l}$) to produce homozygous $RT1^{av1}$, $RT1^{l}$ or heterozygous $RT1^{av1/l}$ offspring. Genotyping of the F2 generation pups revealed that RT1 haplotype frequencies were 23% $RT1^{av1}$, 23% $RT1^{l}$, and 54% heterozygous $RT1^{av1/l}$ (Figure 16).

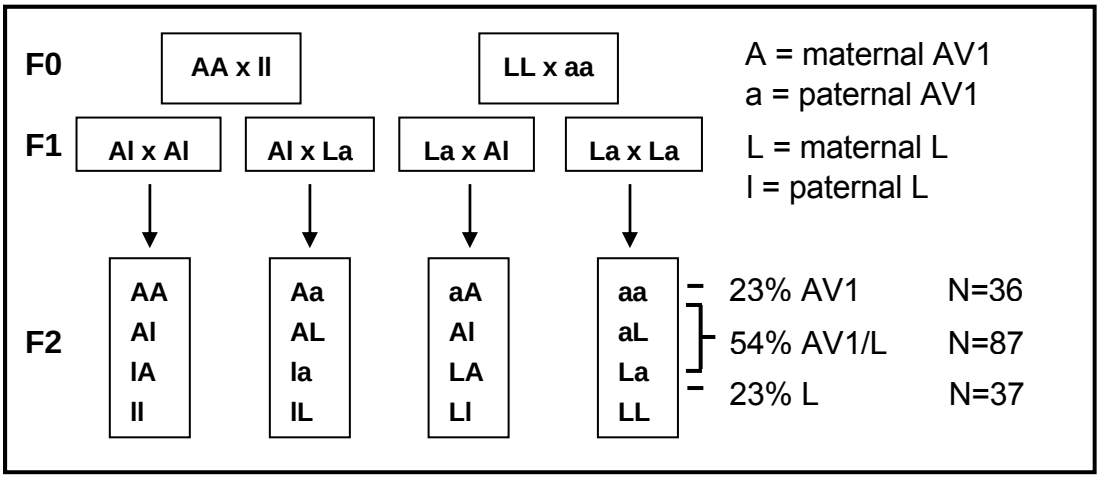

Figure 16. Schematic of F2 cross mating. F0 are the founders and F2 are the pups born to the immunized F1 rats.

ECG was performed at birth to detect heart block in the pups of the Ro52-immunized mothers. Analysis of prolonged PR intervals revealed that a homozygous $RT1^{l}$ or heterozygous $RT1^{av1/l}$ genotype in the pup correlated with significantly higher PR intervals than in homozygous $RT1^{av1}$ pups ($p<0.05$). This indicates that the MHC allele $RT1^{l}$ in the pups in fact confers a higher susceptibility to heart block induced by

Ro52 antibodies than $RT1^{av1}$ alleles in the pup. Taken together with the results from the previous immunization study, it has been demonstrated that the $RT1^{av1}$ MHC-encoding region is associated with maternal antibody specificity, and the $RT1^{l}$ MHC-region is associated with fetal susceptibility to the disease CHB.

4.2.2.3 Serum transfer as confirmation of MHC associations

In order to confirm these results of a separate MHC-association with maternal antibody specificity and fetal susceptibility, a transfer of Ro52-immune sera was performed in DA ($RT1^{av1}$) and LEW ($RT1^{l}$) rats (Figure 17). Ten DA rats had been immunized and sacrificed and their sera pooled for transfer to the DA and LEW rats. Non-immune DA rat sera was also transferred to DA and LEW rats as a control.

Passive transfer of these pathogenic Ro52 antibodies from DA ($RT1^{av1}$) rats induced AV block in LEW ($RT1^{l}$) rats but not in DA ($RT1^{av1}$) rats (**Paper IV**). Surprisingly, the DA rats were not affected in this passive transfer model, even though it was possible to induce AV block in the DA rats in the immunization model. This can possibly be attributed to the lower levels of antibodies in the transfer model compared with the immunization model. The levels of antibodies in the injection sera in the transfer model was equivalent to that of the immunization model maternal rat sera, but the levels of antibodies in the pup circulation are lower in the passive transfer model than the immunization model (**Paper IV**). The levels of antibodies in the DA ($RT1^{av1}$) and LEW ($RT1^{l}$) pups were however the same indicating that the LEW ($RT1^{l}$) pups were more susceptible to disease.

The results of the Ro52-immune serum transfer study confirm that allelic variants within the MHC complex both affect the maternal ability to produce pathogenic antibodies, as well as a fetal susceptibility to develop congenital heart block in response to these antibodies. The data also shows that these traits are linked to different alleles in the mother and child. This MHC-association of antibody specificity may be related to class II antigen presentation of peptide antigens. Differences in the peptide binding spectra of RT1 B and D molecules (de Graaf 1999) of $RT1^{av1}$ and the $RT1^{l}$ haplotypes have been demonstrated, as well as differences in pathogeneicity in MS models (Forsthuber 2001).

The fetal MHC-association with susceptibility to CHB most likely involves genes within the MHC, but probably not genes involved in antigen presentation. At least 220 genes have been identified within the rat MHV (Hurt 2004), and complement genes and TNF are genes within the complex which have immune functions and could be possible candidates for the observed susceptibility to CHB.

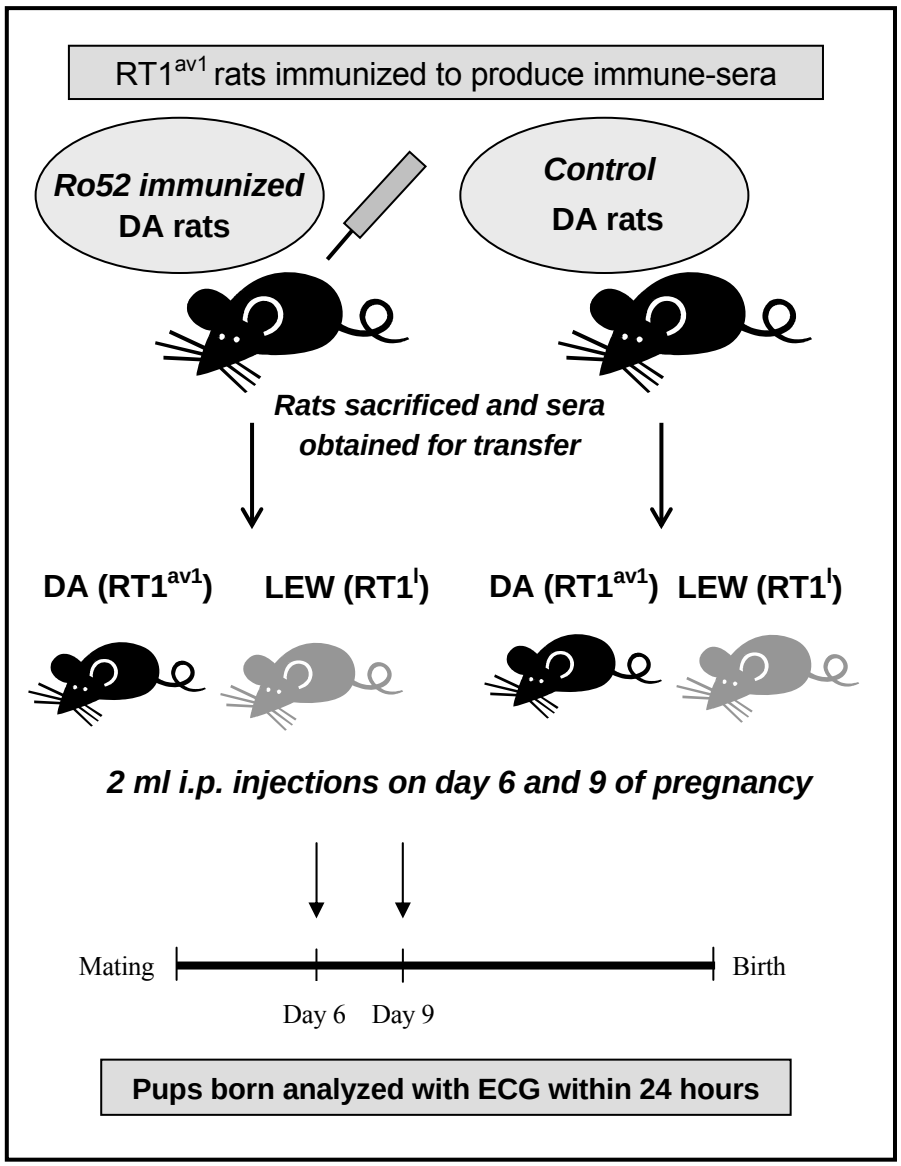

Figure 17. Transfer of Ro52-immune sera to RT1^{av1} and RT1^{l} rats. Sera from DA rats were pooled and transferred to DA and LEW rats on day 6 and 9 of pregnancy. ECG was performed on pups at birth and PR intervals analyzed.

In humans, the Ro/SSA and La/SSB antibodies have been associated with the A1, B8 and DR3/DQ2 haplotype in several studies (Nakken 2001, Gottenberg 2003). These genetic associations are seldom present in the CHB children (Siren 1999a), and the children seldom produce the Ro/SSA and La/SSB antibodies themselves. Fetal susceptibility to CHB in humans has been suggested to be associated with HLA-Cw*03 (Siren 1999a) and TNF-α -308 polymorphisms (Clancy 2003).

HLA associations are found in many autoimmune diseases such as in multiple sclerosis (MS) (Khare 2005), in type I diabetes mellitus (Pociot 2002) as well as rheumatoid arthritis (Zanelli 2000) and confirmed in experimental models of respective diseases. Identification of genes involved in autoimmune diseases is difficult because they are such complex multifactorial diseases which occur in heterogeneous populations. One complicating factor in investigating linkage within the HLA region is the strong linkage disequilibrium between neighboring genes and the fact that several HLA genes acting in concert may determine disease susceptibility.

4.2.2.4 Epigenetic influences in CHB development

In the past, susceptibility to disease was believed to be determined exclusively by inheritable information carried on the primary sequence of DNA. Recently it has become clear that epigenetic effects on gene expression play an equally important role in the development of disease (Santos-Reboucas 2007). The term epigenetics actually means "outside conventional genetics" and was coined by the developmental biologist Conrad Waddington (Van Speybroeck 2002).

Currently epigenetics is defined as heritable changes in gene expression that occur without alterations in DNA sequence. These epigenetic modifications are mitotically and transgenerationally inheritable (Santos-Reboucas 2007). There are so far three distinct mechanisms identified to regulate epigenetic effects; (1) small interfering RNAs, (2) DNA methylation, and (3) histone modifications (Morris 2005, Cheung 2005, Esteller 2005). They regulate cellular functions such as genome stability, X-chromosome inactivation, gene imprinting, and reprogramming of non-imprinting genes (Tang 2007). All this leads to select phenotypes based on whether or not genes are silenced or activated and in what tissues.

Several different studies have suggested the contribution of abnormalities in DNA methylation in the development of SLE (Sekigawa 2006) and epigenetic mechanisms have also been associated with susceptibility to experimental autoimmune encephalomyelitis (EAE), an experimental model of MS (Sobel 2000, Encinas 2001, Teuscher 2006).

Although we were able to demonstrate associations of maternal antibody specificity with MHC-encoding region genes and fetal susceptibility to CHB with a separate MHC-encoding region, we observed a pattern of inheritance in the F2 cross which was not consistent with traditional Mendelian inheritence. Analysis of pup PR intervals in the F2 cross experiment revealed that there may be an additional epigenetic effect in susceptibility to CHB. When analyzing PR intervals with respect to both the pup genotype as well as the F1 generation groups, a pattern was observed (Figure 18).

The F1 groups are denoted by indicating the maternal founder to each F1 female/male pairs which were mated. When the mated female and male rats had $RT1^{av1}$ maternal founders the F2 pups had normal PR intervals. In the F1 group with L/L (indicating $RT1^{l}$ maternal founders) rats mated, the F2 pup PR intervals are prolonged. Within this L/L F1 group it is even evident that the pup genotype adds to the susceptibility to PR interval prolongation. This can also be observed in the F1 groups AV1/L and L/AV1, where there is at least one female $RT1^{l}$ founder to these mated rats.

However, the difference is most obvious when not including the AV1/L and L/AV1 groups. Further analysis of this effect is necessary before any conclusions can be drawn, but this may indicate that the MHC (RT1) haplotype of the female founder may be important for susceptibility to heart block. This could indicate a complicated genetic inheritance of susceptibility in humans and explain why simple associations with MHC genes have not been observed in human studies of CHB.

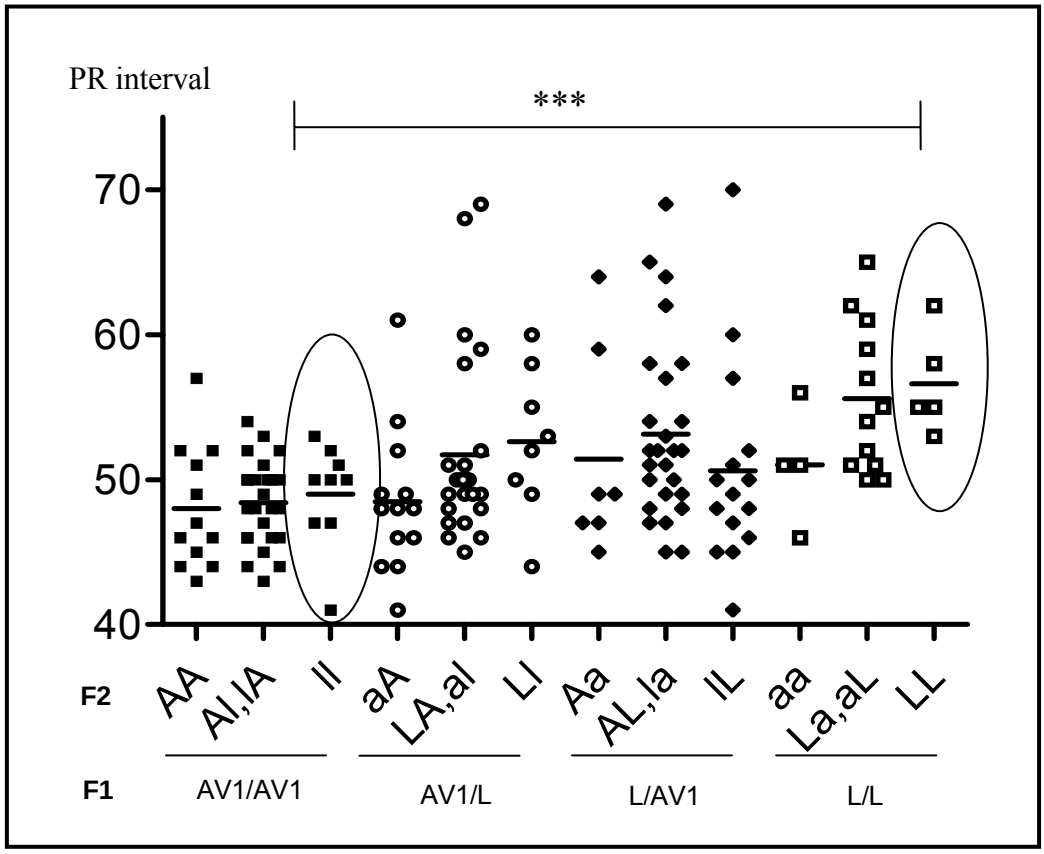

Figure 18. Epigenetic effects observed in analysis of F2 cross. Pup PR interval analysis with respect to pup genotype and F1 groups revealed a possible epigenetic effect. F1 groups are denoted by maternal founder to each female/male pair. Ovals help to indicate comparison of ll paternal founder inheritance and LL maternal founder inheritance with statistically significant differences.

4.2.3 Summary Part II:

We have presented a case report of a CHB girl who develops autoimmune disease later in life, indicating that there may be a need to follow these children. However, because of the low incidence of congenital heart block, genetic associations in humans are difficult. Genetic associations of CHB with MHC genes have been suggested, but they have not been demonstrated experimentally. With an experimental rat model of CHB, we demonstrated that maternal antibody specificity was linked to a specific MHC haplotype, whereas fetal susceptibility to development of CHB was linked to a separate MHC haplotype in the fetus. The inheritance patterns observed in the study also suggested that complex inheritance may be involved in susceptibility, and explain why simple associations with MHC genes have not been observed in human studies of CHB.

4.3 PART III. FUNCTION OF THE Ro52 AUTOANTIGEN

In order to understand the biological role of Ro52 in the diseases Sjögren's syndrome and SLE and to determine its involvement in CHB it is important to first understand the cellular function. While DNA binding activity and associations with transcriptional processes have been suggested previously, these reports have not been confirmed and the biological function was unknown until recently.

4.3.1 Function of Ro52

Ro52 contains a RING finger motif, a B-box, and a coiled-coil domain (Reymond 2001) which includes it in the tripartite motif (TRIM) protein family, and thus Ro52 is also referred to as TRIM21 (Reymond 2001). Many RING finger proteins have E3 ligase activity and are involved in modifying proteins by the addition of ubiquitin. E3 ligases mediate the transfer of the activated ubiquitin from an E2/Ubc to a substrate protein, and depending on whether it is poly- or monoubiquitinated it is degraded by the 26S proteosome, targeted for lysosomal degredation, or functionally modified (Pickart 2001, Weissman 2001).

Previously it was demonstrated that Ro52 was modified by ubiquitin after ectopic expression of Ro52 and ubiquitin (Fukuda-Kamitani 2002), and it has been demonstrated that Ro52 interacts with the deubiquitinating enzyme UnpEL in a coiled-coil dependent manner (Di Donato 2001). Both these findings suggest that Ro52 has E3 ligase activity.

4.3.1.1 Ro52 is a RING-dependent E3 ligase

Since many E3 ligases have the ability to autoubiquitinate themselves, we investigated whether Ro52 was able to autoubiquitinate itself *in vitro* (**Paper V**). FLAG-Ro52, or FLAG-Ro52ΔRING, were co-expressed with 6x His-ubiquitin in HEK293 cells. After purification of proteins modified by 6x His-ubiquitin, immunoblotting with anti-FLAG antibodies was performed to detect ubiquitin modified Ro52 (Paper V). Full-length Ro52 was modified by polyubiquitination while the Ro52ΔRING mutant lacking the RING finger was not. This suggested that Ro52 autoubiquitinated itself in a RING-dependent manner. This data is consistent with other E3 ligases of the RING family, where this domain has been shown to mediate the interactions with E2/Ubcs.

To confirm these results an *in vitro* ubiquitination assay was established where purified Ro52, E1, E2/Ubc, as well as ATP and ubiquitin were added together. Polyubiquitination was demonstrated with several E2/Ubc:s (UbcH2, UbcH5a-c, UbcH6, UbcH7) but was most effective with UbcH6. Absence of any of these reagents resulted in a loss of polyubiquitination, confirming that Ro52 is a RING-dependent E3 ligase. Ro52 polyubiquitination was mediated with several E2/Ubcs *in vitro*, and as has been observed with other E3 ligases, Ro52 probably has other Ubc partners.

In 2006 three independent groups have demonstrated that Ro52 is an E3 ubiquitin ligase (**Paper V**, Wada 2006, Sabile 2006). Results indicate that Ro52 ubiquinates itself in cooperation with the E2 ubiquitin-conjugating enzyme UbcH5b (Wada 2006, Espinosa 2006), but also with UbcH2, UbcH5a, UbcH5c, UbcH6, and UbcH7 (Espinosa 2006). This ubiquitination is suggested to monoubiquitination (Wada 2006) or polyubiquitination (Espinosa 2006), demonstrated *in vitro*. This could

indicate that Ro52 may be involved in endocytosis, trafficking, or lysosomal degredation, as well as being targeted for proteosomal degradation (Bloom 2004). However, it is difficult to determine the exact role in an *in vitro* system under normal biological conditions.

Substrates which have been suggested to be ubiquitinated by Ro52 include IgG (Rhodes 2006), Skp2 and Cul1 (Sabile 2006). In 2007 Kong and colleagues suggested one more substrate which Ro52 associates with and demonstrated that Ro52 ubiquitinated the substrate interferon regulating factor-8 (IRF-8), *in vivo* and *in vitro* (Kong 2007). IRF-8 is a transcription factor which regulates the function and development of macrophages, dendritic cells and B cells (Tamura 2005, Wang 2000, Lee 2006). IRF-8 plays a role in expression of proinflammatory cytokines such as IL-12p40 and type I IFN genes (Tamura 2005, Wang 2000, Tsujimura 2003).

4.3.1.2 Increased expression of Ro52 in Sjögren's syndrome and SLE patients

The expression pattern of the Ro52 in cells of patients with Sjögren's syndrome and SLE has not been previously investigated. For the first time it has been demonstrated that mRNA expression of Ro52 in peripheral blood mononuclear cells (PBMCs) of these patients are significantly increased compared to healthy controls (**Paper V**).

Cells from twenty patients with Sjögren's syndrome and 18 patients with SLE were investigated for mRNA and protein levels. The highest mRNA expression of Ro52 was found in B cells, then monocytes and the lowest levels were found in T cells. From the B cell population, cell sorting revealed that the highest expression was in the naïve $CD19^{+}$ $CD27^{-}$ B cells. This however was not the case for protein expression, where the highest expression of Ro52 was in T cells and monocytes and not B cells, determined by western blot.

4.3.1.3 Overexpression of Ro52 leads to decreased cell proliferation and cell death

In order to investigate the effect of this overexpression of Ro52 in B cells, a mouse B lymphoma cell line (A20) was stably transfected with mouse Ro52-GFP, Ro52ΔRING-GFP (mutant lacking RING-finger), or GFP alone (**Paper V**). The overexpression of Ro52 seemed to have significant effects on proliferation; the plating efficiency and colony size were significantly decreased in the Ro52-GFP transfected cells. But this was not so in the Ro52ΔRING-GFP, or the GFP-transfected cells, indicating it is a RING-dependent mechanism.

Anti-CD40 has been described to increase cell death in transformed cell lines (Hess 1996 J Exp Med), and therefore would be useful in investigation of proliferation following cell activation in Ro52 overexpressed cells. Anti-CD40 was added to normally expressing Ro52 cells as well as the Ro52-GFP, Ro52ΔRING-GFP, and the GFP transfected cell lines. Cell death was increased in all cell lines after anti-CD40 stimulation, but it was significantly higher in the Ro52-GFP transfected cell line. This indicated that the RING domain is important for mediating CD40 induced cell death. The induction of cell death was also demonstrated to be dose dependent.

Together these data indicate that overexpression of Ro52 in B cells may lead to decreased cell proliferation and increased cell death. The overexpression of Ro52 in patient PBMC may possibly explain the decreased proliferation and increased apoptosis of PBMCs in SLE and Sjögren's syndrome.

Other studies by Sabile and colleagues have demonstrated a somewhat different role for Ro52 in the process of p27 turnover and S-phase progression in HeLa cells and as a defining component of a ubiquitin ligase complex (Sabile 2006). They showed that the knockdown of Ro52 expression in HeLa cells with small interfering RNAs causes the accumulation of p27 and the failure of cells to enter S phase. In the study by Kong and colleagues, Ro52 was demonstrated to be an interaction partner of IRF-8 and demonstrated that it ubiquitinated IRF-8 (Kong 2007). Increased Ro52 led to enhanced expression of a target gene IL-12p40 rather than promoting IRF-8 degradation. Their results indicate a role for Ro52 in promoting cell transformation and oncogenesis, as well as activation of cytokine gene expression, whereas our results indicate a tumor suppressor role for Ro52.

RING finger E3 ligases have been shown to function as both tumor suppressors as well as oncogenes. BRCA1 and Fbw7, have been shown to be tumor suppressors in breast cancer, whereas for example Mdm2 has established roles in regulation of cell cycle and apoptosis (Chen 2006). Notably, Ro52 is chromosomally localized to a region containing tumor suppressor activity, on chromosome segment 11p15.5 (Kim 2000).

4.3.2 Cellular localization of Ro52

Conflicting data has been published on the cellular localization of Ro52 (Keech 1995, Pruijn 1997, Pourmand 1998a). In two separate studies we have now confirmed the localization to be primarily cytoplasmic with some weak nuclear staining (**Paper V**, **Paper VI**).

In **Paper V**, different constructs of Ro52 were made and HeLa cells were transfected and observed with immunofluorescence. A full-length construct (Ro52-GFP), as well as a deletion mutant lacking the RING-finger (Ro52ΔRING-GFP) both localized to the cytoplasm, and also gave weak nuclear staining with spared nucleoli. These results were also confirmed in transfection studies of the human B cell line A20 with the mouse Ro52-GFP constructs. Since GFP might alter the localization of Ro52, the localization of endogenous Ro52 was investigated with monoclonal anti-Ro52 antibodies in HeLa cells and in primary human B cells (**Paper V**). Once again a predominantly cytoplasmic localization was observed with weak nuclear staining. In **Paper VI**, Ro52 monoclonal antibodies specific for the zinc-finger region, the coiled-coil region, as well as the B30.2 region all demonstrated this predominantly cytoplasmic staining in HeLa cells with immunofluorescence.

Although a number of studies have reported that Ro and La translocates to surface blebs during apoptosis (Casciola-Rosen 1994, Lawley 2000, Ohlsson 2002), it is still controversial whether these autoantigens are displayed at the surface and available for binding by antibodies. Recently, Reed *et al* has specifically shown that Ro60 is bound on the surface of Jurkat cells during early and late apoptosis (Reed 2007). La antibodies on the other hand bound only during late apoptosis, and Ro52 antibodies did not bind at all to these apoptotic cells.

Different effectors have also been demonstrated to cause surface expression of the Ro/SSA and La/SSB antigens. UVB stimulation, but not UVA (Golan 1992) estradiol, but not other steroid hormones (Furukawa 1988), heat shock stimulation (Jones 1992) as well as infection with adenovirus (Baboonian 1989).

4.3.2.1 IFN-α induces translocation of Ro52

IFN-α has been demonstrated to be associated with both Sjögren's syndrome and SLE pathogenesis. IFN-α serum levels are elevated in SLE patients, and correlate with disease activity (Kim 1987, Kirou 2005). Patients treated with recombinant human IFN-α for malignancy and chronic viral hepatitis have developed *de novo* SLE, which typically resolves after the IFN-α is discontinued (Rönnblom 1990, Niewold 2005). It has also been demonstrated that SLE patients exhibit overexpression of IFN-α induced genes in their peripheral blood mononuclear cells (PBMCs) as compared with healthy individuals and patients with other inflammatory diseases such as rheumatoid arthritis (Bennett 2003, Kirou 2004, Baechler 2003). IFN-α has also been detected in the salivary glands of Sjögren's syndrome patients (Båve 2005) and microarray analysis of biopsies from affected glands revealed an activation of IFN-pathways (Gottenberg 2006). A link between the SLE and Sjögren's syndrome autoantigen Ro52 and a transcription factor which regulates type-I interferons (IRF-8) has been made (Kong 2007) and understanding their relationship and the consequence of the ubiquitination of IRF-8 is the next step in understanding the pathology of these diseases.

An increase in the expression of tri-partite motif (TRIM) proteins upon stimulation with interferon-α has also been demonstrated (Der 1998, de Veer 2001, Pfeffer 2004) and studies have also demonstrated upregulation of Ro52 by IFN-α from microarray studies in patients (Bennett 2003, Baechler 2004, Gottenberg 2006). In **paper VI** we also demonstrate with a panel of Ro52 monoclonal antibodies that Ro52 translocates from the cytoplasm to the nucleus in HeLa cells upon stimulation with IFN-α. After 24 hours there was an increased expression of Ro52 in the cytoplasm compared to non-stimulated cells. After 48 hours of stimulation, a translocation from the cytoplasm to the nucleus had been induced. This translocation was observed upon staining with monoclonals towards the zinc-finger region, the coiled-coil region, the B30.2 region as well as with affinity purified Ro52 antibodies from human serum. This suggests that deletion or extensive modification of any of these domains is not required for the translocation of Ro52. Apoptotic cell death in these IFN-α stimulated cells was also investigated. The translocation of Ro52 from the cytoplasm to the nucleus occurred before apoptosis, as demonstrated by caspase-3 activation in double stained cells, and an increase in TUNEL positive cells occurring after translocation to the nucleus in parallel cultures. These results indicate a nuclear substrate for Ro52.

4.3.3 Summary Part III:

Our results indicate that Ro52 is predominantly a cytoplasmic protein which can translocate to the nucleus and may play a role in IFN-α mediated apoptosis. We demonstrate that Ro52 is a RING-dependent E3 ligase and that expression is increased in PBMCs of Sjögren's syndrome and SLE patients. Transfection of B cell lines with Ro52 leads to decreased cell proliferation and apoptosis indicating that Ro52 may play a role in the pathogenesis of Sjögren's syndrome and SLE, diseases characterized by decreased proliferation and increased apoptosis.

4.4 HYPOTHESIS

IFN-α has been implicated in the pathogenesis of the diseases SLE and Sjögren's syndrome. IFN-α has also demonstrated to induce increased expression of Ro52 in cells and to induce the translocation of Ro52 from the cytoplasm to the nucleus (**Paper VI**). Since Ro52 has been demonstrated to be a cytoplasmic protein, with translocation to the nucleus, it is probable that it does not play a role in the initiation of congenital heart block, as heart block inducing antibodies bind to the cell surface.

Most likely the autoantibodies involved in CHB cross the placenta to the fetus and cross-react with a protein on the surface of the cardiomyocytes leading to inflammation and fibrosis of the conduction system. Levels and subclass profiles have not been demonstrated to play a role in the initiation of disease (**Paper I**), but it may be difficult to detect subtle differences in these small heterogeneous populations studied. An association with MHC genes has been demonstrated in an experimental model of CHB (**Paper IV**).

It has also been proposed that once an inflammatory reaction is initiated that the possible apoptosis may lead to surface expression of the Ro/La antigens. This in turn may lead to binding of the Ro/SSA and La/SSB antibodies present in the circulation to these antigens and possibly it could perpetuate the disease by causing increased inflammation, and possibly decreased clearance of apoptotic cells due to immunoglobulin binding. Macrophages have been suggested to have a role in the subsequent fibrosis seen in complete AV block by secreting transforming growth factor- β (TGF-β) (Clancy 2003, Clancy 2002).

4.4.1.1 Ro52 antibodies and genetic influence in CHB

This hypothesis proposes that maternal MHC genes determine the specificity of the maternal antibodies produced (Figure 19). Ro52 antibodies do not lead to CHB unless they have the special p200-epitope binding capabilities we have described (**Paper II, Paper IV**). The outcome of cases where mothers, transferring these pathogenic Ro52-p200 antibodies to their fetus, is dependent on fetal MHC genes which determine the fetal susceptibility to induction of CHB. An AV block I, II or III may be induced depending on the sum of genetic susceptibility genes present in this fetus. An epigenetic effect contributes, where even the path of inheritance of certain genes may play a role, adding to the complex penetrance of this disease.

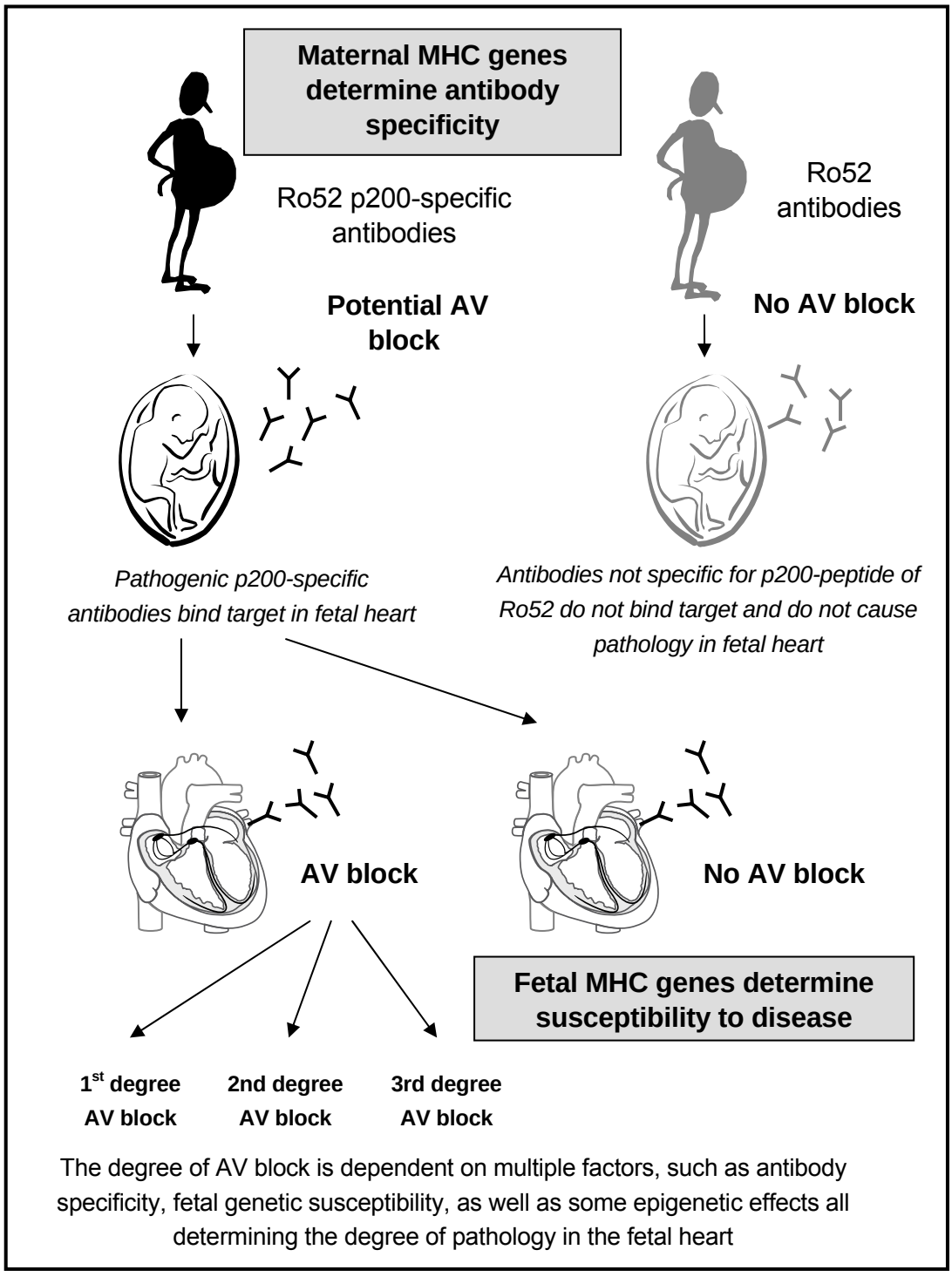

Figure 19. The role of Ro52 antibodies and a genetic influence in CHB.

4.4.1.2 The Ro52 autoantigen in Sjögren's syndrome and SLE

The anti-proliferative and pro-apoptotic effects of IFN-α, as well as data indicating an upregulation of Ro52 by IFN-α from microarray studies in SLE and Sjögren's syndrome patients (Bennett 2003, Baechler 2003, Gottenberg 2006), taken together with our data implicating Ro52 as an E3 ligase (**Paper V**) and the translocation of Ro52 from the cytoplasm to the nucleus upon IFN-α stimulation **(Paper VI**) suggests that there may be a link between Ro52 and apoptosis in these autoimmune diseases.

The overexpression of Ro52 in these patients may lead to increased ubiquitination of IRF-8, which is a transcription factor for macrophages, dendritic cells (DCs) and B cells. This may also lead to activation of type-I interferons, which are regulated by IRF-8, and thus possibly perpetuating the condition (Figure 20).

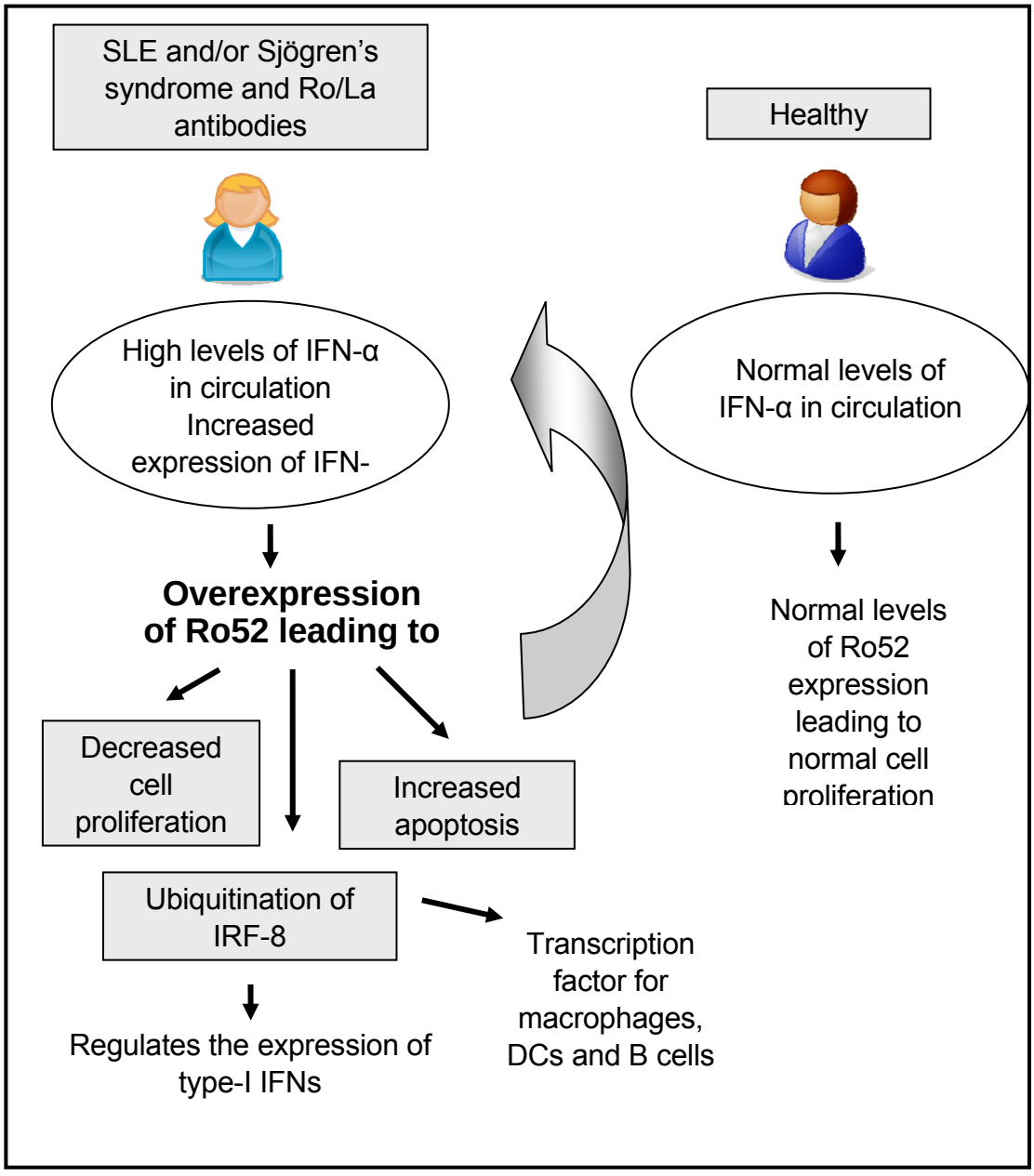

Figure 20. Ro52 function and a role in Sjögren's syndrome and SLE

5 CONCLUSIONS

Studies analyzing Ro52-p200 antibodies in three national cohorts revealed that Ro52-p200 antibodies may be useful as a second step analysis of Ro/SSA and La/SSB mothers with Ro52 antibodies to predict risk of congenital heart block. The levels of Ro52-p200 antibodies were significantly higher in second- and third-degree AV block pregnancies compared to mothers with fetuses with normal heart rate. In the Swedish cohort we were also able to demonstrate that pregnancies which show signs of first-degree AV block also have significantly higher Ro52-p200 levels than controls. This may indicate that these mothers can have an affected fetus in subsequent pregnancies, and therefore surveillance during susceptibility weeks is necessary. Detection of first-degree AV block cases may even be beneficial for understanding of the biological process of the disease. In the Swedish cohort we were able to calculate that using p200-antibodies as a second step analysis in Ro52-positive pregnancies increased the positive predictive value for fetal cardiac involvement (first-, second-, or third-degree AV block) with 36%.

Investigations of Ro/SSA and La/SSB antibody levels as well as Ro52 subclass antibodies and their transfer to the children at birth revealed that antibody levels did not change significantly and that no associations with antibody levels or specific subclasses with congenital heart block could be detected. It is still possible that antibody levels are important for the development of heart block, but since it is most likely a combination of factors, it would be difficult to see any significant differences in small studies.

Clinicians and researchers have speculated for a number of years that there may be a fetal genetic factor responsible for the low incidence of CHB in mothers which have these pathogenic Ro52 antibodies, and even have given birth to a previously affected child. This is the first time anyone has demonstrated an association of maternal MHC genes with antibody specificity and a separate MHC association with fetal susceptibility to the disease. An experimental model with inbred congenic rats was a first step in identifying important genetic associations with this disease. The results of this study are in agreement with human studies which have only been able to indicate that the children with CHB do not seem to have the same genetic associations as the mothers. The genetic associations in the mothers are usually associated with the Ro/SSA and La/SSB antibodies and this is exactly what we have seen in the experimental model, that the antibody specificity is associated with maternal genes. Through the observed pattern of associations, this model has also revealed a possible epigenetic mechanism in fetal susceptibility, however further studies are necessary to dissect these mechanisms.

It is important to understand the function and cellular localization of Ro52 in order to determine whether Ro52 is involved in the pathogenesis of CHB, or whether the Ro52 antibodies involved in the disease are recognizing a completely different protein with cross-reactive epitopes. The Ro52, Ro60 and La autoantigens may be involved in CHB in some other way, such as increasing the inflammatory injury and perpetuating the disease once an injury is initiated by these cross-reactive antibodies.

The Ro52 protein has been demonstrated to be an E3 ligase which can ubiquitinate IRF-8. This could indicate a role in regulation of type-I IFN genes as well

as modulation of a transcription factor in B cells, DCs and macrophages. Expression levels of Ro52 were increased in SLE and Sjögren's syndrome patient PBMCs, and overexpression of Ro52 in a B cell line *in vitro* led to decreased cell proliferation and increased cell death. Taken together these findings may indicate a role for Ro52 in SLE and Sjögren's syndrome which are diseases characterized by decreased cell proliferation and apoptosis.

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