

From THE DEPARTMENT OF MEDICINE
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Being born with congenital heart block

- Risk factors, growth and development

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ABSTRACT

Congenital heart block (CHB) is a rare but life-threatening disease associated with the presence of Ro and La autoantibodies in the mother of the child affected. CHB is one of the manifestations of neonatal lupus erythematosus (NLE) that may also include several other manifestations such as a skin rash and cytopenias. Although the link between Ro and La autoantibodies and the risk for CHB has been recognized for decades, a recurrence rate of 12-17% in subsequent pregnancies suggests that other risk factors also contribute to disease pathogenesis. Furthermore, as CHB is rare disease, little is known about outcome and health in these children. The aim of this thesis was therefore to investigate outcome, health and antibody levels in children with CHB as well as risk factors for CHB development.

In our results we reveal that increased maternal age and seasonal timing of birth are novel risk factors for CHB in autoantibody-positive pregnancies. Furthermore, when analyzing antibody levels in children born to mothers with Ro/SSA autoantibodies, we have demonstrated that maternal autoantibodies decrease rapidly in the infant circulation during the first few weeks of life among both breast-fed and non-breast-fed infants and are not correlated to NLE skin manifestations. We have also demonstrated that an autoantibody-associated complete CHB diagnosis after the neonatal period is possible, advocating testing of maternal serology at the time of diagnosis.

Our results demonstrate that newborns with fetal signs of atrioventricular block (AVB) II-III have a significantly lower weight at birth than those with AVB I or normal conduction and do not show signs of catch-up during the first 12 months of life. Fetuses with AVB I or normal atrioventricular conduction have significant but smaller weight retardation at birth, but showed a rapid catch-up during the first two postnatal months, indicating that they have a good prognosis. Looking at long-term growth, we demonstrate that children with complete CHB are weight restricted both in comparison to their siblings without CHB and the Swedish reference standards from birth to 2-3 years of age, when a catch-up is initiated. From the age of 9-11 they have normal body measurements as a group and do not significantly deviate from the reference standards. When investigating neurodevelopment, our data indicate that in addition to well established factors such as male sex and being born preterm, both maternal SLE and CHB may influence neurodevelopment as learning impairment was significantly influenced by maternal SLE ($p < 0.005$), while attention deficits was influenced by both maternal SLE ($p < 0.05$) and CHB in the child ($p < 0.05$).

In conclusion, our results indicate that maternal age and seasonal timing of birth are risk factors for CHB development, information that may be useful to consider when pregnancy is planned. As antibody levels decreased in the infants and were not correlated to NLE skin manifestations, we conclude that there is no reason not to recommend breast feeding in children born to anti-Ro and or La positive women. Although children with CHB are weight retarded during their first years of life, they appeared to spontaneously initiate a catch-up in weight around the age of 2-3 years. However, as the group of children with CHB did not reach the reference standards until the age of 9-11, careful follow-up of individuals with CHB regarding nutrition and growth is recommended. In addition, follow-up of neurodevelopment should be considered for children with CHB, especially if the mother is diagnosed with SLE. An early diagnosis is one way to help these children overcome their difficulties during childhood and school years and make sure that they obtain the support needed.

LIST OF PUBLICATIONS

The thesis is based on the following studies which will be referred to with their Roman numerals in the text.

- I. **Skog A**, Wahren-Herlenius M, Sundström B, Bremme K, Sonesson SE. **Outcome and Growth of Infants Fetally Exposed to Heart Block-Associated Maternal Anti-Ro52/SSA Autoantibodies.** *Pediatrics.* 2008 Apr;121(4):e803-9
- II. Ambrosi A, Salomonsson S, Eliasson H, Zeffer E, **Skog A**, Dzikaite V, Bergman G, Fernlund E, Tingström J, Theander E, Rydberg A, Skogh T, Ohman A, Lundström U, Mellander M, Winqvist O, Forede M, Ekblom A, Alfredsson L, Källberg H, Olsson T, Gadler F, Jonzon A, Kockum I, Sonesson SE, Wahren-Herlenius M. **Development of heart block in children of SSA/SSB-autoantibody-positive women is associated with maternal age and displays a season-of-birth pattern.** *Ann Rheum Dis.* 2012 Mar;71(3):334-40.
- III. **Skog A**, Eliasson H, Tingström J, Källberg H, Salomonsson S, The Swedish Congenital Heart Block Study Group, Sonesson SE, Wahren-Herlenius M. **Long-term growth of children with autoantibody-mediated congenital heart block.** *Article in press.*
- IV. Klauninger R*, **Skog A***, Horvath L, Winqvist O, Edner A, Bremme K, Sonesson SE, Wahren-Herlenius M. **Serologic follow-up of children born to mothers with Ro/SSA autoantibodies.** *Lupus.* 2009 Aug;18(9):792-8
- V. Bergman G*, **Skog A***, Tingström J, Ottosson V, Hoxha A, Ambrosi A, The Swedish Congenital Heart Block Study Group, Salomonsson S, Wahren-Herlenius M. **Late diagnosis of complete atrioventricular block may be immune-mediated and congenital of origin.** *Manuscript.*
- VI. **Skog A**, Tingström J, Salomonsson S, Sonesson SE, Wahren-Herlenius M. **Neurodevelopment in children with and without congenital heart block born to anti-Ro/SSA-positive mothers.** *Acta Paed.* 2013 Jan; 102(1):40-46.

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LIST OF ABBREVIATIONS

ADHD	Attention deficit-hyperactivity disorder
APL	Antiphospholipid syndrome
AV	Atrioventricular
AVB	Atrioventricular block
BMI	Body mass index
CHB	Congenital heart block
CT	computed tomography
CTD	Connective tissue disease
ECG	Electrocardiography
ELISA	Enzyme-linked immuno sorbent assay
GA	Gestational age
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HC	Head circumference
IVIG	Intravenous immunoglobulin
JA	Juvenile rheumatoid arthritis
kD	kilo Dalton
MANOVA	Multivariate analysis of variance
MHC	Major histocompatibility complex
NC	Normal atrioventricular conduction
NLE	Neonatal lupus erythematosus
OD	Optical density
OR	Odds ratio
pSS	Primary Sjögren's syndrome
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
SGA	Small for gestational age
SLE	Systemic lupus erythematosus
SS	Sjögren's syndrome
sSS	Secondary Sjögren's syndrome
TGF	Transforming growth factor
TNF	Tumor necrosis factor
WHO	World Health Organization

1 BACKGROUND

1.1 INTRODUCTION

Congenital heart block (CHB) is a rare condition characterized by blocking in the transfer of the electrical impulses that control the pumping action of the heart. In a normal heart, these electrical impulses (indicated by arrows in Figure 1), travel from the sinus node to the atrioventricular (AV) node which helps to synchronize the pumping action of the atria and ventricles. In a person with an atrioventricular block (AVB) (also referred to as heart block), the signals are however slowed down or blocked as they travel through the AV node, which may lead to a slow or very slow heart rate (bradycardia).

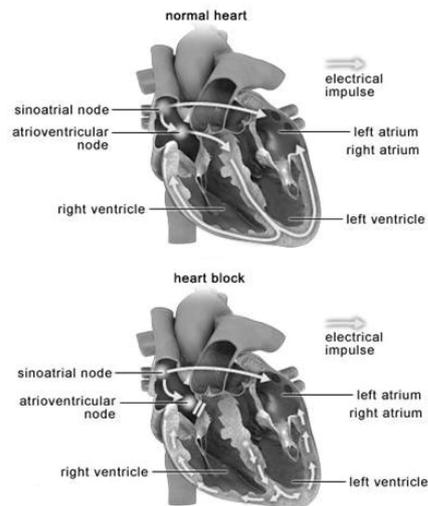


Figure 1. The normal heart and a heart with heart block (AV-block).

A heart block can develop or be acquired by various causes during life including diseases, surgery, medicines, ischemia and infarction. The focus of this thesis is however heart blocks that are congenital of origin and develop during fetal life. In general, an AVB is defined as congenital if it is diagnosed *in utero*, at birth or within the neonatal period (0-27 days after birth) (Brucato et al., 2003). These heart blocks are potentially fatal conditions with conduction abnormalities in a structurally normal heart (Buyon and Clancy, 2003) and may be mediated by both autoimmune and non-autoimmune mechanisms.

Heart blocks mediated by autoimmune mechanisms are characterized by transfer of Ro/SSA and La/SSB autoantibodies from the mother to the fetus during pregnancy. They are considered to be a form of passively acquired autoimmunity, and represent the most serious consequence of an immune-mediated cascade referred to as a neonatal lupus erythematosus (NLE) (Buyon et al., 2004; Wahren-Herlenius and Sonesson, 2006). These blocks usually develop during a risk period of week 18-24 of gestation and are in most cases diagnosed *in utero*, but may also be diagnosed later. Among AV-blocks that are diagnosed *in utero*, the majority of the blocks are autoimmune-mediated and a ~90-95% association between anti-Ro and/or anti-La autoantibodies has been demonstrated (Eronen et al., 2004; Jaeggi et al., 2002).

1.2 CONGENITAL HEART BLOCK

There are three different degrees of AV blocks; all illustrated with the electrocardiogram (ECG) curves in Figure 2A. In a first-degree AVB (AVB I), the electrical impulses are slowed down as they travel along the AV node. This type of AVB is thus characterized by a prolonged interval between atrial contraction and ventricular contraction (the A–V interval) in the presence of normal atrial and ventricular rates (Jaeggi and Nii, 2005). The AV conduction time can be visualized as the PR interval on an ECG (Figure 2B).

In second-degree AV block (AVB II), some but not all impulses reach the ventricles. Three different types of AVB II exist; Mobitz type I (Wenckebach) characterized by progressive lengthening of the AV conduction, Mobitz type II with blocking of an isolated impulse without prior lengthening of the AV conduction time, and 2:1 second-degree AVB characterized by a blocking of every second atrial impulse. Additionally, both 3:1 and 4:1 second-degree AVB may occur, although rare.

Third-degree (complete) AVB (AVB III) is the most serious type of heart block and results in bradycardia. It is characterized by a complete dissociation between atrial and ventricular events with no AV conduction at all. While ventricular rates in complete AVB usually range between 50 and 70 beats per minute, the atrial rates are usually normal (>120 beats per minute). In contrast to AVB I and II that both are potentially reversible, third-degree AVB is considered to be permanent and usually results in a need of a pacemaker implant after birth (Eronen et al., 2004). A recent publication of a reverting AVB III has however challenged the permanency of the third-degree block (Trucco et al., 2011).

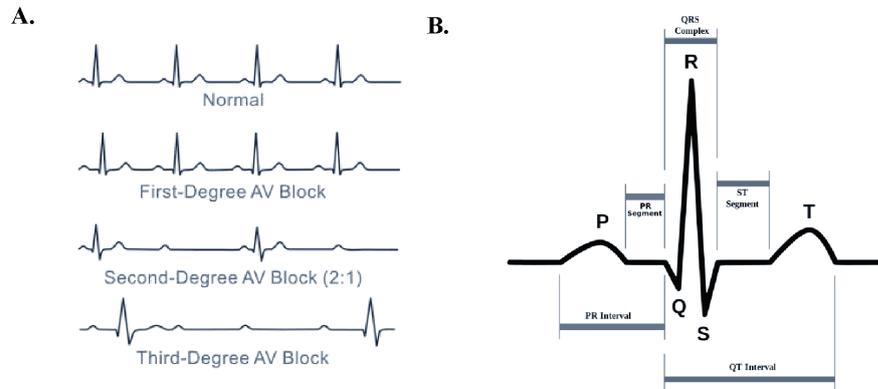


Figure 2 (A): ECG's showing a normal sinus rhythm, first-, second, and third-degree AV-block. **(B):** A schematic representation of a normal ECG curve. The PR interval is measured from the start of the p-wave (depolarization of the atria) to the beginning of the QRS complex (depolarization of the ventricles).

There is accumulating evidence that CHB is a progressive disease and that the AVB may be initiated as a first degree block (Sonesson et al., 2004). Immunoglobulin and complement deposits are found in the fetal heart, and after mononuclear cell infiltration, fibrosis and calcification of the cardiac tissue, the block may progress to a complete third degree AVB (Clancy et al., 2004; Litsey et al., 1985).

1.2.1. Incidence, recurrence rate and mortality

CHB is a rare disease, with an incidence of 1 in 15 000-20 000 newborns in the general population (Michaëlsson and Engle, 1972). The incidence among fetuses born to anti-Ro/SSA-positive women is however higher, and third-degree AVB is suggested to occur in 1% to 2% of these pregnancies (Brucato et al., 2001; Cimaz et al., 2003b; Costedoat-Chalumeau et al., 2004b). Furthermore, CHB has been suggested to be even more frequent in women in whom the anti-Ro activity is targeted to the 52-kd component of the Ro antigen (Buyon et al., 1993; Julkunen et al., 1998b; Salomonsson et al., 2002a). Among mothers who have already given birth to a child with CHB, the recurrence rate for CHB in following pregnancies is only around 12-17% (Ambrosi et al., 2012; Buyon et al., 1998; Julkunen and Eronen, 2001; Llanos et al., 2009; Solomon et al., 2003) despite persisting maternal autoantibody levels (Strandberg et al., 2006), indicating that other factors than the autoantibodies influence disease outcome.

While the incidence for complete heart block is 1 in 15 000- 20 000 newborns, the incidence of AVB I and II is still under investigation and to date, there is conflicting data regarding the incidence of AVB I ranging from 3-14% in newborns to anti-

Ro/SSA positive women, (Bergman et al., 2009; Friedman et al., 2008; Rein et al., 2009), while as many as one third of fetuses in Ro52 positive pregnancies show signs of AVB I *in utero* (Sonesson et al., 2004)

The complete CHB is a potentially lethal condition that is associated with a substantial mortality of 15–20% (Buyon et al., 1998; Eliasson et al., 2011; Eronen et al., 2000; Izmirly et al., 2011; Jaeggi et al., 2002). In fetuses diagnosed with complete AVB *in utero*, the mortality is suggested to be even higher and a 40-50% mortality rate has been reported (Jaeggi et al., 2002; Schmidt et al., 1991).

1.2.2. Pathogenesis

The pathogenesis of CHB is not fully understood. The major known risk factor for CHB is maternal autoantibodies towards the Ro (Ro52 and Ro60) and La proteins. While both Ro60 and La antibodies have been implicated in the pathogenesis of CHB, there is accumulating evidence that the Ro52 antibodies are the initiating autoantibodies mediating the heart block (Buyon et al., 1993; Dörner et al., 1995; Fritsch et al., 2006; Julkunen et al., 1993b; Salomonsson et al., 2002a).

The heart block-associated Ro52, Ro60, and La proteins are all localized intracellularly, something that has challenged researchers in the search for the molecular mechanisms that lead to CHB. How come that even though the target antigens of the antibodies are not within reach for the antibodies, the antibodies still have a pathogenic effect? Through experimental research, two different hypotheses for CHB development have however been proposed; the apoptosis hypothesis and the cross-reactivity hypothesis (Ambrosi and Wahren-Herlenius, 2012).

In short, the apoptosis hypothesis suggests that the maternal antibodies gain access to their target antigen when it is exposed on the surface of apoptotic cells. It has been demonstrated that La and Ro 60 can be present on apoptotic cardiomyocytes (Miranda et al., 1998) and Ro52 has also been shown on the surface of apoptotic cells, however not on live cardiac cells (Clancy et al., 2006). The physiological apoptosis that takes place during heart development is suggested to lead to a brief exposure of the Ro and La proteins of apoptotic cells, allowing the maternal antibodies to enter the fetal circulation to bind the associated antigen. In this scenario, it is possible that the removal of debris from a normal non-inflammatory pathway is diverted towards the engulfment by macrophages through opsonization. The activation of the phagocytic cells will then lead to a production of pro-inflammatory cytokines, recruitment of leukocytes and complement components and subsequently the establishment of an inflammatory

reaction that will damage the target tissue irreversibly (Clancy et al., 2004; Miranda-Carús et al., 2000).

The cross-reactivity hypothesis suggests that the maternal anti-Ro/La antibodies, or at least a subset of these, are able to bind to cardiomyocyte membrane proteins involved in the control of electric signal generation and/or conduction and hence interfere with their function. In support of this hypothesis, Salomonsson et al (2005) have demonstrated that monoclonal human Ro52 antibodies cloned from patients and specific for the p200 epitope (recognizing amino acid 200–239 of Ro52) bind to the cell surfaces of cardiomyocytes in culture, causing dysregulation of calcium homeostasis, calcium overload and eventually apoptosis.

Together, these two hypotheses propose a two-phase model for CHB development (shown in Figure 3), where maternal antibodies cross-react with a fetal cardiac molecule involved in calcium regulation in the heart, initiating conduction disturbances, seen as a first-degree AV block. The disruption of cardiac calcium homeostasis may then lead to increased apoptosis in the fetal heart and an improper clearance of apoptotic debris may lead to a sustained inflammation in the fetal heart, eventually leading to irreversible damage of the heart and a complete CHB (Ambrosi and Wahren-Herlenius, 2012).

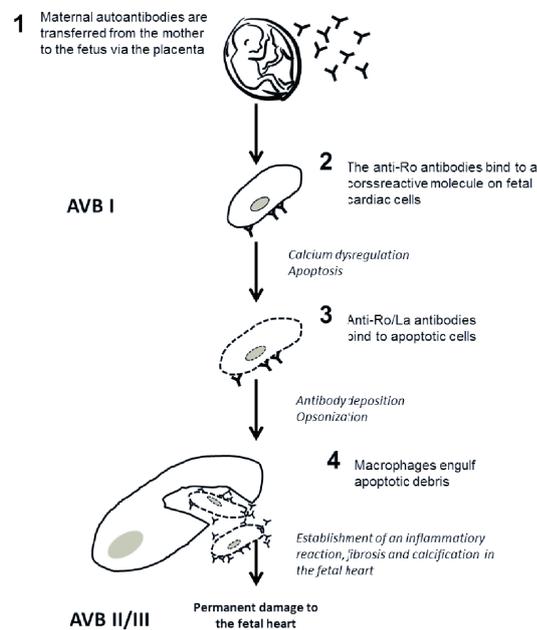


Figure 3. A two-phase model for CHB development. Figure adapted from Ambrosi & Wahren Herlenius, 2012.

1.2.3. Surveillance and prevention/treatment of CHB during pregnancy

In the absence of a prognostic marker, the major risk factors for CHB development are the Ro/SSA and LA/SSB autoantibodies. Women known to be positive for these autoantibodies are therefore followed by fetal monitoring during the risk period for CHB development occurring during week 18-24 of gestation at selected specialist centers. Several methods can be used for fetal surveillance including echocardiography (Doppler flow velocity and Doppler tissue velocity), electrocardiography and magnetocardiography. Fetal echocardiography using m-mode and Doppler techniques are the most commonly used methods as it uses standard equipment and thus is available at most clinics (Wahren Herlenius et al., 2012).

A complete third-degree CHB is generally considered permanent (Breur et al., 2004), and up till date, no effective therapy for CHB exist. Several different prenatal management approaches are however used to improve the outcome in fetuses with CHB, including anti-inflammatory treatment with steroids (Bierman et al., 1988; Buyon et al., 1987; Carreira et al., 1993; Jaeggi et al., 2004; Saleeb et al., 1999) intravenous immune-globulins (IVIG) (Kaaja and Julkunen, 2003; Kaaja et al., 1991; Trucco et al., 2011), and plasmapheresis (Barclay et al., 1987; Makino et al., 2007; Miyakata et al., 2001; van der Leij et al., 1994).

Among these management approaches, fluorinated steroids (betamethasone and dexamethasone) are the most widely used. These are administrated to reduce inflammation in the fetal cardiac conduction system and myocardium to avoid or diminish injury to the cardiac tissue. Since the steroids are fluorinated, they gain access to the fetus as they are able to cross the placenta without being metabolized (Blanford and Murphy, 1977). Transplacental steroid treatment of fetuses with complete CHB might decrease myocardial inflammation and increase fetal cardiac output and as a complement to steroids, beta-sympathomimetics may be useful to increase the fetal heart rate and myocardial contractility (Hutter et al., 2010).

Treatment with fluorinated steroids may be used for both complete and incomplete CHB, but may be more justified in incomplete AV-blocks since it has been demonstrated to inhibit the progression or even reverse some cases of first- (Friedman et al., 2008; Rein et al., 2009; Vesel et al., 2004) and second-degree AVB (Buyon et al., 1995; Carreira et al., 1993; Eliasson et al., 2011; Raboisson et al., 2005; Rosenthal et al., 1998; Saleeb et al., 1999; Theander et al., 2001). Steroid treatment during pregnancy is however associated with risks for both mother and child. Maternal risks of steroid treatment include infection, osteoporosis, hypertension, glucose intolerance and

preeclampsia while risks for the fetus include growth retardation, oligohydramnios and negative effects on neurologic development (Friedman and Shinwell, 2004; Hutter et al., 2010).

In addition to transplacental steroid treatment, several other therapies can be used, although evidence-based knowledge on the efficacy of these treatments are lacking. Injections of immunoglobulins (so called IVIG treatment) was hypothesized to be a useful adjunct to block maternal autoantibody expression in the fetal heart, especially in cases with myocardial inflammation and fibrosis (Hutter et al., 2010), although studies demonstrating no benefit of IVIG in preventing complete AVB exist (Friedman et al., 2010; Pisoni et al., 2010). Plasmapheresis treatment is a way to decrease the pathogenetic maternal Ro and La autoantibodies from the mother-fetus circulation, possibly preventing potential damage to the fetal heart, but only a few studies have been performed, always in combination with transplacental steroid treatment (Barclay et al., 1987; Buyon et al., 1987; Ruffatti et al., 2012; Saleeb et al., 1999), making it difficult to evaluate any potential effect.

1.3 NEONATAL LUPUS ERYTHEMATOSUS

Autoantibody-mediated CHB is only one of the manifestations of the passively acquired autoimmune condition neonatal lupus erythematosus (NLE). While the complete heart block is the most serious manifestation, children with NLE may also have other manifestations that all can occur both in the presence and absence of CHB.

1.3.1 Cutaneous manifestations

The two most common manifestations of NLE are the CHB and the cutaneous manifestations (Buyon and Clancy, 2003). Even though cutaneous manifestations (typically in the form of a malar rash) are a common symptom of NLE, reported to be present in around 15-25% of infants with NLE, the true incidence is unknown since they are likely to be underreported and be mistaken for another neonatal rash (Lee and Weston, 1997).

1.3.2 Other cardiac manifestations than AVB

While the isolated AVB is the most commonly observed cardiac manifestation in NLE, but other cardiac abnormalities may also be found in the fetus or infant affected. These include atrial and ventricular arrhythmias and other conduction abnormalities,

myocarditis, as well as, cardiomyopathy commonly presenting also with endocardiofibroelastosis (Hornberger and Al Rajaa, 2010).

Studies have shown that 15–20% of fetuses with CHB develop diffuse myocardial disease before birth, and that other infants may have myocardial dysfunction after birth even though they receive a pacemaker insertion (Jaeggi et al., 2002; Moak et al., 2001; Nield et al., 2002a; Nield et al., 2002b).

1.3.3 Liver and hematological involvement

Liver involvement in NLE is usually observed as asymptomatic elevated laboratory liver function related tests (typically alanine aminotransferase and/or aspartate aminotransferase) (Silverman and Jaeggi, 2010). Cimaz et al (2003b) reported an elevation of liver enzymes in 26% of children born to anti-Ro/SSA positive mothers and according to some studies, enlargement of the liver, spleen, or of both, has been noted in ~20% to 40% of infants with NLE (Draznin et al., 1979; Watson et al., 1984). However, Lee et al (Lee et al., 1993; Lee et al., 2002) found that liver disease occurred in 9-10% of cases of NLE with CHB or skin manifestations, with transient cholestasis as the major feature.

Hematologic abnormalities such as anemia and thrombocytopenia may also occur in NLE (Selander et al., 1998; Watson et al., 1988; Wolach et al., 1993). In a study by Cimaz et al (2003b), neutropenia was reported to be more common than thrombocytopenia, occurring in up to 23% of children born to mothers with anti-Ro antibodies and in total hematologic abnormalities were found in 27% of the children investigated.

1.3.4 Neurologic manifestations and chondrodysplasia punctata

Apart from the cutaneous, cardiac, liver and hematological manifestations, NLE has also been suggested to involve neurologic manifestations. Nakayama-Furukawa et al (1994) described two cases of hydrocephalus secondary to NLE and when prospectively examining 87 infants and Boros et al (2007) found that 8% of children born to mothers with Ro/SSA autoantibodies had hydrocephalus, concluding that hydrocephalus and macrocephaly are “new” manifestations of NLE, not previously associated to the NLE syndrome. Other studies have reported on mild abnormalities on computed tomography (CT) scans and ultrasound, non-specific white matter changes and calcification of the basal ganglia, macrocephaly, vasculopathy, and transient

myasthenia gravis (Cabañas et al., 1996; Kaye et al., 1987; Peñate et al., 2009; Rider et al., 1991; Wang et al., 1995).

Another condition described in children with NLE is chondrodysplasia punctata. This is a rare condition characterized by radiographic evidence of stippling of the epiphyses and/or the spine. Up to date more than 15 cases of chondrodysplasia punctata have been associated with maternal autoantibodies (Austin-Ward et al., 1998; Chitayat et al., 2008; Elçioglu and Hall, 1998; Honda et al., 2008; Kelly et al., 1999; Kozłowski et al., 2004; Shanske et al., 2007), which indicates that this condition may be a skeletal manifestation of NLE, although rare.

1.4 RO/SSA, LA/SSB, AUTOIMMUNITY AND RHEUMATIC DISEASE

As described above, when being pregnant, women with Ro/SSA and La/SSB autoantibodies are at risk of having a child with neonatal lupus erythematosus (NLE) (Scott et al., 1983). The mothers positive for these autoantibodies are often diagnosed with an autoimmune disease, most commonly SLE or Sjögren's syndrome, but may also be undiagnosed or asymptomatic. As NLE is a result of autoimmunity in the mother, we need to understand the immune system and autoimmunity in order to understand NLE.

The immune system has an amazing ability to distinguish between self and dangerous non-self antigens. Autoimmunity and autoimmune diseases are however a result of an inappropriate immune response against substances normally present in the body, resulting in the production of autoantibodies that target and damage tissues or organs in the body (Abbas and Lichtman, 2011).

NLE is the result of autoantibodies specifically directed towards the Ro/SSA and La/SSB proteins that are produced by the immune system in the mother of the child affected and then transported from the mother to the fetus via the placenta. The Ro/SSA and La/SSB proteins are described in more detail below.

1.4.1 Ro52, Ro60 and La

The Ro and La proteins were first identified as antigens bound by sera of rheumatic patients (Alspaugh and Tan, 1975). The proteins have been conserved through evolution and can homologues be found in mammals and xenopus.

The Ro/SSA antigen refers to two non-homologous proteins; the 52 kilo Dalton (kD) protein Ro52 and the 60 kD protein Ro60. Ro52 consists of 475 amino acids and as illustrated in Figure 4 contains a RING and a B-box, a coiled-coil domain and a

B30.2 (or PRYSPRY) region in the C-terminal end and is expressed predominantly in immune-related organs and cells.

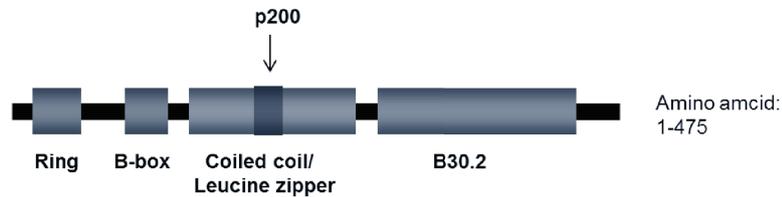


Figure 4. The structure of the Ro52 protein.

The RING, B-box and coiled-coil motif places Ro52 within the family of tripartite motif proteins (TRIMs) (Reymond et al., 2001). As several other TRIM proteins, Ro52 has been demonstrated to have E3 ligase activity and to act in the process of ubiquitination (Espinosa et al., 2006), which is a mechanism of post-translational modification of proteins that allows eukaryotic cells to control biological processes such as protein degradation, trafficking and activation (Hershko et al., 1983). Many of the proteins in the TRIM family play an important role in innate immunity and anti-viral responses (Nisole et al., 2005; Yap et al., 2004), but also in the regulation of immune responses by targeting key molecules involved in cell proliferation, survival or death (McNab et al., 2011). As a TRIM protein, Ro52 is also denoted TRIM21, and *TRIM21* is the official name of the Ro52 gene (Reymond et al., 2001).

As reviewed by Wolin et al (2006), Ro60 is a ubiquitously expressed RNA-binding protein shaped like a doughnut with an inner hole. In the cell, Ro60 binds a family of small cytoplasmic RNAs, hYRNA 1-5. Even though the function of Ro60 was unknown for many years, experiments have now demonstrated that this protein binds misfolded non-coding RNAs in vertebrate cells and likely functions in a pathway by which defective RNAs are recognized and targeted for degradation (Chen et al., 2003; Labbé et al., 1999; O'Brien and Wolin, 1994). In addition, studies have shown that the Ro60 protein is important for cell survival after ultraviolet irradiation (Chen et al., 2000; Chen et al., 2003).

The La protein can also associate with the hYRNAs and has been suggested to have a function in transcription termination and may also be involved in virus replication (Wolin and Cedervall, 2002). Interestingly, while antibodies towards Ro52 seem to be the major link to CHB, La autoantibody levels are higher in mothers of children developing cutaneous manifestations rather than heart block (Silverman et al., 1995).

1.4.2 Systemic lupus erythematosus and Sjögren's syndrome

As mentioned previously, Ro/SSA and LA/SSB autoantibodies are common in women with autoimmune rheumatic diseases such as systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). Both SLE and SS are chronic systemic diseases, typically treated with immunosuppressive medications that can decrease the immune response but not cure the patient.

In SLE, nuclear components of the cells are the main targets of the autoimmune reaction and SLE may affect almost any organ of the body. The disease is much more common in women than in men (80-90% are women), and the disease onset usually occurs during childbearing years (Petri, 2002). The clinical manifestations of SLE are heterogeneous and organ system involvement can occur in virtually any organ including the heart, lungs, kidneys, and central nervous system (Klippel, 1997). Symptoms are equally diverse and include skin rash, arthritis, fever, decreased appetite, fatigue, and joint pain (Askanase et al., 2012).

Sjögren's syndrome (SS) is characterized by lymphocytic infiltration of exocrine glands and progressive tissue destruction. Typical symptoms are dryness of the mouth (xerostomia) and eyes (keratoconjunctivitis sicca) (Jonsson et al., 2000), and the disease may also include a range of other manifestations such as vaginal and nose dryness, kidney, blood vessel, lung, liver, pancreas, and central nervous system involvement. SS can exist as a disorder on its own (primary Sjögren's syndrome, pSS) or may develop years after the onset of an associated rheumatic disorder, such as SLE or rheumatoid arthritis (RA) (secondary SS, sSS). The disease is more common in women than in men, especially for the primary form, in which 9/10 are women.

1.4.3 Pregnancy complications and outcome in women with autoimmune diseases

Pregnancy in women with autoimmune diseases is often more complicated than pregnancies in women who are healthy. Several studies have demonstrated that pregnant women with connective tissue diseases have an increased risk for preeclampsia, perinatal death, preterm delivery and giving birth to a child small for gestational age (SGA) (Skomsvoll et al., 1998; Skomsvoll et al., 1999).

As discussed by Tincani et al (2006), women with SLE were for a long time discouraged from becoming pregnant, partly due to the risk of aggregation of maternal disease and partly because the risks of negative influence of disease itself and/or the maternal treatment on neonatal outcome (Doria et al., 2006; Petri et al., 1991). The

management of SLE patients has however improved during later years and more and more women are now becoming pregnant. A key to successful pregnancies in SLE appears to be good disease control before entering a pregnancy. Today, the majority of maternal flares in SLE pregnancies are mild, and in most cases only require a minor change of the treatment (Tincani et al., 2006). Renal flares during pregnancy are the most frightening complications and the SLE pregnancy may also be complicated by the high incidence of preeclampsia with consequent proteinuria, hypertension and other symptoms resembling renal flares (Gordon et al., 2004). Preterm birth and intrauterine growth retardation are more frequently observed in women with SLE than in healthy women (Aggarwal et al., 1999; Julkunen et al., 1995), and studies on pregnancy outcome in women with SLE show that pregnancy loss (10-50%), preterm birth (21-52%), intra-uterine growth restriction and intra-uterine death are commonly seen (10-30% and 8-36%, respectively) (Clowse et al., 2006; Petri, 1994; Tincani et al., 2002; Witter and Petri, 2000; Yasmeen et al., 2001).

While studies suggest that fetal outcome is affected in SLE pregnancies, several studies have indicated that women with SS give birth to infants who are not more premature or growth retarded than newborns of healthy women (Haga et al., 2005; Julkunen et al., 1995; Takaya et al., 1991). A recent Swedish study did however demonstrate a significantly lower mean birth weight in offspring of pSS mothers in comparison to babies of control mothers (Hussein et al., 2011). In addition two studies have demonstrated increased rates of fetal loss and spontaneous abortions in pregnancies before SS diagnosis (Julkunen et al., 1995; Siamopoulou-Mavridou et al., 1988).

1.5 RISK FACTORS FOR CHB DEVELOPMENT

The major known risk factors for CHB are the maternal autoantibodies toward the Ro and La antigens (Julkunen et al., 2004; Salomonsson et al., 2011; Scott et al., 1983; Taylor et al., 1988). Although the risk for CHB is only around 1-2% in anti-Ro positive pregnancies, anti-Ro/La-positive women who have already given birth to a child with CHB have an increased risk for giving birth for another child with CHB. The rate of recurrence is however 12-17% (Ambrosi et al., 2012; Buyon et al., 1998; Julkunen and Eronen, 2001; Llanos et al., 2009; Solomon et al., 2003) and not a 100% despite persisting autoantibodies (Strandberg et al., 2006), suggesting that other factors play a role for CHB development.

Factors that so far have been demonstrated not influence the risk include gender (Buyon et al., 1998) and maternal disease activity (Eronen et al., 2004; Llanos et al., 2009). A number of other potential risk factors will be described below.

1.5.1 Genetic factors

The first report regarding genetic factors influencing susceptibility to CHB was published by an American group in 2003 (Clancy et al., 2003) who studied 40 children with CHB using a candidate gene approach and found that the TGF β polymorphism assessed (that is associated with increased IgG binding to macrophages and increased fibrosis) was significantly more frequently found in children with CHB than in their unaffected siblings, whereas the TNF α polymorphism studied was found at an increased frequency in both affected and non-affected children in comparison with healthy controls. In recent years, the same group conducted a genome-wide association study (GWAS) of individuals with CHB born to anti-Ro/La-positive mothers and found a significant association with polymorphisms in the HLA region and at the location 21q22 (Clancy et al., 2010). Comparing CHB cases with healthy controls from the general population, as has been done in the study above, does however have limitations since the observed genetic associations may reflect the genetic bias in the mothers, who are autoantibody-positive and may have a rheumatic diagnosis such as SS or SLE. Individuals affected by SS and SLE are genetically and immunologically different from the general population in terms of MHC haplotype and autoantibody profile.

In addition to the studies above, Strandberg et al (2010) used congenic rat strains and a Ro52 immunization model of heart block to demonstrate an influence of both maternal and fetal MHC genes in CHB development. Furthermore, ongoing studies in our research group are focused on genetic factors in relation to CHB.

1.5.2 Other potential risk factors

Other potential risk factors that have been reported in relation to CHB development include hypothyroidism in the mother (Askanase et al., 2006; Spence et al., 2006) and hypoxia (Clancy et al., 2007). One may also speculate that environmental and lifestyle factors such as smoking could be potential risk factors, but studies of such a relation to CHB are lacking.

Maternal age and parity had not been thoroughly investigated as potential risk factors when the work with this thesis began, although one study of 177 families with

children with CHB found that birth order of the fetus did not predict the occurrence of CHB in a given pregnancy (Solomon et al., 2003).

1.6 OUTCOME OF CHILDREN WITH CHB

Outcome of children born with CHB has been investigated only in a few studies. In a recent European multinational study of 175 fetuses diagnosed with AVB II or III during year 2000-2007 (162/175 born to an anti-Ro/SSA -positive mother), 91% percent of the infants were alive at birth and the survival during the neonatal period was 93% and similar in steroid-treated and non-treated fetuses, regardless of degree of block and/or presence of anti-Ro/SSA. Risk factors that associated with a poor outcome included gestation < 20 weeks, ventricular rate \leq 50 bpm, hydrops, and impaired left ventricular function and the presence of \geq 1 of these risk factors was associated with a 10-fold increase in mortality before birth and a 6-fold increase in the neonatal period independently of treatment (Eliasson et al., 2011).

As discussed by Blank et al (2012) the management of patients with complete CHB has changed during the last decades and the current policy is to pace the majority of the patients. Michaëlsson et al (1995) reported high rates of unpredictable Stoke-Adams attacks and subsequent mortality in individuals with CHB and prophylactic pacing of individuals with CHB is now recommended and has been shown to decrease mortality and morbidity (Balmer et al., 2002). Reasons for early pacing include prevention of morbidity by gradually decreasing ventricular rate, ventricular dialation and dysfunction and mitral regurgitation (Michaëlsson et al., 1995). Another indication for pacing is limited exercise capacity, but data regarding exercise capacity in children with CHB do however suggest that paced children with complete CHB do not perform better than un-paced children (Blank et al., 2012). Despite pacemaker insertion in children with complete CHB, ~10% of these children have been reported to develop cardiomyopathy (Friedman et al., 2007).

1.6.1 Long-term outcome and development

Looking at the long-term outcome of children with autoantibody-mediated CHB, one concern is the risk that the affected individual will develop autoimmune disease later in life. The exact risk of developing a rheumatic or autoimmune disease in these individuals is however still unknown.

Esscher and Scott, Feist et al, Fox et al , Jackson and Gulliver, and Waterworth have all reported on cases where children having NLE developed SLE later in life

(Esscher and Scott, 1979; Feist et al., 2003; Fox et al., 1979; Jackson and Gulliver, 1979; Waterworth, 1980), whereas Hübscher et al, Lanham et al and McCue et al have reported cases of children with NLE who developed juvenile arthritis, SS, undifferentiated connective tissue disease and scleroderma respectively (Hubscher et al., 1997; Lanham et al., 1983; McCue et al., 1977). Interestingly, all of the children in these case reports were females. As discussed by Cimaz (2004), the case reports may however reflect the increased incidence of autoimmunity that exists in the families, rather than an increased risk in children with NLE in particular. In addition to the case studies above, several other studies have also presented data on children with NLE developing autoimmune disease later in life. The findings from these studies are summarized in Table 1.

Table 1. Reports of development of autoimmune disease in individuals with NLE.

	NLE diagnosis in included children (follow-up period)	Number of individuals with later autoimmune diagnosis
(Michaëlsson et al., 1995)	102 cases with CHB (51 diagnosed <i>in utero</i> or in the neonatal period) (mean: 38 y, range: 16-66 y)	2 collagen disease, 2 diabetes mellitus and 2 hypothyroidism
(Neiman et al., 2000)	57 cases of cutaneous NLE (mean: 6.4 y, range: 1m-17 y)	1 Hashimoto's, 2 JA and 1 Raynaud's disease
(Martin et al., 2002)	49 cases of NLE (mean: 4.6 y)	2 with JA, 1 Hashimoto's, psoriasis and iritis, 1 diabetes mellitus and psoriasis and 1 congenital hypothyroidism and nephritic syndrome
	45 unaffected siblings (mean: 15.7 y)	None
(Askanase et al., 2010)	33 cases with CHB and (for all 104 included mean: 14.5 y, range: 5-39 y)	Among the CHB cases: 1 nephritic syndrome, 3 hypothyroidism and 1 psoriasis
	20 cases of cutaneous NLE	Among the rash cases: 1 developed JA and 1 hypothyroidism
	51 unaffected siblings	Among unaffected siblings: 2 inflammatory bowel disease, 1 spondyloarthropathy, 1 alopecia areata and 1 APL
(Brucato et al., 1995)	13 cases with CHB (mean: 18.3 y, range: 2-34 y)	None
(Waltuck and Buyon, 1994)	55 cases with CHB (median: 3.7 y, range: 1 w-20 y)	None

JA: juvenile rheumatoid arthritis, APL: antiphospholipid syndrome

Even though both SLE and SS may include central nervous system involvement with neuropsychiatric and neurologic manifestations in the patient (Adelman et al., 1986; Delalande et al., 2004), several studies have demonstrated that the offspring of mothers with SLE may have impaired neurodevelopment (Lahita, 1988; McAllister et al., 1997; Neri et al., 2004; Ross et al., 2003), and as previously described, NLE has been suggested to include neurologic manifestations (Silverman and Jaeggi, 2010), studies on long-term outcome of children with CHB with regards to neurodevelopment are few. Brucato et al (2006a) studied 13 children with CHB (11 exposed and 2 non-exposed to fluorinated steroids *in utero*) and found that all of them had a normal neuropsychological development, although one of the children had learning disabilities. Askanase et al (2010) studied parental-reporting of neuropsychiatric development in 104 anti-Ro exposed children and 22 healthy friend controls and found that in total, 40% of the 104 anti-Ro exposed children were reported to have a neuropsychiatric disorder, compared with 27% of the friend controls ($p = 0.34$). For 8 (24%) of the CHB children (6 boys, 2 girls) the mothers reported attention problems and among the rash children, 4 (20%) (2 boys, 2 girls) had attention problems. Of the unaffected siblings, 9 (18%) (8 boys and 1 girl) had attention problems, whereas one (5%) of the control children (a girl) had attention problems. There was however no statistical difference in attention problems between the groups ($p=0.120$) and the prevalence of behavioral problems, depression, anxiety, developmental delays, learning, hearing, and speech problems were not significantly different between groups.

When the work with this thesis began long-term growth had not been investigated in children born with CHB. Some studies have reported on a low weight of the children with CHB at birth (Brucato et al., 2002; Eronen et al., 2000; Julkunen et al., 1998a). Furthermore, the presence of anti-Ro antibodies in pregnant women with SLE has been suggested to increase the incidence of intrauterine growth retardation (Leu and Lan, 1992) but this finding was not confirmed in later studies in which no significant differences in gestational age (GA), birth weight, or fetal growth retardation between infants of anti-Ro positive and anti-Ro negative women with connective tissue disease were found (Brucato et al., 2002; Costedoat-Chalumeau et al., 2004a).

2 AIMS

CHB is a rare but potentially fatal condition with conduction abnormalities in a structurally normal heart that develops after placental transfer of maternal anti-Ro/SSA autoantibodies from mother to fetus. In children with CHB outcome and growth has not been thoroughly investigated. Furthermore, studies regarding risk factors for CHB development are few.

The overall aims of this doctoral project has therefore been to investigate the outcome, health and antibody levels of children born to Ro/SSA positive mothers, focusing on children born with a CHB and to identify factors other than the autoantibodies that may increase the risk of getting a child with a CHB.

3 METHODOLOGICAL CONSIDERATIONS

In the following section, methodological considerations will be discussed. For a detailed description of the methods used, please see the *Patients and Methods* section in each of the included papers.

3.1 PATIENTS

The major challenge in studying CHB and NLE is its rarity. In paper I and III, a single-center, prospective study approach was used. This study design included a rather small number of individuals which could increase the risk and effect of random errors but on the other hand, the individuals included were prospectively followed in the same way and detailed info on diagnosis etc. was available as they attended several examinations at Astrid Lindgren Children's Hospital and met doctors and nurses that were involved in the studies and followed a study protocol.

The establishment of a population-based cohort of Swedish CHB patients (Salomonsson et al., 2011) has however facilitated the study of this disease; still the number of patients is few in comparison to studies of many other diseases. The nationwide population-based cohort used in paper II, IV, V, and VI have strengths as it has a high proportion of participating index cases and family members but also some limits. First of all, the age span of the included CHB cases is large since the patients included are born between 1914 and 2009. Many things have changed during these years including the Swedish healthcare system and how patients with CHB are managed. In addition, medical records from child health and school health services were not systematically archived and kept in Sweden until the 1980's and information from the Swedish medical birth register is only available for individuals born from 1973 and onwards. In order to minimize potential confounding factors relating to age or birth year when studying patients with CHB, we have only included individuals born between 1973 and 2009 in paper IV and individuals born 1974-2009 in paper VI. Using the 1973/1974 cutoff, we could obtain information from the Swedish medical birth register and collect medical records as the majority of the records were systematically archived. With this cut-off, we do however get another limitation as the time of follow-up varies among the individuals, with the effect that not all individuals were followed until the end of childhood.

A second limitation with the cohort is that the blood samples from the individuals included were gathered at one time-point only, and for the mothers, this time-point has always been after the birth of the child with CHB. Thus, although likely, we cannot be sure that the mothers who were autoantibody-positive in our analysis had these autoantibodies during pregnancy, which may lead to a risk of overestimating the association with autoantibodies. Furthermore, we do not have access to possible variations in autoantibody levels over time and since our analysis is based on sera from live mothers, there is also a risk of underestimation of autoantibody-positive pregnancies related to a shorter life expectancy for mothers diagnosed with SLE.

The third limitation concerns the number of individuals included. Major efforts have been made to search for all cases of CHB in the Swedish population using several different approaches; still we can never be sure that we have found all existing heart block cases. One can therefore argue that the study cohort may not be truly population-based.

3.2 COLLECTION AND ANALYSIS OF CLINICAL DATA

When interpreting the results from the studies included in this thesis, it is important to keep in mind that the collection and analysis of much of the clinical data has been performed in a retrospective manner.

Although growth data were collected and analyzed retrospectively in paper I and IV, measurements were taken prospectively by trained nurses at child and school health services and even though also experienced nurses obtain divergent results when measuring height, weight and head circumference in children (Strandgren et al., 2001), we have no reason to believe that a potential measurement error would be different for children with CHB as compared to children without CHB. In the study of neurodevelopment (paper VI), there might exist differences in reporting and a reluctance to report on neurodevelopmental impairment and neuropsychiatric diagnoses in the medical records. In addition, neuropsychiatric diagnoses are often set later in life and more often nowadays than during some decades ago which could potentially influence the results. The true incidence of children with impairment in neurodevelopment born to Ro/SSA positive mothers may therefore be higher.

3.3 REFERENCE VALUES FOR LONG-TERM GROWTH

In Sweden, three different scientific reports are recommended for follow-up of growth in children within the Swedish healthcare system; Gothenburg -74 (Wikland et al., 2002), Sweden-81 (Werner and Bodin, 2006) and references from the World Health Organization (WHO). In addition, a more recent study of birth size from more than 800 000 children from the Swedish medical birth register is recommended for children born preterm (Niklasson and Albertsson-Wikland, 2008).

The reference values for growth development used in paper I and IV, are based on the Gothenburg-74 material, consisting of 3650 full-term babies born in 1973-75 who were measured and weighed in high school penultimate and final year in 1992. 75% of the individuals were born in 1974. The results of the study are published in a series of publications in international journals and these references and growth curves are widely used by the Swedish healthcare providers and in two different electrical applications from year 2000 and 2005. The Gothenburg-74 material has also been connected to the birth data from the study of the more than 800 000 newborns described above and merged curves have been created.

From a basic statistical standpoint, Sweden -81 has its advantages. It is representative for the whole country, and is collected at a time when breastfeeding rate was significantly higher than for the Gothenburg-74 material, but not so high as to correspond to the current conditions. Sweden -81 was also collected later, contains higher proportion of children from rural districts and has small losses. All of these characteristics contribute to the occurrence of an increased proportion of overweight and obese children compared with Gothenburg-74. However, as we wanted to use the newly published study of birth size from more than 800 000 children from the Swedish medical birth register that is also linked to the Gothenburg-74 study and as the Gothenburg-74 is the most widely used, we chose to use them as references for our material. When it comes to the WHO references, these include major limitations that make them more suitable as a complement to any of the two Swedish reference values as the material to a large extent is collected in countries with different living conditions and different panorama of infectious diseases than those that characterize Sweden.

3.4 ELISA FOR DETECTION OF ANTIBODIES

In order to detect autoantibodies in sera from the samples collected, Enzyme-linked immunosorbent assay (ELISA) was used. ELISA is a widely used method for antibody detection and in contrast to other methods that can be used including immunodiffusion, counter immune electrophoresis and western blot, the ELISA can not only determine whether there is autoantibody positivity but can also give semiquantitative data on levels/titers.

All samples in our experiments were run in duplicates in order to create validation of the results. The antigen used must however be pure in order for the ELISA to work well, but if this requirement is fulfilled, the method is very reliable. The ELISA method does however have limitations, especially regarding the dynamic range. In addition, in the studies included in this paper, we did not determine titers, but instead used optical density (OD)-values and positive and negative controls.

4 RESULTS AND DISCUSSION

4.1 RISK FACTORS FOR HEART BLOCK DEVELOPMENT

Isolated complete CHB is associated with the presence of anti-Ro/SSA and La/SSB antibodies in the mother. The recurrence rate of 12–17% does however indicate that additional factors are involved in the pathogenesis, but as CHB is a rare disease in the general population, studies regarding risk factors have been difficult to perform. In order to investigate risk factors for CHB development other than the well-established maternal autoantibodies, two studies (paper I and II) were performed within the work of this thesis. The results from these studies, with focus on autoantibody-mediated CHB, will be presented and discussed in the following text. A comparison of autoantibody mediated and non-autoantibody mediated CHB in terms of risk factors is presented in chapter 4.1.5.

4.1.1 Maternal age and parity (paper I and II)

In a first attempt to study risk factors, we conducted a single-center prospective study of 32 pregnancies in anti-Ro52 positive women. In the study, the women were followed prospectively with weekly fetal Doppler echocardiograms during week 18-24 of gestation at the pediatric cardiology unit at Astrid Lindgren Children's Hospital (Stockholm, Sweden). Information regarding maternal age, parity, maternal diagnosis and GA were obtained from perinatal records to characterize the role of maternal age and parity in relation to development of CHB. The 32 pregnancies included 7 cases of AVB II or III, 8 cases of AVB I, and 17 cases with signs of normal atrioventricular conduction (NC).

When analyzing maternal factors influencing the development of CHB, we found that women giving birth to infants with AVB II-III *in utero* were older than mothers of fetuses without AVB II-III ($p < 0.05$). The difference in maternal age was however more pronounced when compared with AVB I pregnancies ($p < 0.05$) than with NC pregnancies where the difference did not reach statistical significance. The age of women with fetal signs of AVB I did not deviate significantly from that found in NC pregnancies.

Furthermore, the risk of having a fetus with AVB II-III also seemed to increase with increasing parity, both compared with the NC and AVB I groups. Fetuses with AVB II-III were, on average, born as the 2.4 ± 1.0 child, whereas fetuses with AVB I

were born as the 1.4 ± 0.7 child and fetuses with NC as the 1.6 ± 0.9 child ($p < 0.05$). Increasing maternal age and parity are however obviously linked but analysis of these two factors independently could not be performed due to the small sample size.

As the study cohort in the first study was small ($n=31$ surviving infants), a second study was conducted to further investigate risk factors using a nation-wide population-based Swedish cohort of CHB patients and their family members including a larger number of individuals with complete CHB than in paper I. In contrast to the included fetuses/children in paper I, the individuals included in this study were not followed systematically during pregnancy and signs of AVB I or AVBII could not be detected. The cohort was thus divided into children with complete AVB and unaffected siblings and the unaffected (unaffected defined as not having a complete, third-degree AVB). The influence of fetal gender, maternal age, parity and time of birth on heart block development was analyzed in 145 families, including 190 Ro/La-positive and 165 Ro/La-negative pregnancies. More specifically, we assessed the possible association of these factors with CHB by comparing CHB cases to healthy siblings in a cohort of families with autoantibody-positive mothers and also by analyzing the same variables in a group of patients with CHB and healthy siblings born to autoantibody-negative mothers.

We found that the mean maternal age in Ro/La-positive pregnancies with a child affected by CHB was significantly higher than in pregnancies resulting in unaffected children: mean (95% CI): 29.5 (28.4-30.6) years vs. 27.5 (26.5-28.5) years respectively ($p < 0.05$). When calculating odd ratios (OR), increasing OR for women 30–34 and ≥ 35 years of age compared to women < 25 years of age was observed (Table 2).

Table 2. Odds ratio of CHB in relation to maternal age in Ro/La positive pregnancies ($n=190$; 85 with CHB and 105 without CHB).

Age	OR (95% CI)	p-value	OR (95% CI) <i>Adjusted for parity</i>	p-value
≤ 24	1.0 (Reference)		1.0 (Reference)	
25-29	1.7 (0.8-3.7)	0.18	1.6 (0.7-3.6)	0.22
30-34	2.0 (0.9-4.5)	0.09	2.3 (1.0-5.5)	0.05
≥ 35	3.2 (1.2-8.4)	0.02	4.2 (1.4-11.9)	0.01

With a larger sample size than in paper I, we were now able to separate the effects of maternal age and parity. By stratifying the pregnancies by parity, we found that anti-Ro/La-positive mothers giving birth to a child with CHB were consistently

older than antibody-positive women who gave birth to a non-affected child, independent of whether it was the first, second, third or higher-birth-order pregnancy. In addition, only maternal age had a significant effect ($p=0.01$; logistic regression) in antibody-positive women, whereas parity did not significantly affect pregnancy outcome ($p=0.35$). In paper II we could thus conclude that maternal age but not parity influences the risk of CHB in anti-Ro/La-positive pregnancies but not in anti-Ro/La-negative pregnancies.

The findings from both studies (paper I and II) are in line with results from another study including 177 families with ≥ 1 child diagnosed with complete AVB (Solomon et al., 2003). In that study, birth order could not be demonstrated to predict the occurrence of CHB, suggesting that the more important factor is maternal age.

An increased risk for AVB II-III in older mothers could depend on several different immunologic and non-immunologic factors. Immunologic factors could potentially include epitope spreading and antibody affinity maturation in the mother over time that may contribute to an increased risk. Increasing maternal age may also reflect the appearance and/or increased serum levels of anti-Ro/La autoantibodies in women over time. Unfortunately, blood samples were only collected and tested at one time-point (always after birth of a child with CHB), meaning that differences over time could not be assessed. Other investigators have however shown that Ro/SSA and La/SSB profiles and levels remain stable in adult patients with established disease (Meilof et al., 1997; Praprotnik et al., 1999; St Clair et al., 1990; Wahren et al., 1998). However, members of our group have previously investigated Ro/SSA and La/SSB profiles and levels before, during and after pregnancy and demonstrated a gradual decline of Ro52, Ro60 and La IgG autoantibody-levels in maternal sera during the progression of pregnancy (Strandberg et al., 2006). The gradual decline over time during pregnancy could however reflect the total IgG level decrease suggested to occur during pregnancy (Amino et al., 1978; Khirwadkar and Kher, 1991).

Non-immunologic factors may include placental growth retardation and placental abruption, both known to increase in frequency with age, (Hansen, 1986; Naeye, 1983) mainly due to insufficient uteroplacental perfusion. Increased maternal age might also be associated with less effective placental function that may lead to episodes of low oxygenation that could then potentially influence the inflammatory reaction in the fetal heart and interestingly, findings from previous studies have suggested that hypoxia amplify the harmful effects of maternal anti-Ro/La antibodies on the fetal heart (Clancy et al., 2007). With regards to placental development and function, it is also known that

in pregnancies of women with SLE, the placenta is small in size and may demonstrate ischemic-hypoxic change, decidual vasculopathy, thromboembolism and villitis, events that all may lead to a disturbed support of the fetus (Lockshin and Sammaritano, 2003).

Another suggested risk factor for CHB that is known to increase incidence with age is hypothyroidism. Two studies have suggested an increased risk for giving birth to a child with CHB in autoantibody-positive women with hypothyroidism (Askanase et al., 2006; Spence et al., 2006) but further studies are needed.

In addition to the above discussed potential reasons for why increased maternal age may increase the risk for CHB, it is also, in general, well-known that the risk for both maternal and fetal pregnancy complications increase with increased maternal age. In the mother these complications may include weight gain, gestational diabetes, chronic and pregnancy induced hypertension, antepartum hemorrhage, placenta praevia, prelabour rupture of membranes and preterm labor whereas complications regarding perinatal outcome include low birth weight, prematurity, fetal distress, and perinatal morbidity and mortality (Montan, 2007).

As increased maternal age increased the risk of having a child with CHB in the autoantibody-positive group, one may also hypothesize that paternal age may influence the risk. We did however not find any significant differences in the age of the father with regards to cardiac outcome in neither the autoantibody-positive nor-negative group (Figure 5).

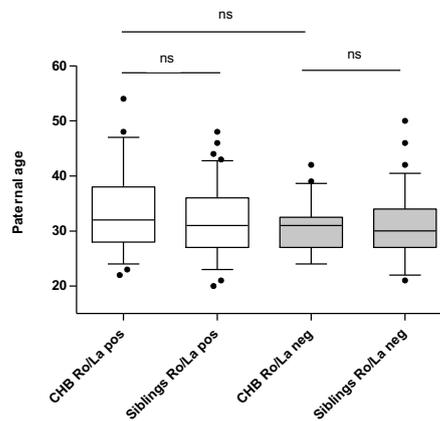


Figure 5. Paternal age at birth of a child with or without CHB in anti-Ro/La-positive or anti-Ro/La-negative pregnancies. Data shown as box plots (25th–75th percentiles) with a line at the median.

4.1.2 Fetal gender (paper I and II)

When it comes to fetal gender as a risk factor for CHB development, we found no gender bias in neither paper I, nor in paper II. In our population-based cohort (paper II), female gender was observed in 52% of affected children and 56% of healthy children born to anti-Ro/La-positive mothers, indicating that fetal gender does not predict the occurrence of CHB ($p=0.661$). These findings thus suggest that, in contrast to many other autoimmune diseases have a strong gender bias, fetal gender is not associated with development of CHB, an observation that has also been made by other investigators but not in a population-based setting as our study (Llanos et al., 2009; Solomon et al., 2003).

4.1.3 Seasonal timing of pregnancy (paper II)

The seasonal timing of pregnancy has to our knowledge not been investigated in relation to CHB before the work with this thesis began. Season of birth has however been investigated in other congenital heart diseases and has been associated with the development of autoimmune diseases such as multiple sclerosis, RA SLE and ulcerative colitis later in life (Disanto et al., 2012; Willer et al., 2005). In order to investigate if seasonal timing of birth could be a potential risk factor for CHB, we used our Swedish population-based cohort of families with ≥ 1 child with CHB to analyze the monthly distributions births (see Figure 6).

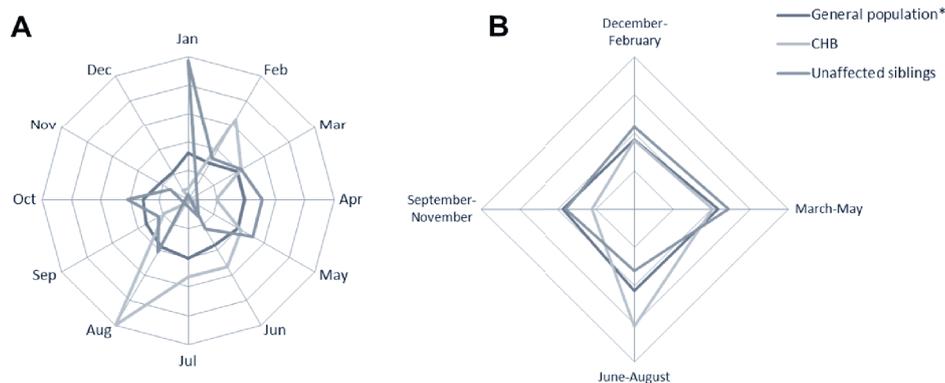


Figure 6. Monthly distribution of births in the general population and in the group of individuals with and without CHB born to an anti-Ro/SSA positive mother (n CHB=87, n No CHB=109). **(A)** Center represents the 5% mark and each additional inner circle represents 1 additional percent up to 10% for the outer circle. **(B)** Center represents the 5% mark and each additional inner line represents 10 additional percent up to 50% for the outer line. *mean proportion of children born per month in Sweden 1998-2011, data from Statistics Sweder (www.scb.se).

When analyzing the pregnancies included in paper II with regards to month of birth further, we found that pregnancies with birth during summer where the CHB susceptibility weeks 18–24 occurred during January–March correlated with a higher proportion of children with CHB. In the Ro/La-positive pregnancy group, children affected by CHB represented 58% of all births in the summer (June-August) and only 39% of all births during the rest of the year ($p=0.015$).

January to March are dark months in Sweden, and we thus speculated that low levels of vitamin D could potentially influence pregnancy outcome. In order to investigate this hypothesis, we analyzed the variation of vitamin D levels over the year based on samples from Swedish women ($n=1068$) that had been taken at different times of the year. In this group of healthy women representing the Swedish female population, Vitamin D levels were higher during the summer and lower during the winter months, with the lowest mean level recorded in March. We found that average vitamin D levels were significantly correlated to the ratio of CHB to healthy pregnancies for which the median susceptibility week (defined as week 21 of pregnancy) fell in that particular month ($p=0.009$). Low vitamin D levels corresponded to a significantly higher proportion of CHB pregnancies.

Interestingly, vitamin D has a well-documented immunomodulatory role with an inhibitory effect on adaptive immune cells (Mora et al., 2008). In addition, serum levels of vitamin D ($1,25(\text{OH})_2\text{VD}_3$) have been found to be significantly decreased in patients with SLE, especially during active disease (Chen et al., 2007), possibly indicating that vitamin D may play a role in the pathogenesis of CHB development. Furthermore, results from a recent study of risk for immune-mediated disease in relation to month of birth and vitamin D levels during pregnancy demonstrated that the risk of immune-mediated diseases in the UK was significantly influenced by the season of birth and that gestational vitamin D deficiency appeared to be a causative agent (Disanto et al., 2012). Even though the vitamin D levels that are used as reference in this study originated from a population of healthy Swedish women and not from the specific mothers of our cohort, it is reasonable to believe that these women follow the same yearly pattern as the women in our cohort.

The winter season is however not only a time of decreased sun exposure and subsequently lower vitamin D levels, but also the peak time for viral infections during the year in Sweden. An increased frequency of viral infections could possibly increase the risk for CHB development. The retrospective character of our study did not allow

us to investigate the occurrence of viral infections in relation to CHB development but this may be a risk factor worth investigating further in a prospective study setup.

4.1.4 Risk for CHB in consecutive pregnancies (paper II)

It has previously been demonstrated that mothers who have already given birth to a child with CHB have an increased risk for giving birth to another child with CHB. In the anti-Ro/La-positive mothers included in our population-based cohort, we observed a recurrence rate of 12.1% in 33 pregnancies immediately following the birth of a child with CHB, and an overall recurrence rate of 8.9% in all siblings born after an affected child. A low overall recurrence rate of 8% has previously been reported by a Finnish group (Julkunen et al., 1993a), but in a study of a larger cohort from the same group, the recurrence rate observed in pregnancies immediately subsequent to a CHB delivery, was higher (18%) (Julkunen and Eronen, 2001). In a study of 87 pregnancies, Solomon et al reported a recurrence rate for CHB in pregnancies immediately following a CHB pregnancy of 13.8% (Solomon et al., 2003) and in another study of 161 autoantibody-positive pregnancies in the USA, an 17.4% recurrence rate in pregnancies occurring immediately following a child with CHB was reported (Llanos et al., 2009). The two reports with high recurrence rates around 17% are however based on cohorts that were built up by recruiting families with cases of NLE (Llanos et al., 2009), or based on registers from tertiary referral centers (Julkunen and Eronen, 2001) which may lead to an over representation of families with multiple CHB cases. Our study cohort is in contrast, collected from national health care registers in an effort to minimize the bias towards the recruitment of families with multiple CHB cases. Apart from differences in recruitment of the cohorts, the differences in recurrence rates could potentially also be due to genetic or other differences between the studied populations.

4.1.5 Differences in risk factors between autoantibody and non-autoantibody mediated CHB

The focus of this thesis has been heart blocks that are congenital of origin in children born to mothers who are autoantibody-positive. The presence or absence of maternal autoantibodies has been suggested to define two separate groups of patients with heart block with different pathogenic mechanism, recurrence rate, prognosis and age at diagnosis (Villain et al., 2006). In our population-based study on risk factors (paper II), we have accordingly analyzed these two groups separately and the differences in risk factors between the groups are summarized in Table 3.

Table 3. Recurrence rate and risk factors for CHB development in relation to maternal antibody status.

	Autoantibody-positive mother	Autoantibody-negative mother
Recurrence rate	12-17%	0%
Season of birth as a risk factor	Yes	No
Increased maternal age as a risk factor	Yes	No
Increased paternal age as a risk factor	No	No
Fetal gender as a risk factor	No	No

4.2 DEVELOPMENT OF CHILDREN WITH CHB FETALLY EXPOSED TO RO/SSA AUTOANTIBODIES

In order to investigate outcome and development in children with and without CHB born to anti-Ro/SSA positive women, several studies were conducted within the work of this thesis. The focus of these studies has been growth (paper I and III), autoantibody levels and breast feeding (paper IV), late development of AVB (paper V) and neurodevelopment (paper VI).

4.2.1 Growth of children with CHB (paper I and III)

Growth development is an important health indicator and used world-wide to monitor the health status and well-being of children. Based on clinical observations of feeding problems and failure to thrive in infants affected by complete CHB who were followed at the Pediatric Cardiology Unit at Astrid Lindgren Children's Hospital, we first initiated a single-center study to evaluate growth in infants fetally exposed to anti-Ro52 antibodies from birth to the age of 12 months. Thirty-two pregnancies in 30 anti-Ro52-positive mothers were included. Seven of these fetuses developed AVB II or III, 8 developed AVB I, and 17 had normal atrioventricular conduction (NC), as diagnosed by using Doppler echocardiography.

The results demonstrated that newborns with fetal signs of AVB II-III had a significantly lower weight, shorter length, and smaller head circumference (HC) at birth than those with AVB I or NC, who, in turn, did not demonstrate any differences in any of these measurements. While the reduction in length and HC at birth seemed to be an

effect of the shorter gestation observed in the AVB II-III pregnancies, birth weight still remained close to 1 SD below Swedish reference standards after correction for GA. The infants with AVB II or III were retarded by 0.98 ± 0.77 SD in weight at birth and did not show any catch-up during the follow-up period of 12 months. In contrast, children with AVB I or normal AV conduction had a smaller, non-significant weight reduction of 0.51 ± 1.01 SD with a catch-up during the first months of life.

Although few individuals were included, the results from paper I indicated that children with AVB II-III were retarded in weight without catch-up during the first year of life. In order to further investigate growth in children with CHB, we used the Swedish population-based cohort of CHB patients previously identified by members of our group (Salomonsson et al., 2011). By using this cohort, we were able to expand the number of individuals included and also study long-term growth of the children from birth to the age of 18. In addition, it enabled us to use the unaffected siblings in the cohort as controls as these children were born to the same parents and in most cases measured by nurses at the same facilities. We used a retrospective study approach and collected medical records for siblings with ($n=72$) and without ($n=60$) CHB born 1973-2009 to anti-Ro/SSA positive mothers from child health care centers and school health services.

The results demonstrated that compared to Swedish reference standards children with CHB were retarded in weight at birth, as previously observed in paper I. This weight restriction persisted for several years, but at 2-3 years of age the CHB group started to catch-up, reaching the level of the reference standards at 9-11 years of age. In addition, the group of individuals with CHB was also retarded in both weight and height, from birth to 9-11 years of age, when compared to siblings without CHB, who did not show any signs of restriction in these measurements.

The results from the paper III thus confirm the findings in paper I showing weight retardation in children with CHB and further demonstrated that the CHB group had a catch-up during childhood years. Using the unaffected siblings as controls, we could reveal that CHB is a more important predictor for the growth restriction than maternal rheumatic disease and anti-Ro/SSA exposure *in utero*, as the siblings included were born to the same set of mothers who all were anti-Ro/SSA positive.

4.2.1.1 *Analysis of long-term growth*

The analysis of long-term growth included repeated measurements and two different statistical methods were used to investigate the longitudinal measurements; Multivariate analysis of variance (MANOVA) and linear mixed model analysis. Using both methods, we could more meticulously investigate the effect of time/age and CHB as predictors on outcome growth variables. While the linear mixed model analysis allows missing data, the MANOVA method only accepts complete data in order for analysis to be performed, thus only individuals with measurements at all time-points were taken into account in the MANOVA. To minimize the effect of differences in the time of follow-up as well as a variation in the timing of examinations after 2 years of age, data were grouped and separated into two analyses; birth to 18 months and 1½ to 11 years of age.

The results from the linear mixed model analysis are presented in Table 4, while the results from the analysis with MANOVA are displayed in Figure 7. In summary, for weight, the results from both statistical methods used demonstrated that there is a statistically significant difference between the two groups but no effect of age during the time-period birth-18 months (CHB n= 36, No CHB n=35 in the MANOVA) (Figure 7A). From 18 months to 11 years, we also observed a significant difference in weight between the groups but also an effect of age in the CHB group (CHB n=35, No CHB n=29 in the MANOVA) (Figure 7B). For height, the results differed slightly between the two statistical methods. The MANOVA demonstrated that there was no significant difference between the two groups from 0-18 months of age (CHB n=28, No CHB n=36) (Figure 7C), but an effect of age in both groups ($p<0.05$ CHB group and $p<0.01$ No CHB group). As seen in Figure 7D, there is however a significant difference between the groups from 18 months to 11 years of age (CHB n=35, No CHB n=31) and during this time period, there is also an effect of age in the CHB group ($p<0.01$). In contrast, the results from the mixed model analysis (Table 4) demonstrated that there was a significant difference between the two groups from 0-18 months of age, while from 18 months to 11 years of age there was also an effect of age. When comparing the results from the MANOVA analysis to the results from the linear mixed model analysis the results from both statistical methods thus demonstrate that weight is the most affected body measurement. In paper IV, the linear mixed model was chosen since it allows a more detailed analysis of the outcome and variables with less time restrictions as individuals without measurements at all time-points also can be included.

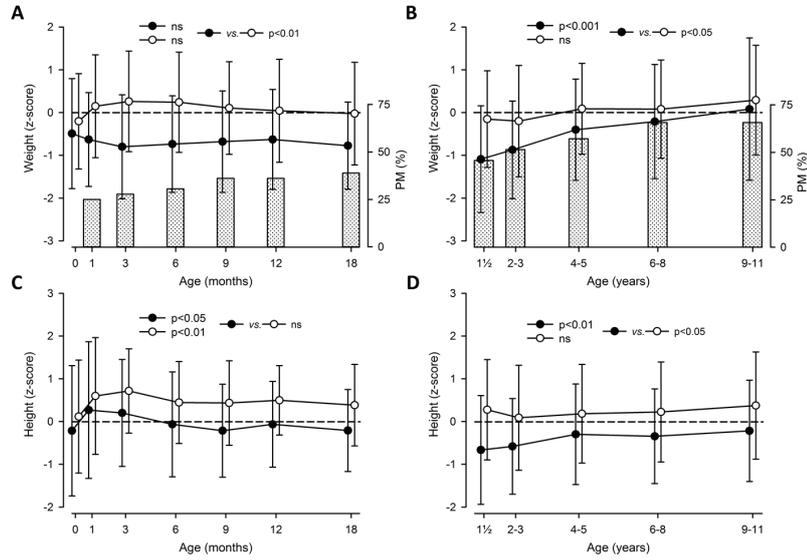


Figure 7. (A) and (B) display the results from the MANOVA in weight z-scores (left axis) for the two investigated groups and with percentages of individuals with pacemaker in the CHB group (right axis) at each time point, while (C) and (D) display the results from the MANOVA in height z-scores.

Table 4. Results from the linear mixed model analysis for weight, height and BMI.

Outcome and variable	Time restrictions	p-value	Comment
Weight			
Time	No	<0.01	Adjusted for gender
Group	No	<0.0001	
Time*Group (Interaction)	No	<0.01	
Time	≤ 18 months	ns	Adjusted for gender
Group	≤ 18 months	<0.001	
Time*Group (Interaction)	≤ 18 months	ns	
Time	≥ 18 months	<0.01	Adjusted for gender
Group	≥ 18 months	0.0001	
Time*Group (Interaction)	≥ 18 months	<0.05	
Time	18 months <age< 11 years	<0.0001	Adjusted for gender
Group	18 months <age< 11 years	<0.01	
Time*Group (Interaction)	18 months <age< 11 years	ns	
Height			
Time	No	ns	Adjusted for gender
Group	No	<0.01	
Time*Group (Interaction)	No	ns	
Time	≤ 18 months	ns	Adjusted for gender
Group	≤ 18 months	<0.01	
Time*Group (Interaction)	≤ 18 months	ns	
Time	≥ 18 months	ns	Adjusted for gender
Group	≥ 18 months	<0.05	
Time*Group (Interaction)	≥ 18 months	ns	
Time	18 months <age< 11 years	<0.05	Adjusted for gender
Group	18 months <age< 11 years	ns	
Time*Group (Interaction)	18 months <age< 11 years	ns	
BMI			
Time	No	<0.0001	Adjusted for gender
Group	No	<0.01	
Time*Group (Interaction)	No	ns	
Time	≤ 18 months	ns	Adjusted for gender
Group	≤ 18 months	0.0001	
Time*Group (Interaction)	≤ 18 months	ns	

Some studies have investigated weight of children with CHB at birth, but to our knowledge no study has investigated the long-term growth of these children. In a Finnish study of 50 infants with CHB, the mean birth weight was found to be -0.34 SD units among children with CHB, but not significantly lower than the birth weight of siblings without CHB born to the same mothers (mean SD= 0.076) (Julkunen et al., 1998a). Friedman et al (2010) reported on weight, height and HC at birth in a cohort of 19 neonates exposed to maternal autoantibodies *in utero*, four of them (2 with CHB and 2 healthy) were born small for GA. In another study of neurodevelopment in 13 children with CHB, 6 had a birth weight below the 10th centile and 3 had a birth weight at or below the third centile, but 5/6 had, however, been exposed to dexamethasone *in utero* (Brucato et al., 2006b). Furthermore, Friedman et al (2009) presented data on weight, height and HC at birth and at 1 years of age in 39 studied children (30 steroid exposed and 9 non-steroid exposed) and found that several dexamethasone treated newborns were premature and/or SGA (40%) with some persistent failure to thrive at 1 year of age, especially among the 22 dexamethasone-exposed children with AVB III.

When evaluating growth in children with CHB, *in utero* treatment with fluorinated steroids must be taken into account as it is a treatment that is suggested to cause intrauterine growth restriction and decreased birth weight in both human and animal studies (Banks et al., 1999; Benediktsson et al., 1997; Bloom et al., 2001; Celsi et al., 1998; French et al., 1999; Jaeggi et al., 2004; Johnson et al., 1979; Moss et al., 2001), which can make it difficult to distinguish effects of steroids from effects of the fetal disease. In contrast, other studies of prenatally steroid-treated children, demonstrate that these children have a normal pre- and postnatal growth (Forest et al., 1993; Lajic et al., 1998; Mercado et al., 1995; New et al., 2001).

In our two cohorts, only a few individuals (n=4 in paper I and n=3 in paper III) with CHB were exposed to fluorinated steroids *in utero* and with such a low number of exposed individuals, it is not possible to investigate whether transplacental treatment affected growth or not. As a significantly lower birth weight was demonstrated in the CHB group even though only a few of the individuals in paper III were exposed, steroid treatment cannot be seen as the cause of the growth restriction observed in the cohort.

4.2.1.2 Possible reasons for growth restriction and the subsequent catch-up

One may speculate on the reason for the growth restriction observed in children with CHB during their first years of life. One explanation is that fetuses with CHB are not able to fully compensate for their usually pronounced decrease in heart rate and to maintain a normal cardiac output and tissue perfusion. Another scenario is that the heart block is a “marker” for a phenotype related to a specific genotype also affecting growth. Interestingly, unpublished results in a mouse model of autoantibody-associated CHB from our group indicate that also mice born with CHB are weight restricted at birth compared to unaffected mouse pups.

Other potential reasons for growth restriction may include problems with breast feeding, and food intake, possibly due to decreased cardiac performance. To be able to evaluate the reasons for the growth restriction observed systematic prospective follow-up studies are however needed.

In relation to our finding of seasonal timing of birth as a risk factor for CHB development, and our interesting observation that this possibly may be due to vitamin D deficiency (paper II), it is also worth noting that vitamin D deficiency in the mother during pregnancy has been associated with fetal growth restriction in the child (Dror, 2011) although studies suggesting no difference in weight at birth exist (Gale et al., 2008).

Interestingly, when plotting birth weight z-scores in relation to month of birth, we found that the lowest mean birth weight in our cohort was found among children who were born in February and March, months when vitamin D levels are low in the Swedish women (Figure 8). However, being born during June-August (with the risk period for CHB occurring during February-March), did not seem to affect the birth weight.

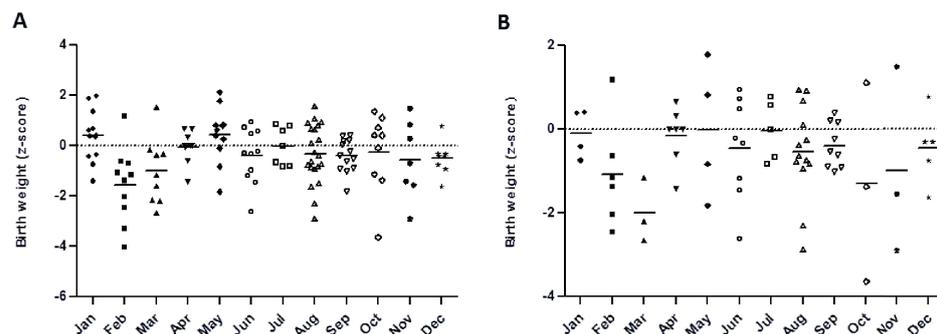


Figure 8. Birth weight z-scores among (A) all included individuals (n=127) and (B) children with CHB only (n= 70).

As the group of children with CHB showed a catch-up in weight, we have speculated what the cause behind this catch-up may be. In contrast to what we initially hypothesized, pacemaker treatment was not correlated to the catch-up in growth. As no obvious cause behind the catch-up could be identified in our material, we speculate that the catch-up in growth may be explained by several or interacting factors, probably including pacemaker implantation and better food intake.

4.2.1.3 Overweight children with CHB

Although the children with CHB, as a group, were significantly retarded in weight from birth and several years onward, they showed an interesting growth pattern as they grew older. After the catch-up in weight, a substantial proportion of the children (12-21%) had a weight that was $> 2SD$ from the reference values (Figure 9). In addition, 14 individuals in the study cohort were overweight with a body mass index (BMI) >25 (9 children with CHB and 5 siblings without CHB). Five of the nine children with a BMI >25 also had a BMI >30 (indicating obesity) for at least one time-point, in comparison to one of the five siblings without CHB. As no child had a BMI >25 before 8 years of age, the unhealthy weight gain occurred after the catch-up period and may possibly suggest that children or youths with CHB are less physically active than youths without CHB. Using special BMI reference values for Swedish children (Karlberg et al., 2001), we found that ten children with CHB had a BMI $>2 SD$ for at least one time-point from 8-18 years. During follow-up of these children it is thus important to continue to measure growth, also when the child has had a catch-up, as overweight is just as important to follow as a restricted weight and may have serious consequences to the health of the individual.

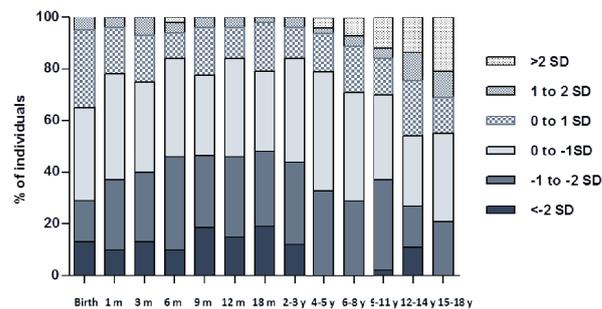


Figure 9. Relative distribution of weight z-scores of children with CHB at different ages.

4.2.2 Breast feeding and antibody levels in children with CHB (paper IV)

During pregnancy, IgG antibodies are transferred from the mother to the fetus via the placenta. These antibodies then circulate in the infant's blood for weeks to months after birth, neutralizing microbes or marking them for destruction by phagocytes - immune cells that consume and break down bacteria, viruses and cellular debris. In addition to the immune protection gained through the placental IgG antibody transfer during pregnancy, maternal antibodies (IgA, IgM and IgG) can also be transferred to the child after birth via breast milk, which can be of great importance during the first few months of life, when the infant often do not have an effective immune response. While antibodies may be of major importance and benefit to the infant in order to cope with foreign substances and infections, *in utero* exposure to maternal anti-Ro and La antibodies may instead harm the fetus and lead to NLE, but whether post-partum transfer of these antibodies may harm the child has not previously been investigated.

In order to evaluate neonatal lupus symptoms and postnatal Ro and La IgG, IgA and IgM antibody levels up to one year of age in infants born to mothers with Ro/SSA antibodies, we prospectively followed 30 mothers and their 32 children. The autoantibody levels were then correlated with NLE skin manifestations, and the role of breast feeding in transfer of autoantibodies from mother to child was evaluated.

4.2.2.1 Maternal autoantibody levels

The majority of the maternal sera contained Ro52, Ro60 and La IgG antibodies. These IgG antibodies also existed in the fetal circulation at birth, thus confirming an IgG transfer from the mother to the fetus during pregnancy. All mothers included in the study were Ro52 positive and as in previous studies (Salomonsson et al., 2002b; Strandberg et al., 2008), Ro52-p200 levels were significantly higher in pregnancies with AVB II and III than in pregnancies resulting in a normal fetal AV conduction. The Ro60 and La autoantibody levels were however not significantly different between mothers of children with AVB and healthy children, which is in agreement with previous studies (Fritsch et al., 2006; Julkunen et al., 2004; Salomonsson et al., 2002b) and supports the role of Ro52 autoantibodies as the pathogenic autoantibodies in CHB.

While our group repeatedly has demonstrated that p200 antibodies are significantly higher in mothers with CHB pregnancies, Clancy et al (2005) have claimed that p200 autoantibody levels do not significantly differ between mothers of children with CHB, mothers of children with skin rash and mothers of children without NLE, and concluded that reactivity to p200 is a dominant but not uniform anti-Ro52

response in women with children affected by CHB. However, when these sera were tested in the lab of our group together with sera from Finnish and Swedish women (total n=515), levels were found to be significantly higher in mothers of children with CHB and are thus suggested as an additional relevant marker in evaluating the risk for fetal AV block (Strandberg et al., 2008).

4.2.2.2 *Autoantibody levels in infants*

The results of the study revealed that Ro52, Ro60 and La IgG autoantibody levels in the infant circulation significantly decreased from birth to 4–5 weeks of age ($p < 0.05$, $p < 0.05$ and $p < 0.01$), a relatively fast clearance of autoantibodies that approximately corresponds to the normal maternal antibody half-life of approximately three weeks and the autoantibody levels in the infants decrease even though more than 70% of the infants were breast fed. Askanase et al (2002b) have previously demonstrated that IgA and IgG autoantibodies specific to Ro52, Ro60 and La can be detected in breast milk and while it is a disadvantage that breast milk of the participating mothers could not be obtained in our study, the significantly lower maternal antibody levels in infant sera indicates that autoantibodies in breast milk do not contribute substantially to titers established *in utero*. Hence, the symptoms associated to NLE are rather related to the placental IgG transfer during pregnancy.

As suggested by previous investigators (Silverman et al., 1995), NLE skin manifestations were associated with anti-La-antibodies and the mothers of children with NLE had significantly higher La IgG levels.

Interestingly, we could see a normalization of the AV conduction time in three neonates affected by AVB I that was parallel with the decrease of the maternal antibody levels in the infant sera. These findings are also shown in a report from Cimaz et al (2003a) who described concomitant disappearance of ECG abnormalities and Ro52 and Ro60 autoantibodies in infants without CHB born to Ro/SSA positive mothers. Although our hypothesis is that the normalization of the AV time is related to a decrease in maternal autoantibody levels in the infant, it is possible that the decrease in IgG antibody titers are not responsible for the normalized AV conduction and that this normalization rather started *in utero*. For example, it is possible that children with AVB I who do not progress to a higher degree block do not express a specific genetic susceptibility that is needed for complete CHB to develop.

In our study cohort, 21/32 infants included were fed by breast milk alone for the first 8 weeks, most of them longer. When relating the infant diagnosis and autoantibody

levels to breast feeding, we found that breast feeding does not delay a conversion from AVB I to normal conduction. Furthermore, NLE skin lesions developed in five infants independently of breast feeding. We consequently conclude that there are no reasons not to advise Ro/SSA and La/SSB positive women to breast-feed their children. These findings are supported by more recent findings from Izmirly et al (2010) who investigated cutaneous manifestations of neonatal lupus and risk of subsequent CHB and found that breast feeding was not associated with the development of NLE skin manifestations after birth ($p=0.91$).

When it comes to the duration of breast feeding, we could not detect a significant difference in duration of breast feeding between infants with AVB and non-affected infants. However, an interesting trend was observed as $> 70\%$ of the AV block II/III infants were breast-fed for ≤ 2 months while $> 70\%$ of the non-affected infants were breast-fed for ≥ 2 months, making it tempting to speculate that the duration of breast feeding correlates to the children's condition i.e. a non-affected child will be breastfed a longer time.

4.2.3 Late development of atrioventricular block (paper V)

Autoantibody-mediated CHB is in most cases diagnosed *in utero* or during the first 27 days of life, whereas non-autoantibody-mediated blocks usually develops later (Villain et al., 2006). In our population-based cohort of Swedish CHB patients, we however found 12 individuals with CHB born to an anti-Ro/La positive mother who got their diagnosis at a later age than 27 days of age. A structured review of medical records from these patients was performed in order to describe the characteristics of these individuals and the progression of disease, a group of patients that has not been described before.

The age distribution at the definite diagnosis of complete CHB the group of 12 individuals diagnosed at a later age than 27 days, ranged from 4 months - 43 years. Interestingly, within the group of 12 individuals with late developing AVB, we could distinguish two different groups with regards to the characteristics investigated (Table 5). As seen in Table 5, the first group of individuals (group 1) consisted of six individuals who were all diagnosed before the age of 5 years. Notably, in these six cases family history of autoimmune disease was significantly more common in this group than in the remaining cases with a complete AVB diagnosis later in life (group 2) ($p<0.05$). In addition, the course of disease differed significantly between the two groups. In group 1, the time from diagnosis to pacemaker implantation ranged 9-46

years, reflecting a course free of significant heart related symptoms. In comparison, repeated episodes of syncope or a significantly low ventricular rate (≤ 20 bpm) was seen more often in case of a complete AVB diagnosis after the age of five years, resulting in pacemaker implantation in close relation to the time of definite diagnosis of complete AVB (range <1 week to 2 months).

Table 5. Characteristics of individuals born to autoantibody-positive mothers and diagnosed with complete CHB after the neonatal period.

	Group 1 (n=6)	Group 2 (n=6)
Age at CHB diagnosis	<5 years of age	9-43 years of age
Family history of autoimmune disease	5/6	1/6
Time from diagnosis to pacemaker insertion*	9-46 years	<1 week to 2 months

*This observation was not explained by mean age at pacemaker implantation in this small case series; 21.3 (range 11-47) years compared to 28.0 (range 12-43) years respectively (ns).

The results of the study indicate that immune mediated AVB may occur later in life. While the maternal autoantibodies may initiate the damage to the fetal heart *in utero*, they may not be required for the block to progress from a lower, to a higher degree block later in life. Some reports have demonstrated that, once established, first- and second-degree AVB may progress to a more advanced block later in life. Askanase et al (2002a) have demonstrated a progression to more advanced blocks in 4 of 9 cases of AVB I, present at birth as well as progression to complete block in 2 out of 4 cases with AVB II present at birth. Furthermore, colleagues in our group have observed that fetuses with signs of AVB I during week 18-24 weeks of gestation, that reverted to normal conduction postnatally then showed a subsequent preschool progression to asymptomatic AVB I (Bergman et al., 2012). In addition, Gordon et al (2001) have published a case report describing a child, fetally exposed to anti-SSA/Ro and with a normal ECG at birth that developed second-degree AVB at the age of 2 in the absence of alternative aetiology.

Cardiologists meeting patients with late development of AVB either during childhood or as adults should rule out other potential causes of the heart block and investigate whether the mother of the individual affected is positive for Ro and/or La autoantibodies to get an insight into the disease itself, the prognosis, therapeutic options

and familial implications. If the mother of the patient indeed is antibody positive, the patient should be notified that he or she cannot pass antibody-associated CHB on to his/her children as long as the patient is not herself autoantibody-positive. It is also important that the patient and his/her family members are notified the increased risk for CHB in siblings born to mothers who already have given birth to a child with CHB.

4.2.4 Neurodevelopment in children with CHB (paper VI)

As described in the introduction, NLE may include several different manifestations and has been suggested to also include neurologic manifestations. For example, case reports of hydrocephalus, non-specific white matter changes, calcification of the basal ganglia, and vasculopathy have been published (Silverman and Jaeggi, 2010). In addition, several studies have demonstrated that the incidence of learning disabilities is higher in children born to mothers with SLE than in those born to mothers without SLE, in particular if the child is a male (Lahita, 1988; McAllister et al., 1997; Neri et al., 2004; Ross et al., 2003). Furthermore, in Ro/SSA-exposed children, parental reporting of neurodevelopment abnormalities, decreased mental and sequential processing and high frequency of dyslexia have been published (Askanase et al., 2010; Behan et al., 1985; Yoshikawa et al., 2010). With this background in mind, we initiated a study of neurodevelopment in children with and without CHB born to mothers with Ro/SSA autoantibodies. The Swedish population-based cohort of siblings with and without CHB born was once again used and individuals born 1974-2009 to an anti-Ro/SSA positive mother were included.

The results demonstrated that impaired neurodevelopment was reported in 16% (18/114) of the children during the follow-up time of 13.0 (8.2-17.5) years, median (quartiles) and that impaired neurodevelopment in any of the studied five categories was more frequently reported in children born preterm, in boys and in children born to mothers with SLE. The reported problems included speech (9%), motor (8%) and learning (8%) impairment, attention deficit (5%), and behavioral impairment (4%).

A comparison of our data with the prevalence of impairment in neurodevelopment in the general population is difficult to perform, partly due to the retrospective character of our study and the lack of studies with comparable study methods, and partly since the described neurodevelopmental problems are neither strict medical nor psychiatric diagnoses and therefore vary in definition over time. The approximate prevalence in the general population can however be used as an indication to evaluate if the children in this study, all exposed to anti-Ro/SSA autoantibodies *in*

utero, have an impaired neurodevelopment in comparison to the general population. Among the five categories observed in this study, speech (9%), motor skill development (8%), and learning impairment (8%) were the most commonly reported problems. The prevalence of language delay/speech impairment range from 1% to 15% in previously reported studies (Law et al., 2000; Tomblin et al., 1997; Westerlund, 1994; Westerlund and Sundelin, 2000), and studies on motor clumsiness and motor coordination performance show a prevalence of around 6% (Kadesjö and Gillberg, 1998; Smyth, 1992) found a prevalence of teacher-reported learning impairment of 12.5% in their Swedish material, while review articles on learning disabilities point to a prevalence of learning disabilities around 5% (Lagae, 2008; Lyon, 1996).

Compared to the above figures, our study does thus not demonstrate any obvious increased risk for impairment in neurodevelopment in anti-Ro/La exposed children if compared with figures of the prevalence in the general population, although some previous studies have suggested that children born to anti-Ro/SSA positive mothers may have an impaired neurodevelopment.

When further analyzing our material, we found that disturbances in motor skill development was more common in boys than in girls and also influenced by GA, while attention deficit was significantly more common in children born to mothers with SLE and in children with CHB. SLE in the mother did also affect learning abilities, and there was a trend towards learning disabilities being more common in children with CHB.

4.2.4.1 GA, gender and steroid exposure in relation to neurodevelopment

From previous studies, it is known that GA and sex may influence neurodevelopment. Children born preterm, particularly children born at a GA of <32 weeks, have an increased risk for problems with cognitive, motor and behavioral skills (Jongbloed-Pereboom et al., 2012). However, only two children in our study were born very preterm at a GA <32 weeks, and none of these two had any impairment in neurodevelopment. As our results from paper III demonstrate that children with CHB are weight restricted at birth, we also investigated birth weight among children with and without neurodevelopmental impairment but found no significant difference in birth weight between the groups.

It is known that boys are overrepresented in neurodevelopmental disorders (Kadesjö and Gillberg, 1998), which was also observed in our study where a significant influence of male sex in the categories motor skill development and behavior was

found. The explanation to the sex differences in neurodevelopmental disorders is unknown, but speculating, one may think of reasons such as sex hormone levels *in utero*, as the fetal brain develops in male direction through a direct action of testosterone on the developing neurons. Other explanations could potentially include epigenetic factors that turn susceptibility genes "on" and "off" during development or perhaps that girls in general have better social skills than boys and so need a bigger "dose" of what causes the neurodevelopmental impairment to cross that threshold to being impaired. In addition, another hypothesis is the theory of maternal immunoreactivity, suggesting that pregnant women produce antibodies towards Y chromosome-associated antigens, possibly affecting the developing brain of male fetuses, with the subsequent expression of developmental disorders (Gualtieri and Hicks, 1985). Interestingly, the theory also suggests that this immunoreactivity response is more common in women with autoimmune or allergic disorders than in other women.

When it comes to *in utero* exposure to fluorinated steroids, several studies have demonstrated that prenatal and postnatal steroid treatment may negatively affect the development and growth of the brain in animals, and the neurodevelopment in children (Abbasi et al., 2000; French et al., 1999; Huang et al., 2001; Jobe and Soll, 2004; Jobe et al., 1998; Matthews, 2000; Spinillo et al., 2004), but in contrast results from Brucato et al (2006b) showed a normal neuropsychological development in children with CHB exposed to high doses of dexamethasone *in utero* (n=11). Furthermore, neither Ross et al (2003) nor Neri et al (2004) found any association between maternal steroid treatment and the incidence of learning disabilities in their children. There are today few follow-up studies on cognitive development of children exposed to steroids *in utero*. In pregnant women with risk for virilizing congenital adrenal hyperplasia, antenatal dexamethasone treatment has been used for more than 20 years, and while some studies report no significant negative effects on developmental outcome (Meyer-Bahlburg et al., 2004), studies from a Swedish cohort demonstrate long-term effects on verbal working memory and certain aspects of self-perception (Hirvikoski et al., 2007). In our study cohort, only one of the children with impairment in neurodevelopment had been exposed to steroids *in utero*, meaning that impairment in neurodevelopment developed independently of steroid exposure.

4.2.4.2 CHB and SLE in relation to neurodevelopment

One category of neurodevelopment, attention deficit, was significantly more common in children with CHB than in their siblings without CHB. Furthermore, there was a trend towards a significant influence of CHB on learning disabilities. When calculating odds ratios (Figure 9, data not shown in paper), we found increasing odds ratios for several categories, but only attention deficit and learning impairment in children born to mothers with SLE was found to be significant, showing a more than tenfold risk of learning impairment and attention deficit in children born to mothers with SLE. However, all children with attention deficit had CHB and the resulting OR of 1.5×10^8 with CI 0-infinity and are thus not depicted.

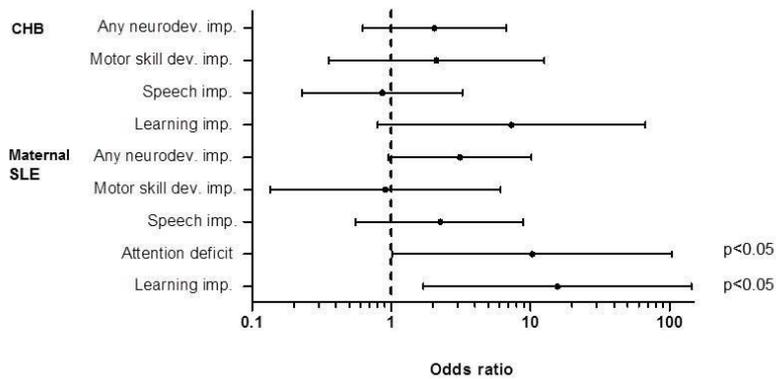


Figure 9. Odds ratios and 95% confidence intervals for neurodevelopment impairment in any category, motor skill development, speech, attention and learning impairment in relation to the predictors CHB in the child and maternal SLE. Analysis of attention deficit in relation to CHB was however not applicable due to small number of individuals and thus not included in the graph. A statistical significance with the logistic regression analysis was observed for the predictor SLE for development of learning impairment ($p < 0.05$) and attention deficit ($p < 0.05$). The number of individuals with behavioral impairment were too small in order for logistic regression analysis to be performed

In our study cohort, four individuals with CHB among whom three had a mother with SLE had a neuropsychiatric diagnosis (dyslexia $n=1$, autism $n=1$, ADHD $n=2$), compared to none of the siblings, further supporting a contribution of both the maternal autoimmune condition and fetal cardiac disease to an impaired neurodevelopmental outcome.

As previously mentioned, studies have shown that the incidence of learning disorders is found to be higher in children born to mothers with SLE than in children born to a healthy mother. McAllister et al (1997) found an association between SLE in

the mother and risk of having a child that develops hyperactivity, reading problems, and attention deficit. Neri et al (2004) found that children of patients with SLE have normal intelligence but an increased rate of learning disabilities, particularly if male. Neither the use of corticosteroids, nor the presence of Ro/SSA and La/SSB autoantibodies, did however have any effect on the children's' intelligence level or the occurrence of learning disabilities. In addition, Yoshikawa et al (2010) showed that children born to anti- Ro/SSA positive mothers have a significantly lower mean score of Sequential Processing and Mental Processing than children born to anti- Ro/SSA negative mothers and Askanase et al (2010) showed that parental reporting of neuropsychiatric abnormalities was high in anti-Ro/SSA exposed children both with and without NLE manifestations, although the results did not reach statistical significance. When investigating children with complete CHB, Brucato et al (2006b) did however show that children with heart block who may or may not have been exposed to high-dose dexamethasone *in utero* have a normal neuropsychological development.

The reasons for the increased risk of neurodevelopmental impairment in terms of attention deficit and possibly learning disabilities in children with CHB and children born to mothers diagnosed with SLE is not known. SLE is characterized by the production of double-stranded DNA antibodies and a subset of these antibodies, present in 40% of the patients, can cross-react with the NR2A and NR2B subunits of the N-methyl-d-aspartate receptor (NMDAR) and have been demonstrated to cause neuronal damage, cognitive dysfunction and fetal loss in the offspring after transfer in mouse models. There is also evidence that maternal IgG can cross the blood brain barrier and cause pathology due to cross-reactivity to a neuronal receptor. Due to this Ca-signalling dysregulation mediating synaptic responses are seen as well (Lee et al., 2009; Wang et al., 2012).

In adults it is difficult for antibodies to cross the blood-brain barrier, but it has been demonstrated that the neonatal Fc receptor is expressed at the blood-brain barrier (Schlachetzki et al., 2002) and that this could provide a portal of entry of anti-Ro antibodies into the central nervous system and subsequent antibody mediated damage to brain tissue, brain microvasculature and choroid plexus may occur (Boros et al., 2007). Children born with CHB may potentially have a phenotype that is more sensitive to these autoantibodies than their unaffected siblings and thus be harmed by the autoantibodies, not only through the development of heart block, but potentially also through neurologic damage. Interestingly, it has been demonstrated that the brain over expresses Ro antigens, just as the heart do (Wang et al., 1995).

Other potential explanations for the impairment in neurodevelopment may include placental dysfunction during pregnancy that may influence the developing fetal brain, a decreased cardiac function in children with CHB with a cardiac output that perhaps is not sufficient to oxygenate tissues and organs in a proper way. The normal head circumference observed in children with CHB does however not indicate that the brain is affected by the decreased cardiac output. Hypothyroidism in the mother; known to affect neurodevelopment and suggested to be more common in mothers to children with CHB, as well as fetal growth or genetic features may possibly also influence neurodevelopment. In studies of neurodevelopment in other congenital heart diseases, low socioeconomic status has been correlated with impaired neurodevelopment, but as the siblings with and without CHB were born to the same set of parents, this did not influence the observed difference between the two groups.

In this study, included individuals were grouped with regard to GA, sex, steroid exposure, CHB, and maternal diagnosis. Unfortunately, we did not have access to information on other potential manifestations of NLE present in the included individuals. When going through case reports on neurological manifestations reported in children with NLE, it is striking that these are reported mostly in girls and often in children with skin rash but seldom in children with CHB. It would therefore be interesting to investigate the individuals included grouped by “skin rash” and “no skin rash” as well as “any NLE manifestation” vs. no “NLE manifestation” and also to perform CT or Magnetic Resonance Imaging scan investigations of the brains of the children to reveal possible neurologic involvement, but this was however not possible in this study.

5 CONCLUSIONS

Complete isolated CHB is a rare but life-threatening disease associated with the presence of Ro and La autoantibodies in the mother of the child affected. Although the link between Ro and La autoantibodies and the risk for CHB has been known for decades, a recurrence rate of 12-17% in subsequent pregnancies suggests that other risk factors also contribute to disease pathogenesis.

In this thesis, maternal age and seasonal timing of birth has been revealed as novel risk factors for CHB development in autoantibody-positive pregnancies. In two different studies (paper I and II), we have demonstrated that increased maternal age is a risk factor for CHB development. We have also demonstrated that parity does not influence the risk for CHB.

Furthermore, analysis of the number of births during the summer (June– August) and the rest of the year revealed a significant difference between affected children and healthy siblings in the Ro/La-positive pregnancy group, with the births of children affected by heart block representing 58.5% of all births in the summer and only 39.0% of all births during the rest of the year ($p=0.015$). Our hypothesis is that events linked to the winter season (such as a decrease in vitamin D levels due to decreased light exposure) occurring during susceptibility weeks 18–24 may affect the outcome of the pregnancy. The novel findings of increased maternal age and seasonal timing of birth as risk factors for CHB may be useful for counseling when pregnancy is considered.

Outcome and development in children with CHB has not been thoroughly investigated previously. In this thesis, we used both a single-center prospective as well as a nation-wide retrospective study approach to study antibody levels in the children, outcome and growth.

We found that maternal autoantibodies decrease rapidly in the infant circulation during the first few weeks of life and that non-detectable levels were reached at one year of age (paper IV). The autoantibody levels were rapidly decreased among breast-fed infants as well. This observation indicates that refraining from breastfeeding does not protect from NLE skin involvement and we thus see no reason not to recommend breast feeding in children born to anti-Ro and or La positive women.

In the single-center study, the results demonstrated that newborns with fetal signs of AVB II-III have a significantly lower weight than those with AVB I or NC and did not show signs of catch-up during the first 12 months of life (paper I). Fetuses without

AVB II-III had significant but smaller weight retardation at birth. These infants did however show a quick catch-up during the first 2 postnatal months, indicating that they, even in the presence of fetal signs of AVB I, have a good prognosis. We then further analyzed growth using the population-based cohort and demonstrated that, compared to reference standards; children with CHB were retarded in weight by 0.75-1.0 SD from birth to 2-3 years of age. Thereafter the CHB children started to catch-up, reaching the reference standards at 9-11 years of age. In addition, the individuals with CHB were retarded in both weight and height from birth to 9-11 years of age when compared to siblings without CHB, who did not demonstrate restriction in these measurements (paper III). Although the children with CHB appeared to spontaneously initiate their catch-up in weight around the age of 2-3 years and the majority of the children in our cohort were found within the ± 2 SD normal reference range regarding weight, the results of this study highlight the importance of careful follow-up of individuals with CHB regarding nutrition and growth.

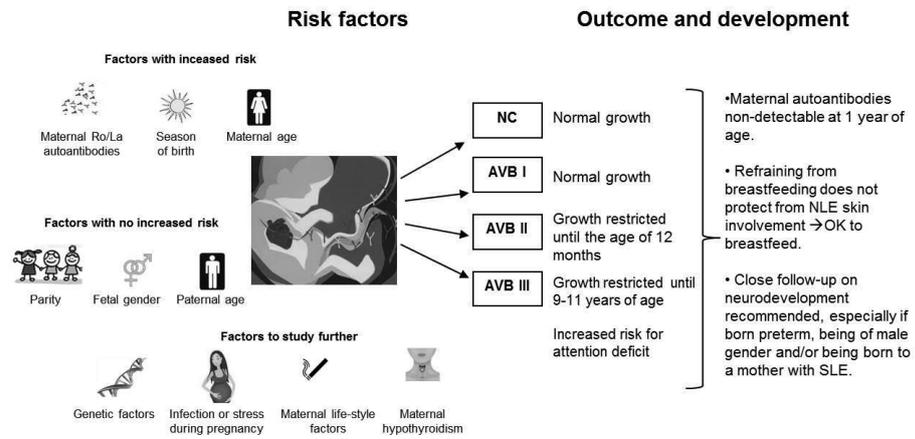
The results of this thesis also demonstrate that autoantibody associated CHB diagnosis after the neonatal period is possible, advocating testing of maternal serology at the time of diagnosis (paper V). Within the group of 12 individuals with late developing complete AVB investigated, we could distinguish two different groups with regard to the characteristics examined. In the first group of individuals (n=6), all diagnosed < 5 years of age, family history of autoimmune disease was significantly more common than in the second group of individuals (n=6) diagnosed with complete AVB diagnosis later in life ($p < 0.05$). In addition, the time to pacemaker differed significantly between the two groups.

When investigating neurodevelopment, our data indicate that in addition to well-established factors such as male sex and being born preterm, both maternal SLE and CHB may influence neurodevelopment (paper VI). Follow-up of neurodevelopment should therefore be considered for children with CHB, especially if the mother is diagnosed with SLE. Careful management of these individuals and early diagnosis is one way to help these children overcome their difficulties during childhood and school years and make sure that they obtain the support needed.

Although the results of this thesis demonstrate a growth restriction and a higher risk of attention deficit among children with CHB than in their siblings, it is important to emphasize that these children, despite their serious heart condition, seem to thrive quite well during childhood and teen-years. The children with CHB appear to spontaneously catch-up in growth during the pre-teen years and the prevalence of

neurodevelopmental impairment does not seem to be higher than the prevalence in the general population. To better understand the potential risk of neurodevelopmental impairment in children exposed to Ro/SSA autoantibodies prospective case-control studies will however be needed.

A summary of the findings of this thesis can be seen below.



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