From the Department of Medicine
Karolinska Institutet, Stockholm, Sweden

RISK FACTORS FOR AUTOIMMUNE-MEDIATED CONGENITAL HEART BLOCK

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Stockholm 2017

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Published by Karolinska Institutet and printed by Eprint AB 2017.

ISBN 978-91-7676-853-2

Risk factors for autoimmune-mediated congenital heart block

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Thesis defense will take place in **Welandersalen**, B1:00,
Karolinska University Hospital, Solna

Friday, December 15th 2017, 9.00 am

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“Everything will be alright in the end. If it is not alright, it is not yet the end.”

(Unknown)

ABSTRACT

Placental transfer of maternal Ro/SSA and La/SSB autoantibodies during pregnancy is associated with conduction disturbances and inflammation in the developing fetal heart, termed autoimmune-mediated congenital heart block (CHB). Maternal Ro/SSA and La/SSB autoantibodies are the main risk factors associated with the fetal cardiac manifestations to date, however, the low recurrence rate despite persisting autoantibodies in subsequent pregnancies indicates that additional factors determine fetal susceptibility. The complex interactions between fetal genetic variants and factors that influence the intrauterine environment are thought to trigger or prevent the onset of CHB in Ro/SSA and/or La/SSB exposed pregnancies. The identification of such variants and factors was the main aim of this thesis.

Genome-wide SNP association studies in families with at least one child affected by CHB and an anti-Ro52/SSA positive mother identified distinct cellular pathways associated with CHB. Exploration of potential candidate genes in the CHB-associated regions identified auxilin as a novel fetal susceptibility gene affecting cardiac excitation-contraction coupling. Discovery of additional CHB-associated variants affecting genes involved in vesicular or transmembrane transport and cardiac function further supported the idea that genetic variants in pathways connected to cardiac conduction and contractility may influence fetal susceptibility to disease. Furthermore, CHB-associated variants affecting genes with function assigned to immune responses emerged from our association studies and are likely to contribute to the inflammatory and tissue destructive processes connected with CHB. Ro/SSA autoantibodies are associated with interferon activation, and we found that cardiomyocyte expression of CHB-associated genes is affected by interferon-alpha stimulation. PBMCs from neonates with CHB and exposed to Ro/SSA autoantibodies *in utero* also displayed differential expression of several CHB-associated genes. Interestingly, expression of auxilin was altered in cardiomyocytes and PBMCs, validating the relevance of this particular gene and its pathway in CHB pathogenesis. We further identified and confirmed distinct class I and II HLA allele associations with CHB implementing potential impact for disease. Among the factors that may influence the intrauterine environment, we found that seasonal timing of pregnancy, infections, outdoor activity and psychological stress associated with the risk for CHB in Ro/SSA positive pregnancies. Finally, we also investigated potential cross-targets for the maternal anti-Ro52/p200 antibodies, and fetal intrauterine exposure to these maternal autoantibody specificities may further influence clinical outcomes of CHB.

In summary, our data expands the current understanding of CHB pathogenesis, and suggests that the overall fetal susceptibility to CHB and degree of disease severity depends on a combination of genetic risk variants, their overall functional consequences, and their interactions with intrauterine factors in addition to the effect of fetal exposure to maternal Ro/SSA autoantibodies.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their Roman numerals

I. **Auxilin is a novel fetal susceptibility gene for congenital heart block that directly impacts fetal heart function**
Sabrina Meisgen, Malin Hedlund, Aurélie Ambrosi, Lasse Folkersen, Vijole Ottosson, David Forsberg, Bo Ding, Luca Biavati, Linn Strandberg, Daniel Ramsköld, Sabrina Ruhrmann, Lauro Meneghel, William Nyberg, The Swedish Congenital Heart Block Study Group, Alexander Espinosa, Robert Hamilton, Anders Franco-Cereceda, Anders Hamsten, Tomas Olsson, Lois Greene, Per Eriksson, Kristina Gemzell-Danielsson, Stina Salomonsson, Vijay K. Kuchroo, Eric Herlenius, Ingrid Kockum, Sven-Erik Sonesson, Marie Wahren-Herlenius
Submitted Manuscript

II. **Genome-wide association analysis of Nordic families with congenital heart block reveals association with intracellular vesicle trafficking, solute carrier, and immune-related genes**
Gudny Ella Thorlacius, Sabrina Meisgen, Stina Salomonsson, Vijole Ottosson, Heikki Julkunen, Marianne Eronen, Gunnar Bergman, Sven-Erik Sonesson, Kristina Gemzell-Danielsson, The Swedish Congenital Heart Block Study Group, Juha Kere, Ingrid Kockum, Marie Wahren-Herlenius
Manuscript

III. **The HLA locus contains novel foetal susceptibility alleles for congenital heart block with significant paternal influence**
Sabrina Meisgen*, Therese Östberg*, Stina Salomonsson, Bo Ding, Håkan Eliasson, Anders Mälarstig, Lars Alfredsson, Lars Klareskog, Anders Hamsten, Tomas Olsson, Tomas Axelsson, The Swedish Congenital Heart Block Study Group, Fredrik Gadler, Anders Jonzon, Sven-Erik Sonesson, Ingrid Kockum, Marie Wahren-Herlenius
J Intern Med. 2014 Jun;275(6):640-51.

IV. **MHC class I and II associations with autoimmune-mediated congenital heart block in European families**
Nikolaos C. Kyriakidis, Ingrid Kockum, Heikki Julkunen, Ariela Hoxha, Stina Salomonsson, Lauro Meneghel, Cathrine Ebbing, The Swedish Congenital Heart Block Study Group, Alexander Dilthey, Marianne Eronen, Sara De Carolis, Torvid Kiserud, Amelia Ruffatti, Juha Kere, Sabrina Meisgen*, Marie Wahren-Herlenius*
Submitted Manuscript

V. **Environmental and lifestyle factors influencing risk of congenital heart block during pregnancy in anti-Ro/SSA positive women**
Sabrina Meisgen*, Joanna Tingström*, Amanda Skog Andreasson, Sven-Erik Sonesson, Ingrid Kockum, Marie Wahren-Herlenius
RMD Open. 2017 Sep 7;3(2):e000520.

VI. **Auxilin-2 is a novel cross-reactive target of Ro52/p200 antibodies in congenital heart block**
Lauro Meneghel, Aurélie Ambrosi, Cecilia Mattsson, Vijole Ottosson, Malin Hedlund, Sabrina Meisgen, Johannes Mofors, Jacob Brandtberg, Alexander Espinosa, Stina Salomonsson, Sven-Erik Sonesson, Peter Nilsson, Marie Wahren-Herlenius
Manuscript

*These authors contributed equally

PUBLICATIONS NOT INCLUDED IN THE THESIS

H1N1 vaccination in Sjögren's syndrome triggers polyclonal B cell activation and promotes autoantibody production
Susanna Brauner, Lasse Folkersen, Marika Kvarnström, Sabrina Meisgen, Sven Petersen, Michaela Franzén-Malmros, Johannes Mofors, Karl A Brokstad, Lars Klareskog, Roland Jonsson, Lisa S Westerberg, Christina Trollmo, Vivianne Malmström, Aurélie Ambrosi, Vijay K Kuchroo, Gunnel Nordmark, Marie Wahren-Herlenius
Ann Rheum Dis. 2017 Oct;76(10):1755-1763.

LIST OF ABBREVIATIONS

AVB	Atrioventricular block
CHB	Congenital heart block
DFAM	Family-based association for disease trait
ECM	Extracellular matrix
EQTL	Expression quantitative trait loci
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
ICT	Isovolumetric contraction time
MHC	Major histocompatibility complex
NLE	Neonatal lupus erythematosus
PBMC	Peripheral blood mononuclear cell
PDT	Pedigree disequilibrium test
SLC	Solute carrier
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Sjögren´s syndrome
TDT	Transmission disequilibrium test

CONTENTS

1 INTRODUCTION

Placental transfer of maternal Ro/SSA and La/SSB autoantibodies during pregnancy is associated with conduction disturbances and inflammation in the developing fetal heart, termed autoimmune-mediated congenital heart block (CHB). Maternal autoantibodies are the main risk factor associated with the fetal cardiac disease to date, however, the low recurrence rate despite persisting autoantibodies in subsequent pregnancies indicates that additional factors determine fetal susceptibility to develop CHB. The complex interaction between fetal genetic variants and factors that influence the intrauterine environment is thought to trigger or prevent the onset of CHB in Ro/SSA and/or La/SSB exposed pregnancies. The identification and functional exploration of such variants and factors was the main aim of this thesis.

1.1 AUTOIMMUNE DISEASES

In autoimmune diseases, the loss of immunological tolerance to self-antigens causes destructive immune responses against self-tissues. Loss of control over immune-recognition and inflammation results in continuous immune activation, governed by periods with disease flares and those with quiescent disease (Valesini et al., 2015). Autoimmunity is considered to result from the complex interaction of genetic and environmental factors, and the common understanding of autoimmune disease pathogenesis is that disease is triggered by an environmental risk factor in genetically predisposed individuals (Colafrancesco et al., 2013).

The observation that the incidence of autoimmune disease in selected families is increased together with a higher concordance for disease in identical twins compared to the risk in the general population has led to the conclusion of a genetic component involved in disease etiology (Becker et al., 1998). Genome-wide association studies (GWAS) have demonstrated that several genetic components in different loci contribute to the complex disease pathogenesis in sporadic cases with little risk contribution from each of the associated disease variants. The complexity of gene-gene interactions and epigenetics have made it further difficult to identify causal genetic components involved in autoimmune disease etiology (Lewis and Knight, 2012).

However, it has also become obvious that most of the genetic associations related to autoimmunity reside within the MHC locus (Matzaraki et al., 2017). Genetic variants may overlap between several diseases; identifying general pathogenic mechanisms in autoimmune disease development *e.g.* break of tolerance. By contrast, associations with non-MHC loci have often been related to one or a specific group of autoimmune diseases. This is exemplified by tyrosine phosphatase PTPN22, involved in lymphocyte activation signaling or the STAT4 transcription factor and their association with several systemic autoimmune diseases (Begovich et al., 2004; Bottini et al., 2004; Gregersen and Olsson, 2009; Kyogoku et al., 2004), while *e.g.* the IRF5 transcription factor polymorphisms associate with systemic lupus erythematosus (SLE) and Sjögrens´s syndrome (SS) (Graham et al., 2006; Miceli-Richard et al., 2007). Moreover, distinct MHC alleles are associated with the production of certain autoantibody profiles, typically of a distinct type of disease and mechanism of pathogenesis (Berg et al., 2000; Morris et al., 2014; Ronnelid et al., 2017), while other MHC alleles, like HLA-DRB1*13 are associated with a variety of autoimmune diseases and considered to mediate similar effects across these diseases (Lundstrom et al., 2009; Vasconcelos et al., 2009; Zeitlin et al., 2008).

Despite the identification of the genetic component contributing to autoimmune diseases, identical twins discordant for disease suggest that an environmental component is involved in disease etiology (Gregersen, 1993). Environmental factors such as infections, smoking, diet and seasonal variation pattern (*e.g.* representative of levels of vitamin D, exposure to ultraviolet light) have been shown to contribute to autoimmune diseases for instance in the context of type-1 diabetes, rheumatoid arthritis and multiple sclerosis (Morran et al., 2015; Padyukov et al., 2004; Willer et al., 2005).

1.2 RHEUMATIC DISEASES IN MOTHERS OF CHILDREN WITH CHB

Rheumatic autoimmune diseases are complex diseases and among others include systemic lupus erythematosus (SLE), Sjögren´s syndrome (SS), rheumatoid arthritis (RA) and as well as the less defined group of undifferentiated or mixed connective tissue disease (UCTD). These autoimmune diseases occur more frequently in women than men, and, among other diagnostic criteria, present with specific autoantibody profiles (Gleicher and Barad, 2007) (Lockshin et al., 2015). From several studies, it has become evident that discrete genetic variants within the MHC region are associated with the production of certain autoantibodies that can be either common for a certain group of autoimmune diseases or specific for a distinct disease (Lockshin et al., 2015; Matzaraki et al., 2017). HLA-DRB1*03 has been associated with the presence of Ro/SSA autoantibodies and placental transfer of these autoantibodies is in turn associated with an increased risk for the development of cardiac and/or non-cardiac manifestations of neonatal lupus erythematosus (NLE) in the child (Litsey et al., 1985; Loiseau et al., 2001; Scott et al., 1983). Commonly, the women carrying Ro/SSA autoantibodies are diagnosed with SLE and/or SS, and to a smaller proportion diagnosed with UCTD (Cavazzana et al., 2001; Chan and Andrade, 1992; von Muhlen and Tan, 1995). However, some women may be asymptomatic and without a diagnosis at the time of pregnancy despite positive autoantibody serology. Because of the association between maternal autoantibodies and fetal disease, NLE is also defined as a passively acquired autoimmune disease.

Both SS and SLE are rheumatic diseases, affecting more women than men (ratio 9:1), and are characterized by B cell hyperactivity, production of autoantibodies, presence of an interferon signature and the increased risk for B lymphoma development (Bennett et al., 2003; Gottenberg et al., 2006; Theander et al., 2006). SS is a relatively common disease affecting the exocrine salivary and lacrimal glands resulting in lymphocytic infiltrates and progressive tissue destruction, which causes dryness of the mouth and eyes (Jonsson et al., 2000). The peak incidence of SS is women between 45 to 55 (Mavragani and Moutsopoulos, 2010) but SS may also affect younger individuals, defining two disease subgroups, and autoantibodies have been described as more prevalent in early onset SS (Haga and Jonsson, 1999). SLE is a more heterogenous disease and destructive processes are characterized by the deposition of immune complexes in a variety of organs including the kidneys, lungs, joints, skin and blood vessels and the central nervous system. Typically, SLE affects more women in their child-bearing years (Klippel, 1997; Petri, 2001).

Pregnancy complications in women with autoimmune diseases, including SS and SLE, are more frequently observed than in healthy women, with increased risk for preeclampsia, perinatal death, preterm delivery and delivery of children small for gestational age

(Skomsvoll et al., 1998; Skomsvoll et al., 1999). Pregnancy management in SLE patients has improved; flares during pregnancy are mild and often require only minor treatment adaptions (Buyon et al., 2015; Tincani et al., 2006). However, renal flares associated with severe pregnancy complications as well as other complications still occur and hence patients require special care during pregnancy (Clowse et al., 2006; Yasmeen et al., 2001). SS is a considerably milder disease compared to SLE, with a slightly later mean time of disease onset, and less pregnancy complications are described for women diagnosed with SS, even if some studies report complications for this group of women (Haga et al., 2005; Hussein et al., 2011; Julkunen et al., 1995).

1.3 NEONATAL LUPUS ERYTHEMATOSUS

Neonatal lupus erythematosus (NLE) is a passively acquired autoimmune disease and presents with cardiac (CHB) and non-cardiac manifestations in individuals exposed to maternal Ro/SSA and La/SSB autoantibodies *in utero.* The population-based incidence of autoantibody-mediated CHB is 1 case in 23.000 live births (Skog et al., 2016).

1.3.1 Non-cardiac manifestations

Cutaneous lesions are one of the most common non-cardiac manifestations, present in approximately 15-25% of the infants with NLE (Buyon and Clancy, 2003b; Lee and Weston, 1997). Hepatic and hematological aberrances are also included in the spectrum of non-cardiac manifestations, all of which are considered transient and resolve after the maternal autoantibodies are cleared from the infant´s circulation (Admani and Krakowski, 2013). Hepatic involvement is usually asymptomatic and presents with elevated liver enzymes (Silverman and Jaeggi, 2010). Incidence numbers vary across different studies and dependent on whether presented in combination with other manifestations range between 10-40% (Cimaz et al., 2003; Lee et al., 1993; Lee et al., 2002; Watson et al., 1984). Hematological abnormalities including neutropenia and thrombocytopenia have been described as additional non-cardiac manifestations of NLE with an incidence of 26% in a larger cohort study by Cimaz and colleagues (Cimaz et al., 2003; Watson et al., 1988; Wolach et al., 1993). Even though neurological and skeletal manifestations are reported among infants exposed to maternal autoantibodies, these are considered very rare conditions compared to the other non-cardiac manifestations (Boros et al., 2007; Nakayama-Furukawa et al., 1994; Shanske et al., 2007).

1.3.2 Cardiac manifestations

The most recognized cardiac manifestation in NLE is a third degree atrioventricular block (AVB) in the absence of major structural heart diseases, which develops when fibrosis and calcification replace the AV node (Buyon et al., 2009; Jaeggi et al., 2002). The majority of cases are diagnosed *in utero* between pregnancy weeks 18 to 24 or during the neonatal period (within 27 days postpartum) (Brucato et al., 2010; Hornberger and Al Rajaa, 2010). A third degree AVB is characterized by a complete block of signal conduction through the AV node resulting in a slow ventricular rate (**Figure 1**).

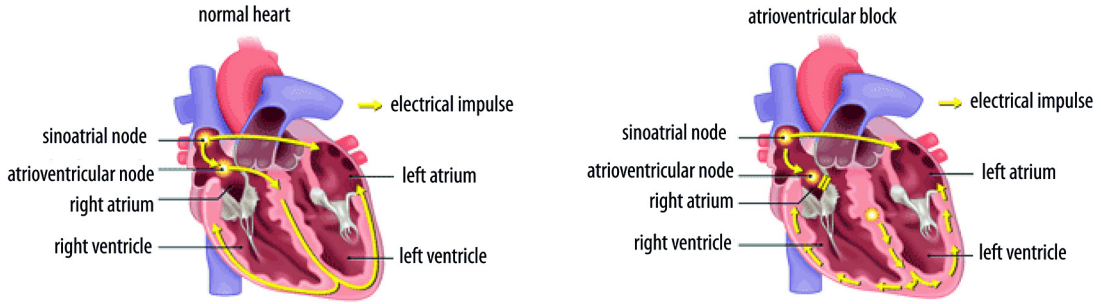

Figure 1. Signal transmission in a normal heart and a heart presenting with an atrioventricular block.

Congenital AVB is also present in congenital heart diseases with structural malformations involving the AV node. This group represents the other major etiology of AVB detected *in utero* (Hitz et al., 2012; Preuss and Andelfinger, 2013; Zaidi and Brueckner, 2017). Of note, mitochondrial diseases are common and often associate with cardiac diseases including heart block, *e.g.* primary mitochondrial cardiomyopathy (Andersson et al., 2011). Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an example of another disease etiology apart from CHB, where cardiac injury may be accompanied by an inflammatory response and repair mechanisms resulting in tissue remodeling contribute to pathophysiology (Svensson et al., 2016).

There is increasing evidence that CHB comprises a broader spectrum of fetal cardiac manifestations affecting both the cardiac conduction system and the myocardium. This evidence comes in part from centers with surveillance of risk pregnancies where it has been recognized that the bradycardia is preceded and paralleled by additional cardiac pathologies (Brucato et al., 2010; Hornberger and Al Rajaa, 2010). In early stages of CHB, these manifestations include sinus node dysfunction, lower degree atrioventricular block and a prolonged isovolumetric contraction time (ICT) and approximately one third of the cases are affected by these potentially reversible conditions (Bergman et al., 2009; Kurita et al., 1992; Sonesson et al., 2004). More diverse myocardial manifestations including myocardial inflammation, have been observed in 15-20% of the fetuses before birth (Jaeggi et al., 2002; Moak et al., 2001) and nearly one-third of the fetuses show signs of junctional ectopic or ventricular tachycardia (Villain et al., 2006; Zhao et al., 2008). Endocardial fibroelastosis and dilated cardiomyopathy (DCM) are the most severe manifestations and associate with a high mortality rate in the fetus (Hornberger and Al Rajaa, 2010). Of note, a late-onset DCM may also occur, but this disease entity is considered different compared to the *in utero* or neonatal DCM (Morel et al., 2017).

In this thesis, CHB refers to the spectrum of fetal cardiac manifestations occurring in neonatal lupus.

1.3.2.1 Incidence, recurrence rate and mortality

Complete congenital heart block in the general population is rare, with 1 case in 15,000-20,000 live births (Michaelsson and Engle, 1972; Siren et al., 1998). Among fetuses exposed to maternal Ro/SSA autoantibodies, the incidence for a third degree AV block is increased, and ranges between 1-2% (Ambrosi et al., 2012b; Brucato et al., 2010; Buyon et al., 2015; Morel

et al., 2017). In anti-Ro52/SSA positive women, the prevalence of CHB has been suggested to be even further increased (Buyon et al., 1998; Salomonsson et al., 2002). The recurrence rate of third degree AVB in following pregnancies is notable, but far from 100%, and ranges between 12-18% despite persisting maternal autoantibodies, thus indicating that additional factors influence disease outcome in the exposed fetuses (Buyon et al., 1998; Salomonsson et al., 2011; Solomon et al., 2003).

In anti-Ro52/SSA antibody positive pregnancies approximately 30% of the fetuses present with signs of first degree AVB, which most often spontaneously reverts before birth (Sonesson et al., 2004). Even though validated numbers for incidence of lower degree of AV block in newborns are still under investigation, the incidence of first degree AV block ranges between 3-14% in Ro/SSA exposed fetuses (Bergman et al., 2009; Friedman et al., 2008; Rein et al., 2009). In a recent study, Sonesson and colleagues could show that the recurrence rate of conduction disturbances in anti-Ro/SSA antibody exposed fetuses is higher than previously described when taking also milder forms of block into account (Sonesson et al., 2017). The mortality in complete AVB is high, ranging between 15-25% (Eliasson et al., 2011; Eronen et al., 2000; Izmirly et al., 2011; Jaeggi et al., 2002; Sharland et al., 1991).

1.3.2.2 Maternal Ro/SSA and La/SSB autoantibodies and CHB

The association between maternal Ro/SSA and La/SSB autoantibodies and NLE in the offspring is known since more than 30 years (Scott et al., 1983; Taylor et al., 1988). The Ro/SSA antigen complex refers to Ro52 and Ro60, two non-homologous proteins named after their mass in kilo Dalton (kD). The Ro52 protein consists of four functional domains, an N-terminal RING, a B-box, a coiled-coil domain and a C-terminal B30.2 region and belongs to the family of tripartite proteins (TRIMs) (Reymond et al., 2001). As other TRIM family proteins, Ro52 (TRIM21) has intrinsic E3 ligase activity and functions in the cellular process of ubiquitination (Espinosa et al., 2006), a posttranslational modification for proteasomal degradation and intracellular signaling (Swatek and Komander, 2016). Ro52 is predominantly expressed in immune cells and organs and plays an important role in innate and anti-viral responses as well as cell proliferation, survival or death (Espinosa et al., 2006; Kong et al., 2007). Ro60 is an ubiquitously expressed protein binding small cytoplasmic RNAs, hYRNAs. Further, Ro60 has been shown to bind misfolded non-coding RNAs and is important for cell survival after ultraviolet irradiation (Chen et al., 2000; Chen et al., 2003). The La/SSB antigen consists of the 48kDa La protein and like Ro60, associates with hYRNA complexes with a suggested function in transcriptional termination and virus replication (Wolin and Cedervall, 2002).

Even though the association between CHB and maternal antibodies directed towards the Ro52, Ro60 and La autoantigens varies across studies, the majority of maternal autoantibodies target the Ro52 protein, present in almost 95% of the mothers to CHB-affected individuals (Buyon and Clancy, 2003a; Eronen et al., 2004; Fritsch et al., 2006; Julkunen et al., 1993; Salomonsson et al., 2002; Sonesson et al., 2017). However, a clear distinction between Ro52 and Ro60 antibody associations has been difficult because of their common co-occurrence, and the individual impact on the development of CHB for the respective antibody is still a matter of debate. The relevance of anti-La antibodies for the development of CHB is controversially discussed and while it was associated with cutaneous NLE rather than CHB (Jaeggi et al., 2010;

Silverman et al., 1995), Gordon and colleagues reported that anti-La antibodies increased the risk of CHB in their study population (Gordon, 2004).

The tight association between CHB and maternal anti-Ro52 antibodies, and the identification of immunodominant epitopes within the Ro52 protein have prompted studies assessing the fine specificity of anti-Ro52 antibodies associated with CHB (Kato et al., 1995; Pourmand et al., 1998). Fritsch and colleagues identified several dominant epitopes within the Ro52 protein in their study cohort representative of specificities in SLE mothers (Fritsch et al., 2006). Epitope mapping in a study cohort with mainly SS or asymptomatic mothers revealed CHB association with maternal antibodies directed towards amino acids 200-239 of Ro52, denoted p200 (Ottosson et al., 2005; Salomonsson et al., 2002; Strandberg et al., 2008). The relevance of the p200 epitope was further validated in a prospective study where higher titers of Ro52/p200 antibodies were correlated with longer AV time in the fetus during the risk period of CHB (Salomonsson et al., 2005). The pathogenic effect of the anti-Ro52 and anti-Ro52/p200 antibodies has been further evidenced by *in vivo* studies showing transfer of these specific antibodies into pregnant rats induced AVB in the offspring (Ambrosi et al., 2012a; Strandberg et al., 2010).

1.3.2.3 Autoantibody cross-target hypothesis and CHB pathogenesis

Despite the association between CHB and maternal anti-Ro/SSA autoantibodies, the mechanisms by which the maternal autoantibodies mediate their pathogenic effects are not fully understood, specifically considering the low cardiac expression of Ro52 as well as its intracellular localization (Espinosa et al., 2009; Espinosa et al., 2006). Related to this, it has been postulated that anti-Ro52/p200 antibodies cross-react with other proteins expressed on the plasma membrane of fetal cardiac cells causing electrophysiological disturbances. In support of this hypothesis, *in vitro* studies demonstrated direct binding of maternal Ro52/p200 antibodies caused disturbances in the calcium homeostasis and subsequent apoptosis in cultured rat cardiomyocytes (Salomonsson et al., 2005). Identification of cardiac L-Type and T-Type calcium channels as potential cross-targets for maternal anti-Ro52/p200 antibodies and functional impairment of these channels in presence of maternal autoantibodies further supported this hypothesis (Boutjdir et al., 1997; Karnabi et al., 2011; Qu et al., 2005; Qu et al., 2001; Strandberg et al., 2013). The $Ca_v1.3$ channel is one example for a potential cross-target identified, and the IgG fraction of antibodies purified from Ro/SSA positive mothers of children with CHB inhibited the $Ca_v1.3$ calcium channel which the authors hypothesize could account for the sinus bradycardia and contractile impairment seen in human CHB (Qu et al., 2005). Another suggested cross-target for maternal autoantibodies is the cardiac 5-HT4 serotoninergic receptor (Eftekhari et al., 2001), although no association between presence of these maternal cross-reactive autoantibody specificities and fetal CHB was detected (Buyon et al., 2002). A recent study identified several discrete epitopes of Ro52/p200 that associated with fetal cardiac conduction system manifestations in rodents, further suggesting that several different antibody specificities and cross-targets may exist and contribute to pathogenic mechanisms related to CHB (Hoxha et al., 2016)

The severe cardiac manifestations during CHB pathogenesis are preceded or paralleled by sustained inflammation of the conductive system and/or myocardium (Friedman et al., 2003). This has been evidenced by the presence of proinflammatory cytokines, leukocyte recruitment, IgG and complement deposition in cardiac histopathology sections from

deceased CHB fetuses who had been exposed to maternal Ro/SSA autoantibodies (Clancy et al., 2004; Nield et al., 2002a). Related to this, it has been hypothesized that the inflammatory processes are partly driven by the activation of macrophages through the engulfment of Ro/SSA immune complexes on apoptotic cardiocytes and subsequent recruitment of leukocytes and production of proinflammatory cytokines (Clancy et al., 2004; Miranda-Carus et al., 2000). Moreover, fibrosis and calcification of the AV node have been described in relation to a third degree AVB as well as endocardial fibroelastosis all part of the spectrum of cardiac manifestations in affected fetuses, and a crosstalk between fibroblast and macrophages has been implicated to drive these tissue destructive processes during pathogenesis (Briassouli et al., 2011; Brito-Zeron et al., 2015; Clancy et al., 2004; Friedman et al., 2003). Even though this suggests that leukocyte infiltration, fibrosis accompanied by TGF-β upregulation and subsequent scar tissue formation are part of the inflammatory and fibrotic cardiac injuries during autoimmune-mediated CHB pathogenesis, the functional mechanism related to the maternal Ro/SSA autoantibodies is not fully understood, especially considering the fast course of destructive processes during disease pathogenesis. Surveillance programs have indeed shown that individuals can progress from a normally presenting heart to an advanced block within a matter of days (Friedman et al., 2009).

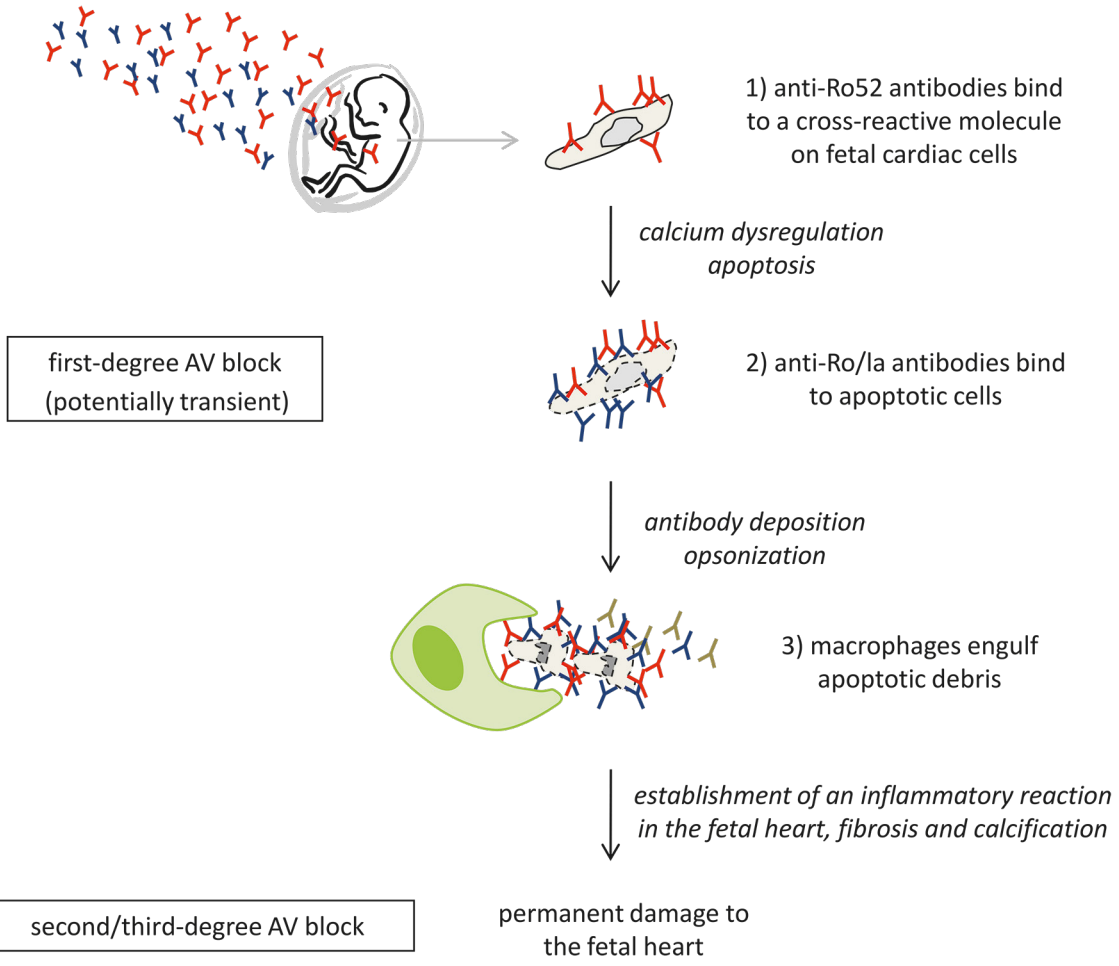

Figure 2. Two-phase model for CHB development (Ambrosi and Wahren-Herlenius, 2012).

Collectively the above-mentioned results gave rise to the two-phase model for CHB pathogenesis (**Figure 2**), where cross-reactive maternal autoantibodies induce conduction abnormalities observed as lower degree AVB, and further dysregulation of the conductive system and uncontrolled apoptosis followed by leukocyte infiltration, inflammation, fibrosis and calcification eventually lead to permanent damage to the fetal heart (Ambrosi and Wahren-Herlenius, 2012). However, while this current model explains some of the direct effects of maternal autoantibodies in fetal cardiac pathogenesis, it does not fully explain why some fetuses with milder CHB progress to a more severe CHB while others revert to a normal heart function despite continuous exposure to maternal autoantibodies.

1.3.2.4 Risk factors and susceptibility to CHB

The low recurrence rate of CHB in subsequent pregnancies despite persistent maternal Ro/SSA autoantibodies indicates that additional factors influence fetal susceptibility to CHB.

Genetic polymorphisms influencing fetal susceptibility to CHB upon exposure to maternal Ro/SSA autoantibodies have been reported in a candidate gene approach and a genome-wide association study (Clancy et al., 2003; Clancy et al., 2010) showing significant associations within the 6q21, 21q22 genomic locations and within the TGF-beta gene locus. While these data indicate fetal genetic factors may be additional factors influencing fetal susceptibility related to CHB, these data still require confirmation. Moreover, one may also be cautious in the interpretation of case control studies in the context of CHB. Comparing frequencies among cases and population-based controls is likely to reflect the maternal disease variants as mothers to CHB cases may have SLE or SS, or even if asymptomatic, are genetically different from the general population. Nevertheless using congenic rat strains, Strandberg and colleagues further supported the hypothesis that fetal MHC and non-MHC genes determine susceptibility to CHB in exposure to autoantibody specificities encoded by the maternal MHC (Strandberg et al., 2010).

Apart from genetic factors influencing fetal susceptibility to CHB, other risk factors including maternal age and hypothyroidism, season of birth and hypoxia have been described (Ambrosi et al., 2012a; Askanase et al., 2006; Clancy et al., 2007; Sonesson et al., 2017; Spence et al., 2006). On the contrary, fetal gender, parity and maternal disease activity did not emerge as risk factors associated with CHB (Ambrosi et al., 2012b; Buyon et al., 1998; Eronen et al., 2004; Llanos et al., 2009; Solomon et al., 2003).

1.3.2.5 Surveillance and in utero treatment/prevention of CHB

Surveillance programs to assess fetal cardiac function during the risk period for CHB (weeks 18 to 24) have been developed (Friedman et al., 2009; Sonesson et al., 2017). For fetal surveillance, several methods can be used including echocardiography, electrocardiography and magnetocardiography whereof fetal echocardiography, using m-mode and Doppler are the most commonly used techniques. (Brito-Zeron et al., 2015). Additionally, hand-held Doppler devices have also been introduced for home monitoring. In addition to the better understanding of the spectrum of fetal cardiac manifestations, this has also led to earlier and potentially more efficient treatment and better fetal outcomes. This is important as a third degree AVB may develop in a matter of days from a normally presenting heart (Friedman et al., 2009) and to date no effective therapy exists (Brito-Zeron et al., 2015).

In utero treatment options have been tested to improve fetal outcomes and include anti-inflammatory treatment using steroids, and transplacental treatment with fluorinated steroids (betamethasone and dexamethasone) (Bierman et al., 1988; Jaeggi et al., 2004). Betamimetics can be used to increase the fetal heart rate and myocardial output (Hutter et al., 2010; Jaeggi et al., 2004), however, as steroids are associated with risks for the mother and the developing child, treatment options should be well justified (Hutter et al., 2010; Tincani et al., 2006). Injection of intravenous immune globulins (IVIG) and plasmapheresis have also been used in treating CHB. Evidence-based treatment is however still lacking (Kaaja and Julkunen, 2003; Makino et al., 2007; Ruffatti et al., 2012).

1.3.2.6 Postnatal outcomes in CHB

Even though the majority of patients diagnosed with complete CHB require a pacemaker implant shortly after birth (Brito-Zeron et al., 2015; Eronen et al., 2000), postnatal outcomes in these individuals are very good, with a survival rate of 96% at follow-up after approximately 9 years of pacing (Eliasson et al., 2015). This is concordant with a previous study reporting survival rates around 95% using similar follow-up intervals (Villain et al., 2006). Low birth weight with catch-up before pre-teen years and attention deficits in children exposed to maternal Ro/SSA autoantibodies *in utero* are outcomes reported, however it should be noted that the vast majority of children, despite their serious heart condition, do well during childhood and teen-years (Skog et al., 2013a; Skog et al., 2013b).

2 AIMS

Fetuses exposed to maternal Ro/SSA autoantibodies are at risk to develop CHB. However, despite persisting autoantibodies in subsequent pregnancies, the recurrence rate for autoimmune-mediated CHB ranges between 12-18% suggesting that additional factors other than the maternal autoantibodies influence risk for CHB development.

The aim of this thesis was to identify such additional risk factors for CHB, focusing on fetal genetic variants and environmental and lifestyle factors that the mother was exposed to or experienced during pregnancy. Specifically, the following aims were set:

- To identify genetic polymorphisms associated with CHB in a genome-wide manner
- To identify MHC alleles associated with CHB
- To understand the influence of maternal body mass index, pregancy weight gain, seasonal timing of pregnancy, infections, sun exposure, outdoor activity and stressful events on the risk of CHB

3 METHODOLOGICAL CONSIDERATIONS

In this section, the strategies to identify genetic and environmental risk factors related to autoimmune-mediated CHB and the screening approach for maternal cross-reactive autoantibodies will be discussed.

3.1 CASES, FAMILIES AND CONTROLS INCLUDED IN THE THESIS

To have a homogenous study population, we used strict inclusion criteria with regard to maternal serology and fetal cardiac manifestations to exclude confounding through sample heterogeneity.

A Swedish cohort of families collected in a population-based manner with at least one child affected by CHB and a mother tested positive for Ro/SSA autoantibodies was the basis for initial risk factor studies and is described within **Papers I, III, V** and **VI**. Families with individuals who had died from CHB at the time of DNA collection were not included, as DNA could not be retrieved from the case.

European families with at least one child affected by CHB and a mother tested positive for Ro/SSA autoantibodies were identified and collected through collaborations with pediatric cardiologists and rheumatologists at several centers in Finland, Norway and Italy, and are described in **Paper II** and **IV**.

3.2 STUDY DESIGN AND ANALYSIS STRATEGIES FOR GENETIC ASSOCIATIONS

The mothers of individuals with CHB carry Ro/SSA autoantibodies and are commonly diagnosed with SLE or SS, and are genetically different from the general population (Gateva et al., 2009; Lessard et al., 2013). With a 50% chance of the fetus to inherit either of the two alleles of a specific gene from the mother, a deviation from the normal population will present in the offspring regardless of CHB or not. Hence an analysis of allele frequency differences between CHB cases and unrelated controls could not be used to identify genetic risk variants for CHB. Therefore, we chose a trio design for the genetic analyses, which includes the parents and affected offspring and uses the transmission disequilibrium test (TDT) which surpasses this problem (Spielman and Ewens, 1996). In complementary analyses approaches, we also used family-based association for disease trait (DFAM) and pedigree-disequilibrium test (PDT) (Laird et al., 2000; Purcell et al., 2007), allowing inference of missing parental genotypes using unaffected sibling genotypes.

3.3 HLA-ASSOCIATION ANALYSIS

Two approaches for HLA typing were used in the papers included in this thesis. In **Paper III**, PCR-based HLA typing was used for identification of HLA-A, -C and -DR alleles. Based on our findings, and with an extended European CHB family cohort, we used imputation of HLA-

A, -B, -C and -DP, -DQ and -DR alleles based on microarray SNP data in **Paper IV**. The *in silico* imputation approach (Dilthey et al., 2016) was used to receive higher resolution HLA-allele types to further fine map causal variants related to CHB, in a time and cost effective manner compared to the classical wet lab HLA genotyping approach. Correct HLA allele designation was ascertained by inclusion of Swedish reference genotypes for the class II region (Zhao et al., 2016) and by confirming alleles according to the Mendelian laws of inheritance in nuclear families.

3.4 PROCEDURES IN GENERATING THE QUESTIONNAIRES

Three approaches were employed for generating the specific questions included in the questionnaire used to identify maternal environmental and lifestyle factors influencing the risk of CHB. These included semi-structured interviews performed with mothers of children with CHB (Tingstrom et al., 2010; Tingstrom et al., 2013), data from our or others previous publications on potential risk or protective factors related to CHB (Ambrosi et al., 2012b; Izmirly et al., 2012) and an extract of questions previously used for risk factor investigation in rheumatoid arthritis and multiple sclerosis (Handel et al., 2010; Ilar et al., 2017). There were controlled case questions, statements to which the answer was given in a four-graded scale from *totally agree* to *totally disagree*, and open-ended questions to which specific comments could be added and space was provided for adding optional extra information. The questionnaires were validated in a test group before use.

Recall bias related to the retro-perspective design is a limitation of the questionnaire-related results. However, memories from pregnancy and delivery are often described as well remembered by mothers (Simkin, 1991) and the comparison of risk factors is between different pregnancies resulting in children with or without CHB from the same mothers possibly leading to even distribution of the remaining limitation between the two outcome groups.

3.5 CROSS-REACTIVE TARGETS FOR MATERNAL AUTOANTIBODIES

A few cross-reactive targets for maternal autoantibodies in CHB have been suggested (Boutjdir et al., 1997; Strandberg et al., 2013; Xiao et al., 2001). To expand the smaller scale cross-target approaches used in previous studies, we used a whole proteome peptide library to screen for additional cross-targets of Ro52/p200 autoantibodies (**Paper VI**). A limitation of our study is the peptide length (12-mers, 6 amino acid overlap), as target epitopes may require direct contribution from distant amino acids or longer stretches of the protein chain to fold correctly.

4 RESULTS AND DISCUSSION

4.1 GENETIC VARIANTS AFFECTING CARDIAC SIGNAL TRANSMISSION AND CONTRACTION

4.1.1 Genes with function in vesicular transport

Overall, cardiac function is dependent on the electrical signal initiation and propagation as well as its subsequent conversion into mechanical contraction. The correct temporal and spatial distribution and function of the proteins and structural elements involved in this complex process is crucial for the rhythmicity of heartbeats. Thus, genetic variants of components that are part of these tightly regulated processes may have pathological consequences. In CHB, prolonged fetal AV-time and ICT are among the major cardiac manifestations observed, suggesting that impairment of the excitation-contraction coupling may be involved in CHB pathology (Bergman et al., 2009; Sonesson et al., 2004). Vesicular transport is a crucial cellular pathway for excitation-contraction coupling (Nori et al., 2004), and was one of the CHB associated pathways we identified (**chapter 4.1**). Therefore, one focus in the course of our studies was to identify and functionally explore genes in vesicular pathways. These are described and discussed in the following chapters.

4.1.1.1 Auxilin is a fetal susceptibility gene for CHB with function in vesicular transport

Several SNPs within the chromosomal region 1p31.3 associated with CHB. The verified top SNP (rs1570868, P_{GAWS}=3x10^{-5}, **Paper I**) is located in an intronic region of the *DNAJC6*/auxilin gene locus which made this gene a very interesting candidate gene to further investigate in the context of CHB. Additional information on the function of auxilin, the protein encoded by *DNAJC6*, in vesicular transport (Scheele et al., 2001) and association of the risk SNP with cardiac-specific expression differences (**Paper I**) further strengthened that *DNAJC6*/auxilin is a relevant candidate gene in the context of CHB. Of note, no other cardiac eQTL effect was observed among the genes investigated within the chromosomal interval 1p31.3 (**Paper I**). Interestingly, we found that cardiac auxilin expression levels were high during fetal development, both before and during the risk period for CHB, and are decreased in adult cardiac tissue (**Paper I**). Moreover, among the fetal tissues tested, fetal cardiac expression is higher compared to expression levels in skeletal muscle or kidney (**Paper I**). Based on these results, we chose *DNAJC6/auxilin* as a novel fetal candidate gene for subsequent functional exploration.

While auxilin was expressed both in the myocardium and conduction system of the human fetal heart, subcellularly auxilin co-localized with clathrin in a vesicular pattern in human fetal cardiomyocytes (**Paper I**). This indicates that auxilin function in the heart is also within clathrin-mediated vesicular transport, a function for this protein previously described in other cell types (Scheele et al., 2001).

Association between cardiac auxilin expression and the CHB risk variant (rs1570868) suggested that the cardiac phenotype in CHB may lead to decreased protein levels. Therefore, we used auxilin-deficient mice as a disease model to further study the impact of auxilin for cardiac function in the context of CHB. After confirmation of auxilin expression in the heart

of wild-type pups, we assessed the impact of auxilin-deficiency on cardiomyocyte function. As Ca^{2+} is one of the main regulators of cardiomyocyte function, we evaluated spontaneous Ca^{2+} oscillations in primary cultures of wild-type and auxilin knockout neonatal cardiomyocytes. Compared to wild-type cells, auxilin-deficient cardiomyocytes showed decreased mean frequencies of Ca^{2+}-transients over time in combination with an increased coefficient of variation, which was indicative of an overall disturbed cellular calcium homeostasis (**Paper I**). Moreover, we found impaired cardiac cell connectivity and communication with cells at greater distance comparing auxilin-deficient and wild-type cardiomyocytes in culture (**Paper I**).

Given the described function of auxilin in clathrin-mediated endocytosis (Scheele et al., 2001) and the finding that auxilin-deficient cardiomyocytes displayed a disturbed calcium homeostasis, we hypothesized that absence of auxilin may impair the recirculation of calcium channels to the plasma membrane of cardiomyocytes. We therefore compared plasma membrane expression of the calcium channel $Ca_v1.3$ in auxilin wild-type and knockout cardiocytes as a functional measure for intracellular recirculation. Flow cytometry revealed that mouse neonatal $Sirpa^+$ cardiocytes (Dubois et al., 2011) had proportions of cells expressing $Ca_v1.3$ comparable in auxilin-deficient and wild-type mice, however $Ca_v1.3$ cell surface expression was significantly lower in $Sirpa^+Ca_v1.3^+$ auxilin-deficient cells compared to wild-type cardiocytes (**Paper I**). Interestingly, cardiac expression levels of $Ca_v1.3$ RNA transcripts were significantly higher in auxilin-deficient neonatal cardiocytes compared to wild-type cells (**Paper I**). This indicates that decreased $Ca_v1.3$ expression on the plasma membrane of auxilin-deficient cells was not due to a general decrease in gene expression, and further suggests that auxilin-deficient cardiomyocytes upregulate the transcription of $Ca_v1.3$ to compensate for decreased protein levels on the cell surface.

Functional relevance for fetal cardiac auxilin in the context of CHB was further supported by results from the analysis of Doppler profiles of mouse pups *in utero*. We found that auxilin-deficient pups *in utero* displayed several different CHB-related cardiac pathologies, including both prolonged AV-time and ICT, abnormal heart rate and arrhythmias including atrial and ventricular ectopic beats (**Paper I**).

Auxilin is involved in clathrin-mediated endocytosis (Scheele et al., 2001) and absence of auxilin may thus impair the recirculation of ion channels or other molecules important for cardiac function to the plasma membrane of cardiomyocytes. $Ca_v1.3$ is important for excitation of cardiac pacemaker cells and subsequent propagation of the signal resulting in contraction-coupling of ventricular cardiocytes (Mangoni et al., 2003) and our results indicated that absence of auxilin results in decreased surface expression of $Ca_v1.3$ that in turn may impact on the overall cardiac function. Decreased cell surface expression of ion channels, including $Ca_v1.3$, in absence of auxilin could also explain the lower cellular connectivity and communication and the decreased and less well-coordinated Ca^{2+} oscillations in auxilin-deficient cardiomyocytes in culture. Interestingly and in relation to our results, $Ca_v1.3$-deficient mice display a pattern of cardiac abnormalities with sinus bradycardia and AV block before birth (Karnabi et al., 2011; Platzer et al., 2000), suggesting that decreased expression of $Ca_v1.3$ on the surface of auxilin-deficient cells may contribute in part to the cardiac abnormalities we observed in auxilin-deficient mice *in utero*.

Fetuses exposed to maternal Ro/SSA autoantibodies have an increased risk to develop CHB (Buyon et al., 1989). Calcium channels, including Cav1.3, have been evidenced as potential cross-targets for the maternal Ro/SSA autoantibodies and binding of these autoantibodies to their antigens expressed by cardiomyocytes inhibited calcium currents inflow

and led to disturbed calcium homeostasis and apoptosis (Karnabi et al., 2011; Qu et al., 2005; Salomonsson et al., 2005; Strandberg et al., 2013). Hence binding of the maternal autoantibodies may additionally affect calcium channel function, blocking and further disrupting the cardiac electrical-excitation coupling. This functional link can therefore explain why genetically predisposed individuals, carrying the auxilin variant, may be more susceptible to CHB in exposure to maternal autoantibodies *in utero*.

Notably, in fetal cardiomyocytes, the sarcoplasmic reticulum is not yet fully developed, and thus excitation-contraction coupling mainly relies on plasma membrane calcium channels at the cell surface (Brillantes et al., 1994; Fisher, 1995). This is reversed in adult cardiomyocytes, where calcium is mainly released from sarcoplasmic reticulum stores. Hence decreased expression of auxilin accompanied by lower surface expression of calcium channels affects the function of fetal cardiomyocytes to a larger extent than adult cells, rendering the fetal heart more susceptible to the pathogenic effects of the maternal autoantibodies. This can explain why cardiac manifestations similar to CHB, despite persisting maternal autoantibodies are not observed in mothers to CHB affected individuals.

Collectively, our data support auxilin as a novel and relevant fetal susceptibility gene for CHB with direct impact on fetal cardiac function through the vesicular transport pathway.

4.2 HLA ASSOCIATIONS

Results from the GWAS and pathway analyses revealed several SNP variants close to genes with function in immune response associate with CHB (**Paper I** and **II**). The association between the MHC locus and autoimmune diseases, including the rheumatic disease of the mothers of the CHB-affected individuals is well-established (Shiina et al., 2004). Together with results from animal studies demonstrating that fetal MHC genes are additional risk factors to exposure to maternal Ro/SSA autoantibodies (Strandberg et al., 2010), we aimed to identify human fetal MHC genes contributing to immune responses influencing fetal susceptibility to CHB.

Parent-offspring trio analysis using PDT in individuals of the Swedish CHB family cohort (n=83 families) showed association of seven SNP markers with CHB in the extended MHC region ($P<0.01$, **Paper III**). HLA-allele typing in Swedish families (**Paper III**) and HLA allele imputation in the Swedish and international families (n=170, **Paper IV**) revealed protective associations of HLA-Cw*06 ($P=0.03$ **Paper III** and $P=0.003$ **Paper IV**) and HLA-DRB1*13 ($P=0.04$ **Paper III** and $P=0.007$ **Paper IV**) alleles across both study approaches. An HLA-DRB1*04 association with CHB ($P=0.03$) was identified in **Paper III** but could not be validated by the joint analysis approach (**Paper IV**).

The expansion of MHC gene loci included in **Paper IV** identified novel suggestive associations for HLA class II genes with DQA1 and DQB1 alleles. Furthermore, the study revealed two novel haplotype associations with CHB, the protective DRB1-DQA1-DQB1 13-01:03-06:03 ($P=0.025$) and the susceptible DRB1-DQA1-DQB1 08-04:01-04:02 ($P=0.022$) haplotype and those include some but not all of the CHB-associated and suggestively associated suballeles (**Paper IV**). Of note, no CHB-associations were observed for HLA-A, -B, -DPA1, -DPB1 and -DRB3, -B4 or -B5 alleles (**Paper IV**).

Even though we observed CHB associations within the MHC region, these associations were less significant compared to the non-MHC associations revealed from **Paper I** and **II**. This is in contrast to study results from Clancy and colleagues showing a strong association

signal within the MHC locus (Clancy et al., 2010). However, in relation to the specific maternal genetic predispositions it is more likely these results reflect the maternal disease traits rather than the CHB-unique traits. Consistent with the maternal predisposition in the HLA region and the well-known HLA-DRB1*03 allele associations with SS and SLE and the production of Ro/SSA autoantibodies, we find significantly higher frequencies of HLA-A*01 (52%; $P< 3x10^{-6}$), HLA-Cw*07 (84%; $P< 5x10^{-6}$) and class II HLA-DRB1*03 (79%; $P< 3x10^{-29}$) and HLA-DRB1*11 (14%; $P< 1x10^{-8}$) alleles, compared to the allele distribution in the general population and concordant to previous reports (Alexander et al., 1989; Miyagawa et al., 1997; Miyagawa et al., 1998) (**Paper III**). Intriguingly, the HLA alleles we found associated with CHB in the offspring of these mothers were different from the alleles associated with the maternal disease (**Paper III** and **IV**) and all associated variants passing thresholds for multiple testing conferred protective effects. We therefore conclude that certain fetal MHC alleles determine susceptibility to the effects of the maternal Ro/SSA antibodies in relation to the destructive immune responses during CHB pathogenesis. However, these fetal MHC alleles are different from the maternal alleles that are associated with the ability to generate the pathogenic antibodies.

The class I HLA-Cw*06 allele was one of the most robust MHC associations with CHB identified in our study cohorts. HLA-C molecule:peptide complexes are potent inducers of cytotoxic responses through the interaction with receptors expressed by NK and $CD8^+$ T cells (Blais et al., 2011). In connection to this, it is interesting that one of these effector cells, $CD8^+$ T cells, are among the mononuclear cells infiltrating the fetal heart evidenced from immunohistology sections of CHB hearts (Nield et al., 2002a; Nield et al., 2002b). This indicates that MHC class I peptide presentation by certain allelic variants of the HLA-C molecule may be involved in disease pathogenesis. Moreover, these fast and specific cytotoxic effector functions are concordant with reports from centers with surveillance programs for pregnant Ro/SSA positive women reporting that severe degree heart block development from a normal appearing heart occurs within days (Friedman et al., 2009). A certain group of HLA-C alleles, sharing a particular amino acid at position 80 and including the HLA-Cw*06 allele, was recently reported as more frequent in siblings affected by CHB than those unaffected by CHB (Ainsworth et al., 2017). A detailed analysis of the HLA-C alleles was not included in this study making comparisons difficult. However, results from our CHB family cohorts did not reveal any associations of the other alleles or group of alleles assigned to the HLA-C group with increased risk to CHB. The protective HLA-Cw*06 allele associations we found are therefore likely to be specific and independent of other allele associations within this structurally connected group of HLA-C molecules. The protective association of the HLA-Cw*06 allele was evident across different European populations and may thus indicate that structural features within the peptide binding groove of this specific allelic MHC variant impact on disease outcome related to CHB. Hypothetically, these structural features may exclude the presentation of peptides needed for activation of cytotoxic T cell responses contributing to tissue destruction in the course of disease.

Part of the immunopathogenesis in CHB indicated the involvement of macrophages driving the inflammatory processes in the fetal heart (Clancy et al., 2004) and it is suggested that an imbalance of the fetal immune system is involved in the initiation of the tissue destructive responses during pathogenesis. We validated protective HLA-DRB1*13 associations across the Swedish and European CHB family cohorts indicating these are robust allelic variants that may determine fetal susceptibility to CHB. Interestingly, in European

populations protective DRB1*13 associations are commonly described, including many different systemic and organ-specific autoimmune diseases such as SLE, RA and Hashimoto´s thyroiditis (Bettencourt et al., 2015; Lundstrom et al., 2009; Vasconcelos et al., 2009; Zeitlin et al., 2008). We therefore hypothesized that the protective DRB1*13 associations for CHB may underlie a general mechanism of protection from inflammation shared among autoimmune diseases. Such a mechanism could be the result of a more proficient self-antigen presentation during thymic selection that would favor efficient clonal deletion of self-reactive $CD4^+$ T cells by the HLA-DRB1*13 molecule (van Heemst et al., 2015).

HLA-DRB1*04 associations with CHB could not be confirmed across both studies and hence, we cannot exclude this results represents a false positive association. Noticeable, for a number of parents, mainly Swedish, no parental genotype information was available, although DRB1*04 alleles were present in the CHB individuals. We could however not collect DNA from these parents to generate data on transmission, which potentially could confirm the association due to the increased number of informative pedigrees. Moreover, the DRB1*04 allele is less common in southern Europe and hence including more families from those countries will probably increase the power to detect that association.

The findings of HLA-DQA1 and -DQB1 associations with CHB, combined in the DRB1-DQA1-DQB1 13-01:03-06:03 protective haplotype are concordant with results from a previous study in a Finnish cohort (Siren et al., 1999) suggesting that similar as for the DRB1 associations, particular DQA1 and DQB1 allelic variants in complex with certain peptides elicit a certain type of immune recognition that may in turn impact susceptibility to CHB. In this context, it is interesting that DQ-restriction has been reported to be involved in the modulation or onset of autoimmune responses (Moustakas and Papadopoulos, 2002; Sugita et al., 1990; Tree et al., 2004). HLA-DQA1*01 and DQB1*06 alleles have also been described previously for their associations with systemic and organ-specific autoimmune diseases, like ANCA-associated vasculitis (Gencik et al., 1999) and T1D (Kiani et al., 2015), often in combination with the DRB1*13 allele.

In summary, we identified specific and discrete fetal MHC alleles that may influence susceptibility to CHB in addition to the maternal Ro/SSA autoantibodies. The results further emphasize that cytotoxic immune responses may contribute to cardiac inflammation. A likely interaction with CHB risk variants outside the HLA locus or variants affected by the proinflammatory cardiac environment (discussed in **chapter 4.3.2.**) may additionally influence disease susceptibility in predisposed individuals.

4.3 CHB AND FACTORS WITH POTENTIAL TO INFLUENCE THE INTRAUTERINE ENVIRONMENT

The intrauterine environment impacts on fetal health (Burton et al., 2016) and in relation to CHB, maternal Ro/SSA autoantibodies are the main risk factors identified that may modulate the fetal environment (Litsey et al., 1985; Salomonsson et al., 2005). The low recurrence rate for CHB despite persisting maternal autoantibodies in subsequent pregnancies however indicates additional factors determine fetal outcome (Ambrosi et al., 2012b; Buyon et al., 1998; Levesque et al., 2015). Given the relatively narrow window of the onset of CHB, defining factors that may influence the intrauterine environment and relate to the risk of CHB are relevant. In the following sections, potential CHB risk factors related to maternal

environmental and lifestyle factors as well as maternal autoantibody specificities will be described and discussed.

4.3.1 Maternal environmental and lifestyle factors and influence to the intrauterine milieu

In order to identify maternal environmental and lifestyle factors related to fetal susceptibility to CHB, we performed a questionnaire-based study in a population-based cohort of women with a positive Ro52 autoantibody serology and who had given birth to at least one child with CHB and may have had unaffected children.

The overall response rate for the questionnaires was high, 89% (n=78/88), returning information from 81 CHB pregnancies and 108 unaffected sibling pregnancies (**Paper V**). As expected given the inclusion criteria of the study, all women were Ro52 positive with a considerably high proportion of Ro60 and La autoantibody positivity (**Paper V**).

No differences in pregnancy outcomes were observed for the investigated maternal factors smoking, body mass index (BMI), and weight increase during pregnancy resulting in children with or without CHB (**Paper V**). Further, we did not observe any variances between medication intake and pregnancy outcomes before and during pregnancy up until gestational week 25 (**Paper V**). Of note, no protective effect for hydroxychloroquine (HCQ), previously suggested reducing neonatal morbidity and the recurrence risk of CHB in women diagnosed with SLE (Izmirly et al., 2012; Leroux et al., 2015), was detected in the study. However, the relatively low number of women on HCQ treatment in our study population still implements a potential effect for CHB development. Additionally, other medication not prescribed for treatment of rheumatic diseases and addressed in our study may influence pregnancy outcomes in the context of CHB (**Paper V**).

We found that common infections during pregnancy, such as infection of the respiratory tracts or influenza, were associated with CHB (P=1.3x10^{-4}, **Paper V**). Even though direct viral transmission via the placenta is rare (Irving et al., 2000), secondary effects in the offspring due to the maternal inflammatory state have been demonstrated and those comprise effects on the brain, occurrence of congenital abnormalities including the heart and may lead to preterm birth (Acs et al., 2005; Shi et al., 2005). This is specifically interesting taking into account the results from **Paper II**, indicating that exposure to maternal Ro/SSA autoantibodies may induce a proinflammatory environment in the fetus affecting the expression of genes in immune responses. Infectious trigger of the maternal inflammatory state contributing to the proinflammatory environment by *e.g.* increased levels of type I and II interferons, together with fetal susceptibilty genes may therefore decrease the threshold and precipitate CHB.

Concordant with results from a previous study (Ambrosi et al., 2012b), we found that seasonal timing of pregnancy emerged as a risk factor for CHB (P=0.02, **Paper V**). Fetal risk for CHB was increased in pregnancies where the risk period (weeks 18-24) was during January to March and might be explained by increased frequency of infections during this season. Multivariate analysis supported the hypothesis that association of seasonal timing may be partly dependent on maternal infection. In addition to common infections, factors such as vitamin D levels (Ambrosi et al., 2012b) and time spend outdoors also underlie seasonal variation. While reports on sun exposure habits were not different among CHB and non-CHB pregnancies in our study, the amount of outdoor activities during daytime emerged as a protective factor in unaffected pregnancies and notably increased with the amount of

time spent outdoors (P_{TREND}=0.01, **Paper V**). Interestingly, multivariate analysis disclosed that the association between CHB and season of birth was not independent of outdoor activity during daytime and hence may denote an additional important factor, apart from infections, underlying the association between CHB and season of birth (**Paper V**). Active time spent outdoors may be an indirect measure of several factors besides mild-to-moderate physical activity indicated in questionnaire examples, which *per se* could favorably influence the pregnancy. Other factors could be a reflection of the women´s general well-being during pregnancy or indicate increased exposure to UV light and related to higher vitamin D levels. In line with studies reporting an important role for vitamin D in the regulation of innate and adaptive immune responses and in relation to the modulation of placental inflammation (Lagishetty et al., 2011; Liu et al., 2011), this is an intriguing result.

Reports of psychologically stressful events, such as the death or severe disease of a close relative, up until week 25 of pregnancy differed significantly with more women reporting such an experience during their pregnancy resulting in a child with CHB (P=0.02, **Paper V**). Indeed, there is evidence from studies showing that experiencing a life-changing event resulting in psychological stress may negatively influence pregnancy outcome (Adam et al., 2013; Laszlo et al., 2013) and may hence also influence CHB pregnancy outcomes. However, it has to be noted that the number of women reporting such events in our study was low and thus confirmation from studies including lager cohorts is needed to assess psychological stress as a potential risk factor in the context of CHB.

In summary, we have shown that certain maternal environmental and lifestyle factors with the potential to modulate the intrauterine environment associated with different pregnancy outcomes related to CHB in presence of Ro/SSA autoantibodies. However, prospective studies will be needed to confirm these data.

4.3.2 Intrauterine exposure to maternal anti-auxilin-2 autoantibodies

Among the anti-SSA/Ro52 maternal autoantibodies, anti-Ro52/p200 antibodies feature the most antigenic epitope within the Ro52 protein and associate with an increased risk for fetal CHB in relation to anti-p200 antibody presence and levels (Ottosson et al., 2005; Salomonsson et al., 2002; Strandberg et al., 2008; Tonello et al., 2016). It has been shown that anti-SSA/Ro52 and anti-Ro52/p200 antibodies may cross-react with targets in the fetal heart; however, the proportion of these cross-reactive autoantibodies specificities in positive tested sera from mothers with CHB-affected children were considerable low (Buyon et al., 2002; Karnabi et al., 2010; Qu et al., 2005; Qu et al., 2001; Strandberg et al., 2013). This indicates that maternal antibody reactivity to one or more additional cross-targets may exist, and these specificities are potentially more explanatory for fetal disease pathogenesis than the previously described cross-reactive autoantibody specificities. We therefore aimed to identify novel targets of anti-Ro52/p200-specific antibodies using a broad proteome library screen and investigate their clinical relevance in CHB.

In line with the cross-target hypothesis, *in vivo* transfer of monoclonal antibodies specific for the p200 region of Ro52 into pregnant mice, led to signs of first degree AV block in the pups even in absence of the Ro52 protein. Signs of AV block were not observed in mouse pups exposed to anti-Ro52 antibodies specific to the C-terminal region of Ro52 (**Paper VI**). This is concordant with previous studies showing anti-Ro52/p200 antibodies, but not other Ro52-specificites, can induce AV block in rat pups after transfer (Strandberg et al., 2010).

With a peptide screen covering the human proteome we found that anti-Ro52/p200 antibodies cross-react with peptides sharing either one of two minimal epitopes "YSDF" (Tyr-Ser-Asp-Phe) or "YSNF" (Tyr-Ser-Asn-Phe) (**Paper VI**). Proteome mapping of those peptide sequences led to the identification of seven potential cross-targets for CHB recognized by Ro52/p200 but no other Ro52 specific antibodies, among those auxilin-2 and thyroglobulin (**Paper VI**). Interestingly, auxilin-2/GAK (Cyclin G Associated Kinase) is a homologue to auxilin-1/*DNAJC6*, which we identified as a novel fetal susceptibility gene for CHB (**Paper I**). Despite their relatively high sequence homology, the "YSNF" epitope maps to a protein kinase domain of auxilin-2, not present in auxilin-1 (**Paper VI**).

We found that 22.4% of the antiRo52/p200 positive mothers who had given birth to a child with CHB displayed cross-reactivity to auxilin-2, which was significantly higher compared to control sera ($P<0.001$, **Paper VI**). Interestingly, high p200 titers correlated with higher anti-auxilin-2 reactivity, showing that reactivity towards auxilin-2 may occur in an anti-p200 level-dependent manner (**Paper VI**). The proportion of maternal anti-Ro52/p200 autoantibodies specific for auxilin-2 was higher in our study compared to other studies showing 14 and 16% of the maternal CHB sera are positive for the Ro/SSA autoantibodies cross-specificities in maternal CHB sera (Buyon et al., 2002; Karnabi et al., 2010). Nonetheless, cross-reactive auxilin-2 antibody proportions are still far below 100%. This indicates other, yet unknown and clinically more relevant cross-targets for anti-Ro52/p200 antibodies may exist. This hypothesis is supported by a recent study demonstrating that different fine specificities within the Ro52/p200 region are found in sera from mothers having children with CHB (Hoxha et al., 2016).

Risk carrier frequencies of auxilin-1, the functional homologue of auxilin-2 and a novel fetal susceptibility gene for CHB (**Paper I**), were not associated with abundance or titers of maternal auxilin-2 cross-reactivity in children affected by CHB (**Paper VI**) suggestive of both risk factors acting in independent pathways influencing the course of disease.

Interestingly, we found that ventricular septal defects (VSD) associated with maternal anti-auxilin-2 cross-reactivity in individuals affected by CHB ($P=0.02$, **Paper VI**) indicating this specific cardiac manifestation of CHB may relate to maternal anti-auxilin-2 antibodies.

Collectively, this study suggests that the intrauterine exposure to maternal anti-auxilin-2 autoantibodies may contribute to susceptibility to CHB, however in a clinically minor manner.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, fetal genetic variants and factors influencing the intrauterine environment and their relevance for fetal susceptibility to CHB were investigated.

We identified distinct cellular pathways to be associated with CHB and further explored genes in these pathways in relation to the fetal cardiac disease. In one of the CHB-associated pathways, vesicular transport, we identified and functionally characterized auxilin as a novel fetal susceptibility gene for CHB affecting cardiac excitation-contraction coupling. In addition, we have shown that several other variants affecting genes with a function in vesicular or transmembrane transport and cardiac contraction were associated with CHB. Thus, fetal genetic variants involved in cellular processes sustaining cardiac excitation-contraction coupling may determine the clinical outcome in Ro/SSA autoantibody exposed fetuses.

We furthermore identified several CHB-associated variants affecting genes with a function in immune responses. We have shown that a proinflammatory environment, related to the maternal Ro/SSA autoantibodies or interferon alpha, affects the expression of these genes in cardiomyocytes and PBMCs. Moreover, we found several distinct HLA alleles to be associated with CHB. Collectively, the fetal genetic variants with assigned function in immune responses identified are likely to interfere with the inflammatory and tissue destructive processes during CHB pathogenesis. Furthermore, the interaction of these fetal genetic variants with the proinflammatory intrauterine environment determined by the maternal Ro/SSA autoantibodies may additionally influence fetal susceptibility to CHB.

We have shown that infections, seasonal timing of pregnancy, outdoor activity and psychological stress are associated with CHB and effects of these factors on the intrauterine environment may additionally influence fetal susceptibility to CHB in Ro/SSA positive pregnancies. Finally, we have shown the existence of cross-targets for the maternal anti-Ro52/p200 antibodies and that fetal intrauterine exposure to these maternal autoantibody specificities may influence the clinical outcomes of disease.

Further studies are needed to confirm the relevance of each of these factors in the context of CHB pathogenesis, and several of the identified factors should be possible to explore in terms of prevention and treatment of the disease. In line with this, prospective studies combining clinical parameters on fetal conduction disturbances and factors identified to determine fetal susceptibility will further elucidate crucial clues to CHB pathology and treatment options.

Taken together, the results from our studies expand the current model and understanding of CHB pathogenesis. In addition to the pathogenic effects of the maternal Ro/SSA autoantibodies, the overall fetal susceptibility to CHB and degree of severity will depend on the combination of genetic risk variants, their functional consequences, and their interaction with intrauterine risk factors (**Figure 9**).

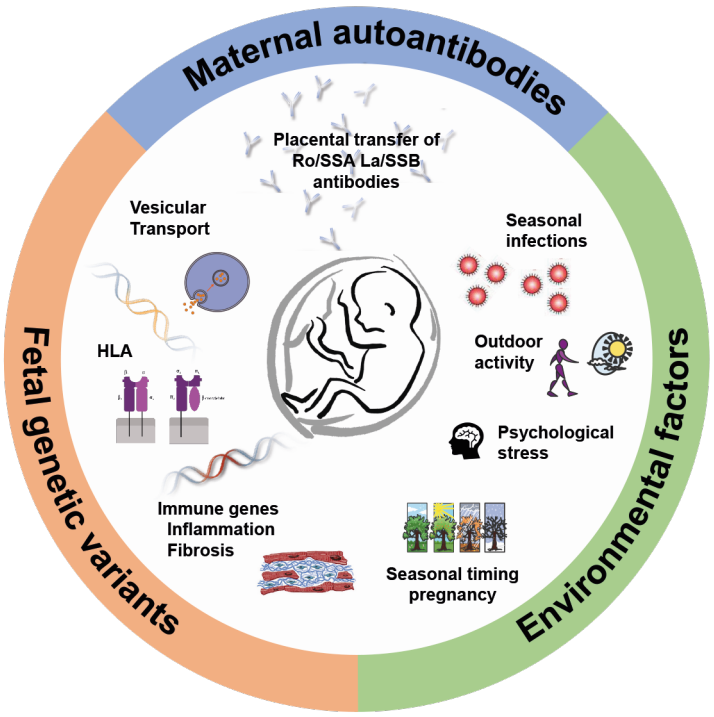

Figure 9. Risk factors for autoimmune-mediated CHB.

6 ACKNOWLEDGEMENTS

In a way, this is the most important chapter of my thesis. Not only because this part is guaranteed to be read by everybody, but also because my PhD project involved many people who have contributed.

Marie Wahren-Herlenius, my main supervisor, thank you for guiding and supporting me throughout my PhD education. I have learned a lot from you; you gave me the opportunity to constantly broaden my knowledge and work on challenging research questions. I truly appreciated you always taking the time for enlightening scientific discussions, which I enjoyed very much. You always kept the big picture in mind and brought me back on track when I got lost in details. With your enthusiasm for science and your ability to go the relevant paths, you have guided me on this journey. Thank you that I could be part of your group.

Ingrid Kockum, my co-supervisor, thank you for sharing your knowledge on epidemiology and genetics, and for your endless patience answering my questions. I enjoyed working with you; thanks that I could always count on you.

Sven-Erik Sonesson, my co-supervisor, thank you for sharing your cardiology expertise with me and contributing with so much interesting clinical input to my projects. I will always remember recording Doppler profiles from mouse pups *in utero* with you.

Stina Salomonsson, my co-supervisor, thank you for your support and always having an open ear for me concerning science and outside of science matters.

Andor Pivarsci, my mentor, thank you for sharing your scientific, but also your parenting experiences with me.

The current and previous Head of the Rheumatology Unit: *Lars Klareskog*, *Ingrid Lundberg* and *Anca Catrina,* for making the lab at CMM such an inspiring place to work.

Present and former members of the MWH group: *Aurélie*, my unofficial co-supervisor. Our scientific discussions and those beyond science have been a pleasure and were so valuable for me. Thanks a lot that I could always count on you no matter if in Sweden or New York! *Amina,* thank you for your support with all the different methods, it was fun to explore all of it with you. Thank you also for the chocolate cookies and all the good laughs. *Vijole,* thanks a lot for all your help with the animal experiments, but also for keeping up the positive atmosphere by taking good care of everybody at the lab. *Amanda* and *Joanna*, my dear friends from "två dörrar till höger", thanks for all the laughter and the great collaboration on our joint project. Our Istanbul trip always makes me smile. Skål to Susie Q, Barbara Ann and Peggy Sue! *Maria,* I really enjoyed sharing the same office with you, thank you for your positive attitude, your friendship and our conversations about science and life outside the lab. *Åse,* thank you for introducing me to the group and patiently teaching me to speak Swedish. *Nånnis,* thank you for introducing me to the ELISA method when I first came to the lab and all the nice chats throughout these years. *Malin,* thank you for the nice conversations in between microscopy and cell culturing, your enthusiasm and energy. *Alex* thanks for sharing your knowledge of molecular biology with me, and for the critical scientific discussions. *Lasse O.,* still considered as a group member, thanks for all your support with computer issues and letting me help you emptying your chocolate drawer. Thanks also to all present and former group members, *Jorge, Nikos, GudnyElla, Rita, Lauro, Albin, Lara M., William, Alina, Therese, Johanna, Elisabeth,*

Jakob, Tomas, Kinga, Lara A., Johannes, and Ariela. Thank you for being great colleagues and keeping up the nice atmosphere at the lab.

All present and former members of the Rheumatology and Neuroimmunology groups: Thank you for scientific discussions and keeping up the friendly, creative and positive environment! Especially *Marianne and Eva J.* for sharing your impressive knowledge of histology. *Heidi,* for always caring and taking the time for a smile and a nice word. *Sabrina R.,* thanks for all the chats and laughter and always having an open ear. *Jessica* thanks for your friendship from my very first day at CMM. *Anna T., Lina and Karine,* thank you for all the friendly conversations. *Izaura, Magda and Jenny,* thank you for adopting me to the epidemiology discussion groups and being so friendly and supportive. To all present and former people in the writing room including *Maria S., Peter, Sabrina R., Jenny, Christina, Aase, Too, and Espen,* thank you for the nice atmosphere.

All Pediatricians at Astrid Lindgren Hospital, especially *Håkan* and *Gunnar*, thank you for great scientific discussion and sharing your knowledge and expertise from the clinical point of view.

All co-authors and all members of The Swedish Congenital Heart Block Study Group, thank you for all scientific input. Especially to *David, Eric and Lasse F.* for your expertise and enthusiasm.

Gunnel, Stina N., *Veronica and Lillemor,* thank you for all your help with administrative tasks; it was always a pleasure to come over to you. Science includes a lot of computer issues; thanks to the *IT-Department* for help. Thank you also to the *present and former CMMities from the second floor*, I have always felt welcome. Thanks to the *Glada Restaurangen Crew* for providing an endless source of coffee, but also for always taking the time for a nice chat.

A special thanks to all *patients and their families* who participated, without you, this would not have been possible!

My lovely and dear friends in Stockholm and Uppsala, thank you for being there and filling my life with laughter, joy and adventure!

Anna McC., we had much to share and I am so happy for all the wonderful moments we spent together, I miss you!

My lovely friends in Germany, you know who you are! Thank you for being there, even if we do not see each other very often. I feel grateful to have you in my life! *Sebi,* special thanks for designing the cover picture.

My family in Bonn and Leverkusen, and my extended family-in-law, thank you for your support and always believing in me wherever I have been or whatever I have been doing!

Opa, thank you for all the things you taught me and your unconditional love. Words cannot express how much you mean to me. I deeply miss you!

My precious and beloved family *Florian, Felicitas, Mathilda and Charlotta*, I feel gifted to have you in my life and I am looking forward to all the adventures that are lying ahead of us!

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