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Cross-reactivity of anti-Ro52 antibodies and genetic susceptibility in congenital heart block

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"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza "

(Dante Alighieri)

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Abstract

Introduction. Congenital heart block (CHB) is a passively acquired autoimmune disease associated with transplacental transfer of maternal anti-Ro/SSA and/or anti-La/SSB antibodies mediating inflammation and subsequent fibrosis of the atrio-ventricular node in the fetal heart. Despite the long-established association of maternal antibodies in directly inducing CHB both with *in vitro* and *in vivo* studies, the pathogenic mechanisms involved remain unclear and identification of cross-reactive targets of anti-Ro52 antibodies in the fetal heart is matter of debate. CHB occurs in 1–2% of anti-Ro/SSA antibody-positive pregnancies and has a recurrence rate of 12–20% in a subsequent pregnancy, suggesting that additional factors, such as genetic and environmental components may determine the outcome in terms of CHB development in autoantibody exposed fetuses.

Objectives. This thesis is divided into two parts and investigates the pathogenic mechanism and the genetic association with CHB. The first study aims to identify cross-reactive targets of anti-Ro52/p200 antibodies by screening a library of peptides covering the whole human proteome and using monoclonal antibodies specific for the Ro52/p200 region and to confirm the reactivity at peptide level and on the whole antigen level. The second study aims to identify fetal susceptibility genes among the Human Leucocyte Antigen (HLA) locus in anti-Ro/SSA autoantibody-mediated CHB from DNA samples collected from a multi-centric European cohort of families in which children affected by CHB were born.

Part I. A whole proteome microarray revealed 17 peptides to be significantly cross-reactive with a Ro52/p200 monoclonal antibody (Ab31) and two linear motifs ("YSDF" and "YSNF) were shared among these sequences. Among the targets, cross-reactivity was studied in detail for TG and GAK, for which reactivity was

showed both at the peptide and whole protein level with anti-p200 monoclonal antibodies and with sera from mothers whose children have CHB, suggesting that reactivity to these protein could represent a risk factor for development of CHB in the fetuses in anti-Ro/SSA-positive pregnancies.

Part II. DNA from 636 individuals of 173 European families in which children affected from CHB were born (119 Swedish, 38 Finnish, 2 Norwegian and 14 Italian families) was genotyped and imputation of HLA class I and HLA class II loci was performed. From the analysis, HLA-Cw*06, -DRB1*13, -DQA1*01 and -DQB1*06 emerged as a protective alleles associated with CHB development, while HLA-DQA1*04 transmissions were associated with susceptibility. Furthermore, haplotype analysis revealed that the DRB1-DQA1-DQB1 13-01:03-06:03 haplotype significantly associated with protection from CHB development, while DRB1-DQA1-DQB1 08-04:01-04:02 was significantly associated with CHB susceptibility. A parent-of-origin effect was seen for the following alleles: a lower maternal transmission to affected children was associated with Cw*06, while lower paternal transmission was observed for DQB1*06 and DRB1*07. With these findings, we propose that HLA typing in anti-Ro52-positive pregnancies might be a useful tool to assess the risk of CHB in the fetuses.

Riassunto

Introduzione. Il blocco avtrio-ventricolare (BAV) congenito è una malattia autoimmune passiva associata al trasferimento transplacentare di anticorpi materni anti-Ro/SSA e/o anti-La/SSB, causando infiammazione e fibrosi del nodo atrio-ventricolare con successivo blocco cardiaco nel feto. Nonostante l'associazione diretta degli anticorpi materni nell'indurre il BAV sia da lungo nota, i meccanismi patogenetici coinvolti rimangono poco chiari e l'identificazione di bersagli di cross-reattività degli anticorpi anti-Ro52 nel cuore fetale è oggetto di dibattito. In aggiunta, Il BAV si verifica nel 1-2% delle gravidanze in cui sono presenti anticorpi anti-Ro/SSA e ha un tasso di ricorrenza del 12-20% nelle una successive gravidanze, suggerendo che fattori aggiuntivi, quali genetici e ambientali sono coinvolti nello sviluppo del BAV.

Obiettivi. La prima parte della tesi riguarda uno studio che ha avuto lo scopo di identificare i bersagli di cross-reattività degli anticorpi anti-Ro52/p200 attraverso uno screening di una libreria peptidica coprente l'intero proteoma umano e l'utilizzo di anticorpi monoclonali specifici per la regione p200 della proteina Ro52. Ulteriore obiettivo è quello di confermare la reattività a livello di peptide e a quello di intero antigene. Il secondo studio ha lo scopo di studiare l'associazione genetica del locus "*Human Leucocyte Antigen*" (HLA) con il BAV in una coorte di famiglie europee in cui sono nati bambini con BAV.

Parte I. Lo screening su base proteomica ha individuato 17 peptidi significativamente cross-reattivi con un anticorpo monoclonale specifico per la regione p200 (Ab31). Tra le 17 sequenze sono stati trovati due motivi lineari condivisi ("YSDF" e "YSNF). Tra i target positivamente legati dall'anticorpo Ab31, la cross-reattività è stata ulteriormente studiata per TG e GAK, per i quali la

reattività è stata dimostrata sia a livello peptidico, che e a livello di proteina intera, usando anticorpi monoclonali anti-p200 e sieri di madri i cui figli hanno il BAV. Reattività verso queste proteine potrebbe rappresentare un fattore di rischio maggiore per lo sviluppo del BAV nei feti in gravidanze positive per gli anticorpi anti-Ro/SSA.

Parte II. Campioni di DNA provenienti da 636 individui da 173 famiglie europee in cui sono nati bambini affetti da BAV (119 svedesi, 38 finlandesi, 2 norvegese e 14 italiane) è stato genotipizzato ed è stata eseguita l'imputazione dei loci HLA di classe I ed HLA di classe II. Dall'analisi è emerso che gli alleli HLA-Cw*06, -DRB1*13, -DQA1*01 e -DQB1*06 conferiscono protezione dallo sviluppo BAV, mentre la trasmissione ai feti di HLA-DQA1*04 conferisce suscettibilità alla malattia. Inoltre, l'analisi ha rivelato che l'aplotipo DRB1-DQA1-DQB1 13-01:03-06:03 è significativamente associato con la protezione dallo sviluppo BAV, mentre DRB1-DQA1-DQB1 08-04:01-04:02 è significativamente associato con suscettibilità al BAV. Infine, è' stato osservato anche un effetto di origine parentale per questi alleli: -Cw*06 è associato una minore trasmissione materna ai bambini affetti, mentre DQB1*06 e DRB1*07 sono associati ad una inferiore trasmissione paterna. Con questi risultati, proponiamo che la genotipizzazione di questi alleli in gravidanze anti-Ro52-positive potrebbe essere un utile strumento per valutare il rischio di sviluppo del BAV nei feti.

1. Introduction

1.1 Congenital heart block

Congenital heart block (CHB) is a manifestation of neonatal lupus erythematosus (NLE), a passively acquired autoimmune condition caused by the transplacental transport of maternal autoantibodies (anti-SSA/Ro and anti-SSB/La) into the fetal circulation. Mothers usually present with systemic lupus erythematosus (SLE), Sjögren syndrome (SS), or undifferentiated connective tissue disease (UCTD), but may be also asymptomatic (1). Beside CHB, other manifestations of NLE are skin rash, hepatitis and cytopenias (2-5). Skin lesions or CHB occur in about 50% of NLE cases, but occurs concomittantly with CHB in only 10% of NLE cases (6). While CHB is a permanent condition, skin manifestations are transient and will disappear as maternal autoantibodies decrease in the child's circulation to reach undetectable leves 6-8 months after birth (7). NLE occurs in 15-20% of infants born of mothers with SLE and anti-Ro/SSA antibodies (2). Liver involvement may be the only manifestation of NLE. It occurs in 25% of cases as reported in a prospective study (2). Liver involvement is usually transient and resolves within the first months of life (3). Cytopenia, as a manifestation of NLE, appears within the first week of life and tends to resolve by 2–4 weeks of age. It was found that neutropenia was much more commonly seen than thrombocytopenia, and that neutropenia occurs in up to 10–15% of children born to mothers with anti-Ro antibodies (2, 4).

CHB, the most severe manifestation of NLE, is characterized by the delay or block of electrical conduction at the atrioventricular (AV) node that results in atrioventricular block (AVB) (8, 9). It occurs in a structurally normal heart and in

association of maternal anti-Ro/SSA and anti-La/SSB antibodies. CHB usually develops during the risk period between the 18th and the 24th weeks of pregnancy and is diagnosed *in utero* or within the neonatal period (0-27 days after birth) (10). According to the severity of the manifestation, AVB can be classified in three degrees. First-degree AVB is characterized by a prolonged interval between atrial and ventricular contraction, and can be visualized on an electrocardiogram (ECG) by a lengthened PR interval (11). In the second-degree AVB, some of the atrial impulses don't reach the ventricles. Third-degree AVB, the most severe manifestation of CHB, is characterized by complete block of conduction of impulses through the AV node and dissociation between atrial and ventricular contraction, resulting in bradycardia. While I and II degree AVB are reversible, III degree AVB is irreversible and can lead to long-life pacemaker implantation or even death occurring *in utero* or perinatally (12, 13) and it is hypothesised that development of CHB from first to third degree is a progressive process (14).

At the cellular and molecular level, CHB is characterized by deposition of immune complexes, signs of inflammation, calcification and fibrosis at the AV node in the fetal heart (15-17).

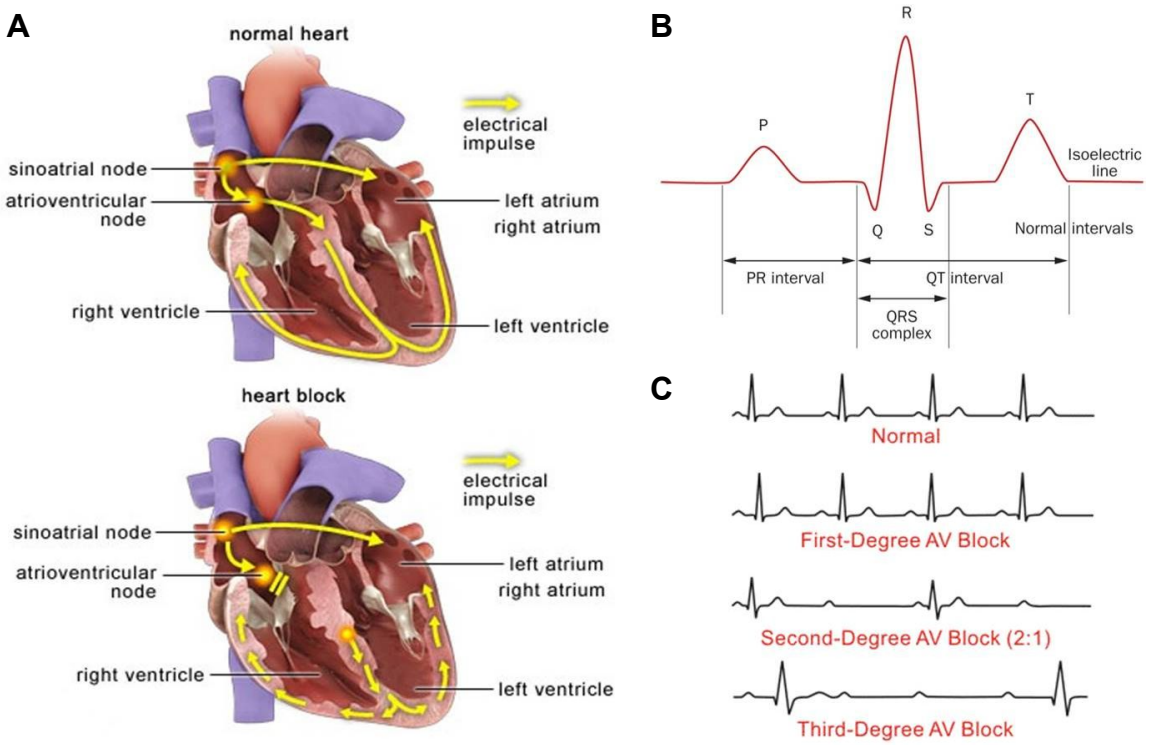

Figure 1. (A) The normal and the CHB-affected heart conduction system. Electrical impulses are generated in the sino-atrial (SA) node and propagated from the atria to the ventricles via the atrioventricular (AV) node, before being conducted through the bundle of His to the bundle branches and the Purkinje fibers, and finally through the entire myocardium. **(B)** Electrocardiogram (ECG) showing the P wave and the QRS complex, which correspond to the atrial and ventricular depolarization, respectively. The PR interval is measured from the beginning of the P wave to the beginning of the QRS complex. **(C)** ECG showing a normal sinus rhythm, I-, II- and III-degree AV block.

1.1.1 Epidemiology

Complete CHB is considered a rare disease with a reported incidence of 1:15000 - 1:20000 newborns in the general population. Recently, a population-based study which considered CHB cases in anti-Ro/SSA positive pregnancies and without structural heart diseases, reported that CHB II-III degree AVB occurs with an incidence of 1:23300. (18). The association of CHB with maternal autoantibodies directed to Ro/SSA and/or La/SSB is well verified (19-21). In women carrying anti-Ro/SSA antibodies during pregnancy, the risk for fetuses to develop CHB is 1-2%

in an anti-Ro/SSA positive pregnancy (22, 23) and the recurrence risk for the following pregnancies increases to 12-20% despite the persistence of autoantibodies (1, 24-26). The incidence of I and II degree AVB is still a matter of debate with contrasting data regarding the incidence of I-degree AVB ranging from 3-14% in newborns from anti-Ro52 positive pregnancies (27-29), while about one third of fetuses of anti-Ro52 positive mothers show typical features of I-degree AVB *in utero* (14). Complete CHB is associated with a mortality rate of 15-20% (1, 30-33), though a recent single-center prospective study reported a substantially lower mortality of CHB in anti-Ro/SSA positive pregnancies at 6% (18).

1.2 Autoantigens and maternal autoantibodies in CHB

Maternal autoantibodies directed to Ro/SSA and La/SSB antigens have been firmly associated with CHB (19-21). In the early '80s the presence of anti-Ro/SSA antibodies to ribonucleoprotein were demonstrated in sera from mothers who gave birth to infants with CHB (34-36). Maternal autoantibodies were also found in *post-mortem* hearts of neonates deceased due to CHB (15, 17). Anti-Ro/SSA and anti-La/SSB antibodies are common in the rheumatic diseases SS and SLE. In particular, in anti-Ro/SSA and anti-La/SSB antibodies are found in 40-95% of SS patients; while among SLE patients, 20-60% of them have anti-Ro/SSA and 10-20% have anti-La/SSB in their serum (37). In the late '80s and early '90s the Ro/SSA autoantigen was shown to consist of two unrelated proteins, Ro52 and Ro60 (38-41).

1.2.1 The Ro/SSA and La/SSB antigens

Ro52

Ro52, is a 475 amino acid (aa) residues long protein with a molecular weight of 52 kDa. It belongs to the tri-parted motif (TRIM) family of proteins (42). It is composed of a RING and a B-box motifs, followed by a coiled-coil (CC) domain, which contains a leucine zipper motif, and a B30.2 (or PRYSPRY) region in the C-terminal end (Figure 2) (43).

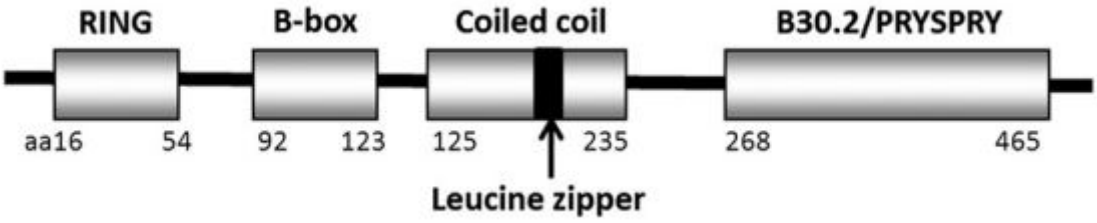

Figure 2. The structural domains of Ro52 (Oke et al., *Journal of Autoimmunity* 2012). The RING, B-box and coiled-coil domain constitute the TRIM motif. The coiled-coil domain contains a leucine zipper motif and the C-terminal of the protein include a B30.2 (PRYSPRY) domain.

As a TRIM protein, Ro52 is also denoted TRIM21, and *Trim21* is the official name of the Ro52 gene (42), which is mapped along 8.8 Kb of chromosome 11 and consists of 7 exons. The gene is expressed mainly in immune cells (http://biogps.org) (44, 45)

Many of the proteins in the TRIM family play an important role in innate immunity and anti-viral responses (46, 47), but also in regulating immune responses by targeting key molecules involved in proliferation, survival or death of cells (48).

Like several other TRIM proteins, Ro52 has E3 ligase activity and acts in the process of ubiquitination (49). Ubiquitination is a process of post-translational modification of proteins, conserved in eukaryotic cells and it is used to control biological processes such as protein degradation, trafficking and activation (50-

52). The process of ubiquitination is a complex three-step pathway requiring energy (ATP). The first step includes binding and activation of the ubiquitin molecule by a ubiquitin activating enzyme (E1). The activated ubiquitin molecule is then transferred to a ubiquitin conjugating enzyme (E2). In the last step, a ubiquitin ligase (E3) mediates the transfer of the ubiquitin to from the E2 to a lysine residue of the target protein (Fig. 3) (51, 52). Ubiquitinated proteins are commonly targeted to the proteasome, where they are degraded. Proteins can be modified by ubiquitination in three different ways: mono-ubiquitination, where a single ubiquitin molecule is attached to the target protein; multi-ubiquitination, where several single ubiquitin moieties are covalently bound to separate lysine residues on the target protein; and poly-ubiquitination, where a chain of ubiquitin molecules is added to a single lysine residue on the target.

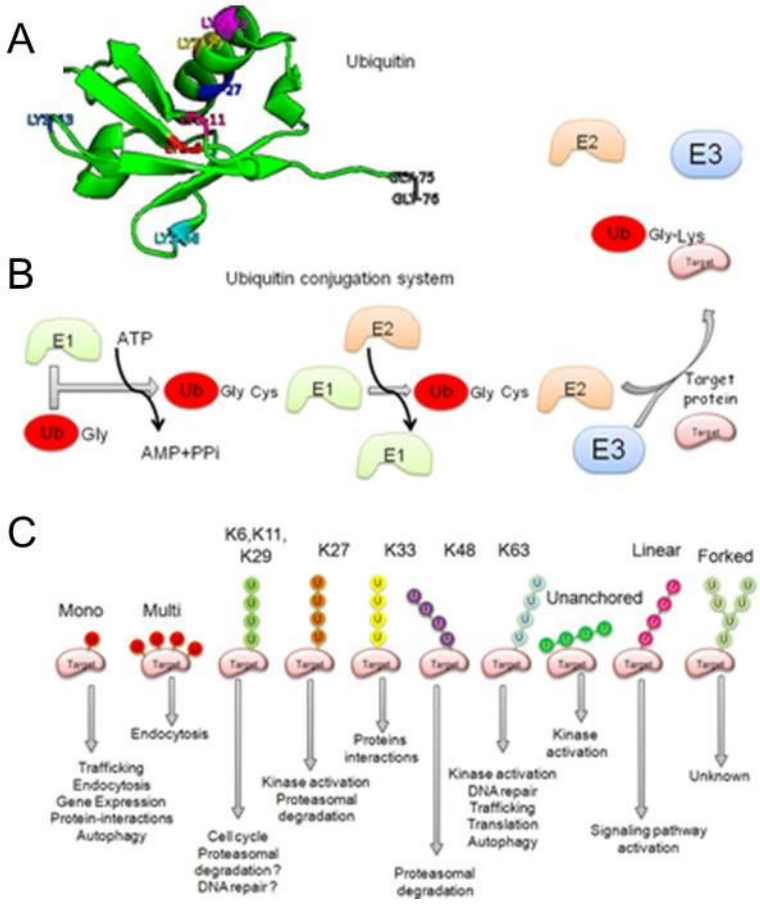

Figure 3. The process and diverse outcomes of ubiquitination. (A) Crystal structure of Ubiquitin showing seven conserved lysine and C-terminal di-glycine motif. (B) Ubiquitination of target protein involves the sequential action of three enzymes: E1, E2 and E3. (C) Different forms of protein ubiquitination and their fate. Proteins can be mono-ubiquitinated with a single Ub, multi-ubiquitinated or poly-ubiquitinated. Poly-ubiquitination can be of different types depending on the Ub lysine residue which is targetted for Ub chain elongation. The different fates of the modified proteins have been shown in the figure. (figure from Tomar *et al*., *Bio Cell* 2015)

It has been demonstrated that Ro52 may regulate both cytoplasmic and nuclear substrates via ubiquitination, interacting either with the E2s UBE2D1 and UBE2E1 located in the cell cytoplasm and nucleus, respectively (49, 53). Targets of ubiquitination mediated by Ro52 include Interferon regulatory transcription factor (IRF) 3, IRF5, IRF7 and IRF8 (45, 49, 54-56), diminishing their activity (56). Ro52, as all the TRIM family proteins, is a critical regulator of innate immune response specifically in antiviral immunity (57, 58). The interferon (IFN) and NF-κB are the

critical pathways in the regulation of innate immune response during viral infection, regulating the expression of various cytokines and other immunity-related genes (52, 59). The stimulation of cells with IFN leads to the accumulation of Ro52 in the nucleus (60) which ubiquitinates IRF8 and enhances cytokine expression, specifically type I IFN (61, 62). It was also shown that Ro52 is involved in regulation of NF-κB, inducing mono-ubiquitination and subsequent autophagic degradation of IKKβ, a positive regulator of NF-κB (63). Hence, degradation of IKKβ suppresses NF-κB activity.

Ro52 function has been studied also in Ro52-deficient mice (45). A distinct role for Ro52 in Th17-related autoimmunity was demonstrated with enhanced production of proinflammatory cytokines that are regulated by the IRF transcription factors, including cytokines involved in the Th17 pathway (IL-6, IL-12/IL-23p40, and IL-17). Disruption of the IL-23/Th17 pathway in the Ro52-deficient mice by deletion of p19, a subcomponent of the heterodimeric IL-23 cytokine, provided complete protection from the skin inflammation and systemic autoimmunity, revealing the importance of Ro52 as a negative regulator of proinflammatory cytokine production (45).

Ro52 is involved also in the regulation of the cell cycle and apoptotic process but discordant findings have been present. Overexpression of Ro52 in stably transfected B cells leads to decreased proliferation and increased sensitivity to CD40-mediated cell death, (49). In contrast to these findings, it was described that knock-down of Ro52 leads to impaired progression of the cell cycle caused by accumulated p27 and inability of cell to enter S-phase (64). These observations, though, are all made in *in vitro* systems using different cells and experimental

conditions. More investigations are thus needed to allow a better understanding of the role for Ro52 in the regulation of the cell cycle and apoptotic process.

Ro60

60 kDa-Ro60 is an RNA-binding protein (65) encoded by the gene *TROVE2* in humans. The protein is conserved from *Caenorhabditis. elegans* to all vertebrates and found also in the green algae *Chlamydomonas*, several eubacteria and one mycobacteriophage (66). It is ubiquitously expressed and shaped like a doughnut with an inner hole. In vertebrate cells, Ro60 is normally complexed with small noncoding RNAs, known as YRNAs. It has been shown that the protein also binds misfolded, non-coding RNAs that are eventually degraded (67-69) and likely functions in a quality control pathway by which incorrectly folded or defective non-coding RNAs are targeted for degradation (65, 66). In addition, studies of Ro60 in both mammalian cells and bacteria have revealed that the protein is important for cell survival after ultraviolet irradiation (69, 70). Recently it was shown that Ro60 also binds repetitive transposons called Alu retroelements, leading to an aberrant Toll-like receptor (TLR) signalling which is TLR7- and TLR8-dependent (71, 72). Moreover, Alu transcripts were induced by type I IFN and stimulated proinflammatory cytokine secretion by human peripheral blood cells and Ro60 deletion resulted in enhanced expression of Alu RNAs and IFN-regulated genes.

La

The SSB/La antigen is a single 48 kDa nuclear phosphoprotein encoded by *SSB* gene in humans. Expressed ubiquitously in eukariotic cells (73, 74), the La protein associates with the 3’ of many newly sinthesized RNA plymerase III transcripts, including pre-tRNA and pre-5S rRNA and other small RNA. This binding prevents this RNA precursor from digestion by exonucleases and it is important for their

maturation (74-77). Moreover it has been observed that in adenovirus- or Epstein-Barr-infected cells, La can bind viral-encoded RNAs and therefore may be involved in virus replication (76, 78, 79).

1.2.2 Anti-SSA/Ro and anti-SSB/La antibodies

CHB appears to be more specifically linked to the presence of maternal anti-Ro/SSA and anti-La/SSB antibodies rather than to maternal diagnosis, as the mother of an affected child may be also asymptomatic (13, 30, 80).

Regarding the Ro/SSA autoantigen, older studies did not distinguished between Ro52- and Ro60-specific antibodies (38, 40, 41). Subsequent studies regarding the association of maternal antibodies with CHB tried to determine the serum profile of mothers of affected children in relation to the three components Ro52, Ro60 and La and are described below.

Anti-Ro52/p200 antibodies

Anti-Ro52 antibodies are the most common specificity in primary SS where can be found in 66.7% of the patients (81). Co-uccurrence of these antibodies with anti-Ro60 is observed in 52.1% of patients with primary SS, while anti-Ro52 together with anti-La are are present at the same time in 49% of these patients. In SLE, as well as systemic sclerosis and autoimmune myositis patients, anti-Ro52 antibodies are detected in one third of patients (62, 81). In CHB, instead, most studies aiming to screen for a specific maternal antibody profile have found an almost universal presence of antibodies targeting the Ro52 protein (12, 20-22, 82-89). More recently this finding was confirmed in a Swedish cohort of 193 mothers of children with CHB, where 95% of cases of antibody-related CHB were positive for anti-Ro52 (90).

Systematic analyses have been undertaken to identify the subpopulation and specificity of anti-Ro52 antibodies that correlate with congenital heart block and it was revealed that a major antigenic part resides in the central part of Ro52, inside the leucine zipper structure in the coiled-coil domain (Figure 1) (21, 91-95). Epitope mapping using overlapping peptides covering this domain revealed a significant association of autoantibodies against amino acid sequence 200-239 (p200) of the Ro52 protein with a higher risk of developing CHB (21, 87, 89, 96). Another study based on structurally derived mutations in different p200 syntetic peptides and circular dichroism allowed a better definition of the borders of the antigenic p200 region and revealed that binding of human monoclonal anti-Ro52 antibodies to p200 is dependent on a partly α-helical fold within the leucine zipper of p200 (96).

In a prospective study of 25 anti-Ro52-positive pregnant women, maternal antibodies anti-p200 were found to correlate to longer AV time intervals in fetuses (87). In addition, in a retrospectively collected Swedish cohort of mothers of children with CHB, 60% of anti-Ro52-positive sera also bound p200 (90). The association of anti-p200 antibodies with CHB outcome was also re-confirmed recently by an Italian multi-center study (97). In particular anti-p200 antibodies were significantly more frequently positive in mothers of children with CHB child than in women with healthy children (81.0% vs 59.1%, respectevely). Given the low risk of developing CHB in anti-Ro/SSA positive women, it has been suggested that also the levels of maternal anti-Ro52 antibodies are important in prediction of the pregnancy outcome (21). This hypothesis was corroborated also by an another group, which found that cardiac complication is associated with moderate or high

levels of maternal anti-Ro/SSA, but not with low levels in a group of children born by anti-Ro-positive mothers (98).

Also the specific interaction between anti-Ro52 antibodies and the corresponding antigen has been investigated (99). It was found that anti-Ro52-positive patient sera inhibited the E3 activity of Ro52 in ubiquitination assays and that anti-Ro52 autoantibodies inhibited the E3 ligase activity of Ro52 by sterically blocking the E2/E3 interaction between Ro52 and UBE2E1 (99).

Anti-Ro60

Anti-Ro60 antibodies are mostly present together with anti-Ro52 antibodies and many studies still rely on clinical assays which don't distinguish between Ro52 and Ro60 to investigate the presence of anti-Ro antibodies in maternal sera. It is, therefore, difficult to assess the individual clinical value of anti-Ro60 in the development of CHB. However, in a study by Salomonsson and colleagues, anti-Ro60 antibodies were found in 63% of antibodies-positive CHB pregnancy (90), but this antibody occurred in anti-Ro52-negative mothers only in 3% of cases. Anti-Ro60 antibodies have been suggested to have a minor role in predicting the fetal clinical outcome in anti-Ro and anti-La antibody–positive mothers (21, 85, 100), although an association also between these autoantibodies and the incidence of CHB has been demonstrated (85, 101).

Anti-La antibodies

The association of anti-La antibodies to CHB is still matter of discussion. The level of antibodies to the La protein has been found to be higher in mothers of children developing cutaneous lupus rather than heart block (3, 85, 98). However, another study suggested that the presence of anti-La antibodies increases the risk of CHB (101). As for anti-Ro60 antibodies, also anti-La antibodies have been considered

less associated to CHB than anti-Ro52 (50, 55, 56). Anti-La antibodies were found in 59% of antibody-positive women with a CHB baby (90). The current general opinion is that although CHB may develop independently of maternal antibodies against Ro60 and La, their presence might be able to amplify the immunological response that leads to the AV block (102).

1.2.3 Other autoantibodies

In addition to antibodies directed to the Ro and La proteins, several other targets have been investigated for being associated with development of CHB. Antibodies to calreticulin, a protein involved in calcium storage in myocytes, have been found more frequently in sera from mothers of children with CHB than in sera of mothers of healthy children (103). Antibodies recognizing the neonatal heart muscarinic acetylcholine receptor (mAChR) have been thought to have a functional role in the disease supported by binding, and biological effects in *in vitro* studies (104, 105). Antibodies to a cleavage product of α-fodrin has been suggested as an additional serologic marker for CHB, in addition to being commonly found in patients with SS (106). Another suggested antibody specificity associated with congenital heart block is antibodies to p57, which was identified in a child with the disease (86, 107). All these studies, however, were not able to provide a strong association of the proposed antibodies with the involvement in development of CHB (80).

1.3 Pathogenesis of CHB

1.3.1 Evidence of a pathogenic role of maternal anti-Ro/SSA and anti-La/SSB antibodies

Several experimental studies, both *in vitro* and *in vivo*, have provided direct evidence for a pathogenic role of maternal anti-Ro/SSA and anti-La/SSB and especially anti-Ro52 antibodies in the development of CHB. The first demonstration of this link was provided in the '80s by several groups which showed the presence of anti-Ro/SSA antibodies in the cardiac tissue of fetuses with CHB, together with deposition of complement, fibrosis and calcification (15-17, 108, 109). Later, in *in vitro* studies, perfusion of rat or human hearts with the Langendorff technique with maternal IgG containing anti-Ro/SSA and anti-La/SSB antibodies was shown to induce bradycardia and complete AVB (110, 111). The same effect was gained using affinity-purified anti-Ro52 antibodies, showing a direct pathogenic role of maternal autoantibodies specific for the Ro52 antigen.

In vivo studies provided more evidence of the pathogenic role of anti-Ro/SSA and anti-La/SSB antibodies based on models of active immunization of rats, mice or rabbits females before gestation or passive transfer of antibodies in pregnant animals.

Among immunization studies, immunizing BALB/c mice with Ro60 or La, led to development of I-degree AVB in 19% and 7% of the offspring, respectively (112) and similar results were observed in C3H/HEJ mice (113). Instead, in two different studies immunization of the same model of BALB/c mice with human Ro52 led to I-degree AVB and to II/III-degree AVB in 9-25% and in 3.5-10% of offspring, respectively (110, 112). In rats, immunization with human Ro52 induced I-degree AVB in 10-45% of pups, depending on the strain (114). A pathogenic role of

antibodies specific for the p200 peptide has also been shown. After immunization of Dark Agouti (DA) rats with p200 peptide 19% of offspring developed I-degree AVB (87).

Immunization-based experiments present an intrinsic variability that rely on the immune response of each immunized animal, dependent also on factors like genetic background, age and immunization process. In the context of CHB it was shown that induction of the disease depends on the antibody response initiated in the mother and that maternal major histocompatibility complex (MHC) is crucial in the generation of pathogenic antibodies in CHB (114). Injecting antibodies directly into the females during gestation, instead, allows to overcome these problems and provided advantages in knowing the specificity and the amount of antibodies transferred in the animals. It was shown that transfer of affinity-purified anti-Ro/SSA and anti-La/SSB antibodies from mothers of children with CHB into pregnant females BALB/c mice induced I-degree AVB in 47-90% of the offspring, depending on the day of gestation at which the injection was performed (115). Instead, transfer of monoclonal antibodies specific for the p200 region of Ro52 in rats induced AV block in 100% of offspring but injection of antibodies targeting other domains of Ro52 did not (116). In addition, anti-p200 antibodies were shown to disturb calcium homeostasis in cultured neonatal cardiomyocytes, supporting a pathogenic role for anti-p200 antibodies in CHB (116).

1.3.2 Pathogenic mechanism

Despite the big achievement in showing that anti-Ro/SSA and anti-La/SSB have a direct pathogenic role in CHB, the mechanism by which they induce the disease is still not known. The intracellular localization of Ro52, Ro60 and La proteins and the lack of evidence that maternal antibodies can cross the sarcolemma of a

normal cardiomyocyte and bind to the specific antigens are the biggest obstacle in elucidating the molecular mechanism of CHB induction. Based on this fact two hypotheses of the pathogenic mechanisms, not mutually exclusive and each supported with experimental data, have been proposed: the “apoptosis hypothesis” and the “cross-reactivity hypothesis”.

The apoptosis hypothesis

According to the apoptosis hypothesis, the maternal autoantibodies gain access to their target antigens when they are exposed on the surface of apoptotic cells. Supporting this idea were the observations that Ro60 was found to be displaced to the surface blebs in apoptotic keratinocytes (117) and, then, that Ro60 was also present on the surface of early apoptotic cardiomyocytes (102, 118), and therefore exposed to binding by anti-Ro60 antibodies. Conversely, Ro52 was found to be exposed only after plasma membrane breaking in necrotic cells (102) and was bound by anti-Ro52 monoclonal antibodies to a lesser extent than anti-Ro60 and anti-La did (119). This discovery and the observation of exaggerated apoptosis and infiltrating macrophages in the heart of fetuses dying of CHB (109) supported the hypothesis that maternal anti-Ro60 may bind to apoptotic cardiac cells during apoptosis normally occurring in developing fetal hearts and so deviate the removal of apoptotic debris from a non-inflammatory pathway to their ingulfment by macrophages by opsonization. Subsequent activation of the phagocytic cells may lead to production of pro-inflammatory and pro-fibrotic cytokines, recruitment of leukocytes and complement components, and establishment of an inflammatory reaction that will eventually irreversibly damage the targeted tissue (109, 120). Subsequent *in vitro* studies showed that opsonized cardiomyocytes can activate phagocytic cells to produce pro-inflammatory and pro-fibrotic cytokines (120-122).

It is of note, though, that these experiments were performed in ventricular myocytes, not in conduction system myocytes such as AV node myocytes, and the correlation of these findings with the AV conduction abnormalities seen in CHB is not straightforward. According to these *in vitro* studies anti-Ro60 would first bind to apoptotic cells and later anti-Ro52 to necrotic cells, leading to establishment of inflammation and fibrosis in the fetal heart. These findings are, however, not in accordance with the *in vitro* and *in vivo* observation that anti-Ro52 are the most common in mothers of children with CHB (12, 20, 90) and that they have been shown to induce AVB in several animal models of CHB (110, 112, 114, 116).

The cross-reactivity hypothesis

Several studies have shown arrythmogenic effects of anti-Ro52 antibodies either with Langendorff-perfused hearts (110, 111) and with transfer of monoclonal antibodies anti-p200 in pregnant rats (116). Calcium homeostasis disturbance was also shown in cultured neonatal cardiomyocytes after adding anti-p200 antibodies (116). These data, together with the intracellular localization of Ro52 in live cells and the fact that Ro52 is predominantly expressed in immune cells (44, 45) and functions as a ubiquitin E3 ligase (49), support the idea that anti-Ro52 antibodies may mediate their pathogenic effect on the fetal hearts by cross-reacting with another targets available in the extracellular space. This targeted molecules might be involved in the control of electric signal or conduction and cross-reactivity might interfere with normal cardiac function.

Several targets have been proposed as cross-reactive targets and a list of studies is reported below (Table 1). The 5-HT4 serotoninergic receptor (5-HT4R) was suggested by Eftekhari and colleagues (123) as a putative cross-reactive target. In this study they found that antibodies specific for peptide 365-382 of Ro52

recognized residues 165-185 of the cardiac 5-HT4R (123) and that affinity-purified 5-HT4R antibodies could antagonize the serotonin-induced calcium channel activation in atrial cells (124). However, mouse pups born to females immunized with Ro52 peptides cross-reacting with anti-5-HT4R antibodies did not develop any sign of AVB or any other cardiac dysfunction (124). Additionally, others couldn't reproduce this data and were not able to confirm the reactivity to the specific epitope of 5-HT4R in any of 116 sera from mothers with affected children (125), leading to discordant results about this putative target (126).

Other than 5-HT4R, L- and T-type calcium channels were suggested as potential cross-reactive targets, supported by the observation that inhibition or blockade of this channel lead to AV block similar to conduction abnormalities seen in CHB. Specifically, the L-Type calcium channels subunits Cav1.2 (α1C) and Cav1.3 (α1D) are essential for AV node electro genesis and cardiac excitation-contraction coupling, with Cav1.2 crucial for cardiac contractility and Cav1.3 more important for atrial sinus pacemaker activity and signal conduction (127-129). Cav1.3, moreover, was also was shown to be expressed in human fetal heart (130).

In vitro experiments have shown that IgG purified from mothers with CHB-positive pregnancy could inhibit L-Type calcium currents in ventricular myocytes, sino-atrial and AV node cells as well as in exogenous expression systems (111, 130-134). Cross-reactivity of anti-Ro/SSA and anti-La/SSB antibodies with the Cav1.2 and Cav1.3 calcium channels has been proved (130, 133). Maternal antibodies have been proposed to induce, after cross-reactivity, calcium channel internalization and degradation with subsequent inefficient signal conduction, insufficient excitation-contraction coupling and reduction of cardiac contractile function (132). Supporting this hypothesis, mouse pups transgenic for Cav1.2 developed AV

block and sinus bradycardia at a lower frequency than non-transgenic littermates after *in utero* exposure to anti-Ro/SSA and anti-La/SSB antibodies in an immunization model (135). In addition, mouse pups in which the Cav1.3 subunit of the L-type calcium channel has been genetically knocked out exhibit first-degree AV block, albeit at a low frequency, and the occurrence of AV block is increased following immunization of females with the Ro and La protein before gestation (135). This study, however, doesn't show the specific interaction of maternal autoantibodies with the calcium channels. More recently, Strandberg and colleagues (136) found that sera from mothers with CHB affected children were able to bind the T-Type calcium channel subunit Cav3.1 (α1G) and to a lesser extent with Cav3.2 (α1H) and decrease of T-type calcium channel currents was seen in mouse sino-atrial node cells.

Cross-reactivity to α-Enolase, a glycolytic enzyme expressed on the membranes of several hematopoietic, epithelial, and endothelial cells, has been proposed as the target of anti-p200 monoclonal antibodies after a screening of a neonatal rat heart expression library (137). However, only a small proportion of anti-Ro52 positive sera from women with CHB pregnancy were also positive for this target, indicating that these antibodies may represent only a subset of mothers at risk (138). In conclusion, so far, none of the suggested cross-reactivity targets has been confirmed convincingly and showed to be a major target of cross-reaction. Moreover, even if previous studies showed that autoantibodies specific for p200 region interact with cardiomyocytes and disturb calcium homeostasis (87, 116), their cross-reactivity has not been investigate thoroughly.

Taking together the results of these studies, an attempt of merging together the apoptosis and cross-reactivity hypotheses has been made in order to better explain the molecular mechanism leading to CHB (139). The current opinion proposes a scenario in which, after crossing placenta, anti-Ro52 antibodies may cross-react with one or several molecules such as calcium channels on the surface of fetal cardiac cells, inducing disturbancies in calcium homeostasis. Prolonged exposure to maternal autoantibodies may then lead to increased cardiomyocytes apoptosis, followed by exposure of Ro52, Ro60 and La antigens. The consequent autoantibodies binding allows establishment and amplification of inflammatory reaction as described for the apoptosis hypothesis with eventual fibrosis and calcification in the AV nodes and establishment of complete CHB, as depicted in Figure 4.

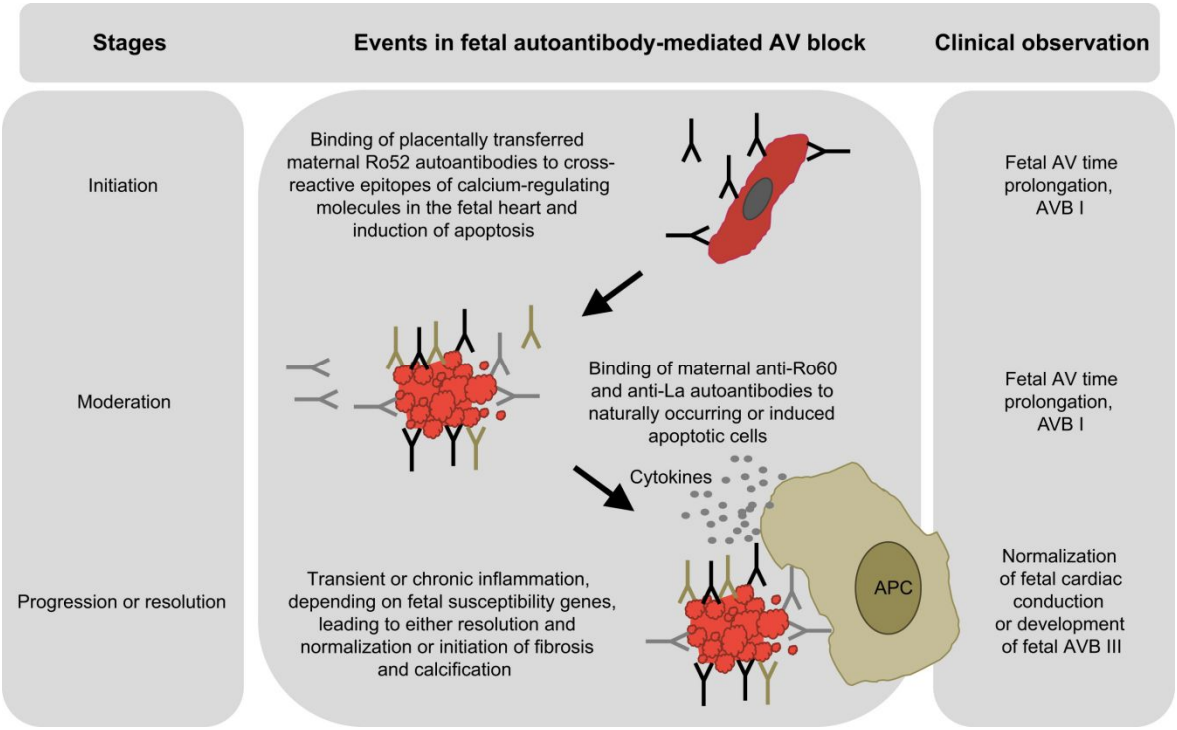

Figure 4. Schematic representation of events in the pathogenesis of congenital heart block. (Image from: (Ambrosi *et al., Exp Cell Res*. 2014;325:2-9)

Cross-reactivity target	Antibodies/method	Major finding	Reference
5-HT4R	Sera from SLE patients anti-Ro52-positive	cross-reaction with second extracellular loop of 5-HT4R	Eftekhari *et al.*, 2000 (123)
5-HT4R	Immunization of mice with 5-HT4R peptides (a); or Ro52 (b)	Mice developed bradycardia, AVB I- and II-degree (a); not developed any symptomps of CHB (b)	Eftekhari *et al.*, 2001 (124)
5-HT4R	Sera from mothers with CHB pregnancy	None cross-reacted with 5-HT4R	Buyon *et al.*, 2002 (125)
5-HT4R	Sera anti-Ro/La positive from mothers with/without CHB children and healty controls	Reactivity to 5-HT4R was found in 16% of mothers with CHB	Kamel *et al.*, 2005 (126)
L-Type calcium channels	Perfusion of rat hearts with IgG from anti-Ro/SSA-positive sera	Inhibition of whole cell L-Type calcium current I(Ca)	Boutjdir *et al.*, 1998 (111)
L-Type calcium channels subunit α1D	Effect of IgG containing anti-Ro/La on I(Ca) in heterologous expression systems	IgG binds directly to α1D Ca channel protein and inhibited I(Ca)	Qu *et al.*, 2005 (130)
L-Type calcium channels subunit α1C	Effect of IgG containing anti-Ro/La on I(Ca) in heterologous expression system	IgG binds directly to α1C Ca channel protein and inhibited I(Ca)	Xiao *et al.*, 2001 (131)
L-Type calcium channels	Immunization of pregnant rabbits with Ro52	Conduction defects and down-regulation of L-Type calcium channels in newborns. Inhibition of I(Ca) through IgG from immunized animals	Xiao *et al.*, 2001 (132)
L-Type calcium channels subunit α1C	Effect of IgG containing anti-Ro/La on I(Ca) in human fetal Purkinje myocytes and in heterologous expression system	IgG bound α1C and inhibites I(Ca) in both systems	Qu *et al.*, 2001 (133)
L-Type calcium channels subunit α1D	Sera anti-Ro/La positive from mothers with/without CHB children and healty controls. Effect of IgG on I(Ca) in heterologous expression system	14% sera from mothers with CHB bound extracellular loop of domain I S5-S6 region of α1D and reduction of I(Ca)	Karnabi *et al.*, 2010 (134)
L-Type calcium channels	Overexpression of L-Type calcium channels in Ro/La-immunized pregnant mice	Rescueing of AVB inMice pups with overexpressed L-Type calcium channels. interaction of maternal autoantibodies with the target not shown	Karnabi *et al.*, 2011 (135)
T-Type calcium channel subunit α1G and α1H	Sera from mothers with CHB baby	Binding to α1G and to a lesser extent to α1H. Decreased I(Ca) in mouse sino-atrial node	Strandberg *et al.*, 2013 (136)

α-Enolase	Anti-p200 monoclonal antibodies	Reactivity shown in ELISA and westernblot	Ambrosi *et al.*, 2007 (137)
α-Enolase	anti-Ro52 positive sera from women with CHB pregnancy	7% of sera positive for α-Enolase reactivity. Preincubation with Ro52 did not inhibit anti-α-enolase reactivity.	Llanos *et al.*, 2009 (138)

Table 1. Summary of papers published so far with aim of investigating possible cross-reaction targets of anti-Ro/SSA and anti-La/SSB antibodies.

1.4 Risk factors for CHB development

As the risk for CHB in anti-Ro/SSA- and/ anti-La/SSB-positive women is only 1-2% (22, 23) and the recurrence rate in the following pregnancies is 12-20%, despite the persistence of maternal autoantibodies (1, 24-26), it is clear that the sole presence of these antibodies is not sufficient to provoke CHB. This fact indicates that additional factors, maternal and fetal, genetic and enviromental, may be necessary for development of CHB in an autoantibody exposed fetus (Figure 5).

1.4.1 Genetic risk factors

Completion of the sequencing of the human genome in the early 2000s and a big improvement in the technology of genotyping and statistical analysis was the basis for researchers to discover that autoimmune diseases have a consistent genetic component, with the Human Leucocyte Antigen locus (HLA) being the most significantly associated with rheumatic disorders (140, 141). Regarding CHB, maternal and fetal genetic susceptibility has been investigated firstly by a Finnish group (142-145), which suggested that the disease-associated HLA alleles were potentially linked, in mothers, with the specificity and/or the levels of autoantibodies, as well as with the competence of immune system to fight infections and, in children, with the specificity of antigen presentation. These

evidence prompted researchers in finding fetal susceptibility and protective genes that could explain the low recurrence rate of CHB (Table 2). A candidate-gene approach was applied by Clancy and colleagues (146) to investigate the fetal susceptibility to CHB of two known single-nucleotide polymorphisms (SNPs) in genes encoding the pro-inflammatory and pro-fibrotic cytokines TGFβ and TNFα. The authors revealed that the SNP in TGFβ was significantly more frequent in the 40 children with CHB than in their 31 unaffected siblings, whereas the TNFα SNP was found at an increased frequency in both affected and non-affected children in comparison with healthy controls. However, these findings have not been replicated yet in a larger cohort of CHB patients.

Several studies with different approaches found that the HLA region is a dominant genetic contributor to CHB development. In particular, a Genome-Wide Association Study (GWAS) of 116 Caucasian children with cardiac manifestations of NLS and of 3,351 healthy controls showed that the most significant associations with cardiac neonatal lupus were found in the HLA region and at location 21q22 (147). However, comparing CHB cases with healthy controls, CHB-specific traits in the MHC region were not distinguished from the maternal ones, who may have SLE, SS and, even if asymptomatic, are genetically and immunologically different from the general population. Thus, the association revealed may represent the genetic bias present in mothers, rather than the true fetal CHB-specific association. Fetal HLA alleles appeared to be determinants in CHB susceptibility also in an *in vivo* study by Strandberg and colleagues (114). Using different congenic rat strains and a Ro52 immunization model of heart block, it was shown that the generation of pathogenic anti-Ro52 antibodies is restricted by maternal MHC, whereas susceptibility to CHB development and fetal disease outcome in

anti-Ro52-positive pregnancies is regulated by fetal MHC. Other evidence in favor to the role of HLA region in CHB were provided in a recent genetic association study by Meisgen and colleagues (148). In a cohort of 86 Swedish families where CHB pregnancies occurred, it was reported that HLA-Cw*06 and HLA-DRB1*13 are protective alleles associated with CHB in children born to anti-Ro/SSA positive mothers, while HLA-DRB1*04 was found to confer susceptibility for the disease.

Finally, a recent study reported that also non-HLA regions may confer susceptibility to CHB. In particular, through a GWAS of >500,000 SNPs in a population-based cohort of families with CHB children, it was found that Auxilin, the putative tyrosine-protein phosphatase encoded by *DNAJC6* is a novel fetal susceptibility gene of CHB, with a decreased cardiac expression in presence of the disease risk genotype (149).

HLA-related studies

Associated HLA alleles/variants	Method	Population	Reference
***B8*, *DR3*, *DQ2* associated to mothers with CHB children**	Susceptibility alleles in mothers. Serological typing of *HLA-A*, *-B*, *-C*, *-DR* and *-DQ*	31 mothers with CHB children, 45 with SLE and 21 with primary SS and healthy children and 900 healthy controls	Julkunen *et al.*, 1995 (142)
***DQB1*03/04* more frequent in affected children**	Susceptibility alleles in siblings. Serological typing of *HLA-A*, *-B*, *-C*; DNA-based typing of *DRB1*, *DQA1*, *DQB1*, *DPB1*	6 CHB children and their 10 healthy siblings in 4 Finnish families	Siren *et al.*, 1997 (143)
***A1*, *Cw7*, *B8* and without *B15* associated to high risk of CHB**	Susceptibility alleles in mothers. Serological typing of *HLA-A*, *-B*, *-C*; DNA-typing of *DRB1*, *DQA1* and *DQB1*	45 mothers with CHB children and 32 mothers with healthy children	Siren *et al.*, 1999 (145)
***DQ1* alleles, *Cw3* associated to CHB**	Susceptibility alleles in children. Serological typing of *HLA-A*, *B* and *C*; DNA-typing of *DRB1*, *DQA1* and *DQB1*	24 children with CHB and 10,000 healthy controls (serological typing); 91 organ donors for DNA-based typing	Siren *et al.*, 1999 (144)

HLA region and 21q22	GWAS based on genotyping of 370,000 SNPs	116 caucasian children with CHB and 3351 healthy controls	Clancy *et al.*, 2010 (147)
Production of anti-Ro52 and fetal disease outcome regulated by MHC	Ro52-immunization in female rats with different MHC haplotypes	Four MHC congenic rat strains	Strandberg *et al.*, 2010 (114)
Susceptibility role for *-DRB1*04* and *-Cw*05*. Protective role for *-DRB1*13* and *-Cw*06*	Low-resolution *HLA-A*, *-Cw* and *–DRB1* allele typing and family-based analysis	86 families where CHB occured	Meisgen *et al.*, 2014 (148)
Non-HLA-related studies			
Associated gene/allele	**Method**	**Population**	**Reference**
None	Susceptibility alleles in siblings. DNA-based typing of *TNF* and *HSP70-HOM*	6 CHB children and their 10 healthy siblings in 4 Finnish families	Siren *et al.*, 1997 (143)
p.Leu10Pro in *TGFβ* more frequent in CHB children	Candidate-gene approach on two known SNPs: c.-308G>A in *TNFα* and p.Leu10Pro in *TGFβ*	88 children (40 with CHB), 74 mothers and 102 healthy controls	Clancy *et al.*, 2003 (146)
DNAJC6	GWAS and cardiac tissue-eQTL analysis on >500,000 SNPs	Population-based cohort of families: 92 CHB children and 256 relatives	Meisgen *et al.*, 2015 (149)

Table 2. Summary of papers published so far with aim of investigating genetic susceptibility of CHB

1.4.2 Environmental risk factors

Several enviromental factors have been suggested to contribute to the development of CHB. Although neither fetal gender nor maternal disease severity has been associated with the disease (12, 25), it has been proposed that maternal age may have an influence over the outcome in anti-Ro52-positive pregnancies with an increased risk for CHB with increasing maternal age (150). Also in a later study by Ambrosi and colleagues (8), it was found that the risk for CHB outcome increased with maternal age in a population-based study. In the same study an association between the development of CHB with the season of bitrh was been described. Infact, there was an increased proportion of affected pregnancies in

which the gestational susceptibility weeks 18–24 occurred during the months from January to March. These months are also associated to lower levels of vitamin D in a sample of Swedish women, suggesting a correlation between CHB and winter season, reduced sun exposure and vitamin D levels. However, other events linked to the winter season, like viral infection in the woman during pregnancy, may be addressed to the onset of CHB. Indeed, in a report by Tsang and colleagues (151), cell surface exposure of the Ro antigen in fetal cardiomyocytes following cytomegalovirus infection has been described.

Another potential risk factor that has been reported in relation to CHB development include hypothyroidism in the mother. In particular, in a group of 87 anti-Ro/SSA-positive women, CHB occurred in 55% of hypothyroid group and in 13% of the normal thyroid function group. The risk for delivering a child with CHB in women with anti-Ro/SSA antibodies and hypothyroidism was found to be 9-fold higher than the risk associated to women with antibodies alone, irrespective of maternal condition (152). An association between hypothyroidism and CHB was also suggested by Askanase and colleagues (153) who found that anti-Thyroglobulin (anti-TG) antibodies were significantly more prevalent in mothers with children with CHB than in women with primary SS. It may also be speculated that environmental and lifestyle factors such as smoking could be potential risk factors, but studies of such a relation to CHB are currently lacking.

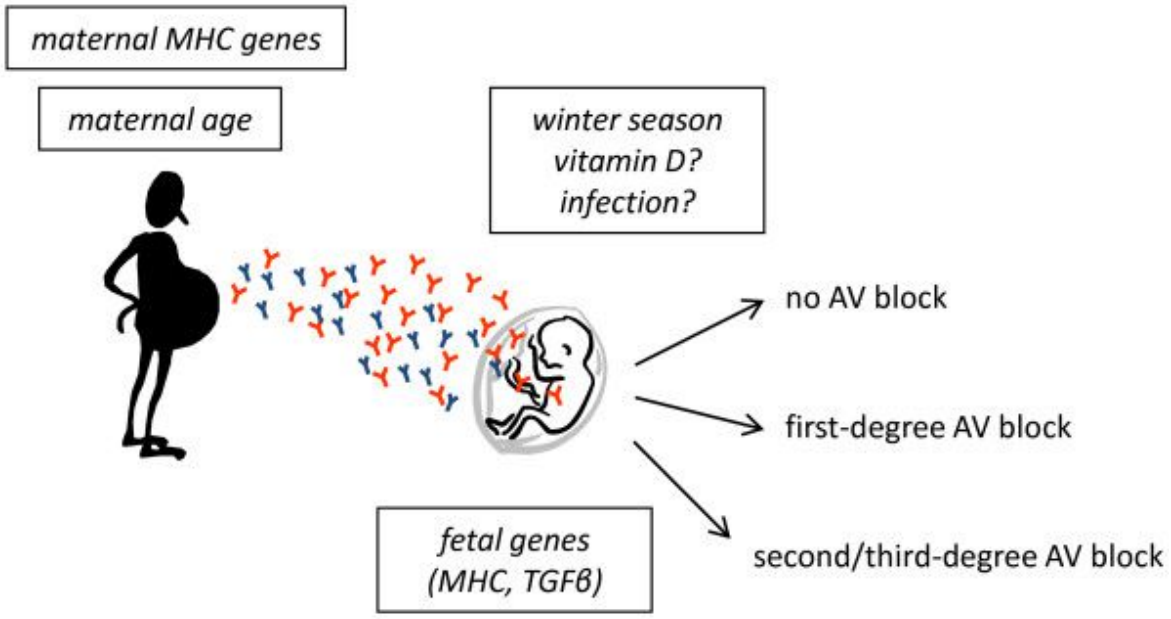

Figure 5. Schematic representation of risk factors in congenital heart block. (Image from: Ambrosi and Wahren-Herlenius *Arthritis Research & Therapy* 2012, 14:208).

2. Aim of the thesis

Part I: Identification of cross-reactive targets of anti-Ro52 antibodies

The aim of this study is to investigate the cross-reactivity target of anti- Ro52/p200 antibodies in the pathogenesis of CHB, and to identify cross-reactive proteins. This was performed by screening a library of peptides covering the whole human proteome and using monoclonal antibodies specific for the Ro52/p200 region. The putative cross-reactive targets are studied at a peptide level, in order to characterize the specific epitope, and on the whole antigen level, in order to determine the accessibility of the epitope to the antibodies.

Part II: Family-based study on HLA associations with CHB

The aim of this multi-center study is the identification of fetal susceptibility genes among the Human Leucocyte Antigen (HLA) locus in anti-Ro/SSA autoantibody-mediated CHB. DNA samples were collected from Swedish, Finnish, Norwegian and Italian families in which children affected from CHB were born. SNPs within the HLA region, the locus most significantly associated with rheumatic disorders, were genotyped and the transmission disequilibrium test was performed in order to identify unexpected parental HLA allele transmission to affected children not following the Mendelian rules.

PART I

IDENTIFICATION OF CROSS-REACTIVE TARGETS OF ANTI-RO52 ANTIBODIES

3. Materials and methods

3.1 Study population

Anti-SSA/Ro and/or anti-SSB/La-positive sera from 49 mothers of fetuses with CHB were selected from a population-based cohort of families with at least one individual affected by complete third-degree CHB (90). As control population, anti-SSA/Ro and anti-SSB/La-negative sera from 40 women without diagnosis of any rheumatic diseases were selected. Antibodies against Ro52, Ro60 and La were detected through a commercial line blot analysis (Inno-Lia™ ANA Update, Innogenetics, Cambereley, United Kingdom), according to the manufacturer's instructions. Anti-Ro52-positive sera were later analysed for reactivity to the p200 epitope of Ro52, using the Wieslab® SS-A p200 ELISA kit (Eurodiagnostica, Malmö, Sweden) according to the manufacturer's instructions. All analyses were performed at the Clinical Immunology Unit, Karolinska Institutet. The sera were stored at -80°C.

3.2 Generation of monoclonal antibodies

In this study several monoclonal antibodies specific for different regions of the Ro52 protein were used. As described by Strandberg and colleagues (60), these antibodies have been previously produced in mouse immunized with recombinant human Ro52 full length protein and with the hybridoma technique.

In particular, two antibodies specific for the p200 region, 7.2H4 (Ab11) and 7.8C7.F10 (Ab31), and monoclonal binding Ro52 but not specific for p200, 7.2F4 (Ab8), were used for this study (Figure 6).

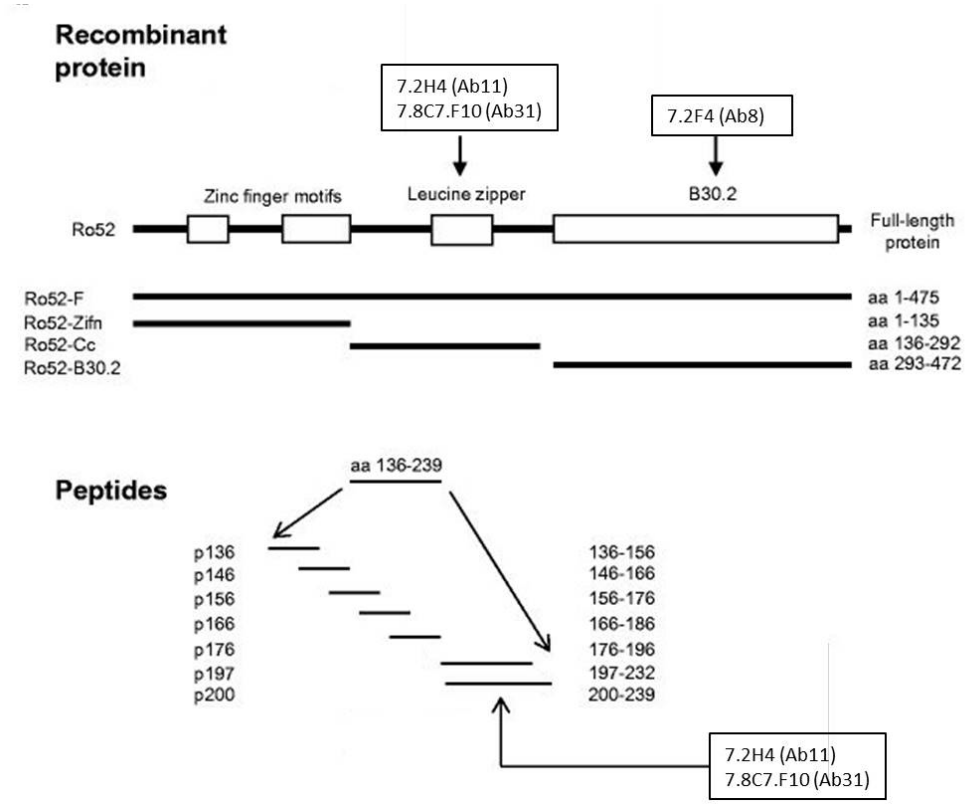

Figure 6. Schematic drawing summarizing the Ro52 binding specificity of the 3 mouse monoclonal antibodies used in this study.

3.3 Whole proteome microarray

The identification of cross-reactive targets of anti-Ro52/p200 antibodies was performed through screening a whole proteome microarray in collaboration with SciLifeLab, KTH, Stockholm, Sweden (154). This technique is based on an *in situ* peptide array synthesis and subsequent antibody binding analysis (Figure 7). The whole human proteome array was designed based on the human Consensus CDS (version 37.1) protein set provided by the National Center for Biotechnology Information (NCBI) (155). To cover the proteome, 2.1 million 12-mer peptides overlapping by six amino acids were randomly distributed on the array.

The peptide library synthesis was accomplished by means of light-directed array synthesis in a Roche-Nimblegen Maskless Array Synthesizer using an amino-

functionalized microscope slide as a substrate coupled with six-amino hexanoic acid as a spacer and amino acid derivatives carrying a photosensitive 2-(2-nitrophenyl)propyl-oxycarbonyl group at the α-amino function. Coupling of amino acids to the spacer was done with pre-activation in 30 mM amino acid, 30 mM activator (HOBt/HBTU), and 60 mM ethyldiisopropylamine in N,N-dimethylformamide. Washings were done with 1-methyl-2-pyrrolidinone, and site-specific cleavage of the photosensitive protector group was accomplished by irradiation of an image created by a Digital Micromirror Device (Texas Instruments Inc., Dallas, TX), projecting light with a 365-nm wavelength. Final treatment of the slide with TFA/water/triisopropylsilane for 30 min cleaved off the side-chain protection of the amino acids. Repeated cycles of selective activation, addition of amino acids, and removal of excess amino acids enables parallel synthesis of peptides with unique sequences.

After peptides synthesis, the slides were washed twice with TBSTT (20 mM Tris, 0.9% NaCl, pH 7.4, 0.1% Tween 20, 0.4% Triton X-100) for 2 min, twice in TBS (20 mM Tris, 0.9% NaCl, pH 7.4) for 2 min, rinsed quickly three times with de-ionized water, and dried. Primary antibody specific for p200 region of Ro52 (Ab31) diluted in binding buffer (10 mM Tris, 0.45% NaCl, pH 7.4, alkali soluble casein 0.5% (Novagen, EMD Chemicals, San Diego, CA)) was injected to the array. The slides were incubated overnight in a NimbleGen Hybridization Station (Roche NimbleGen Inc.). After the primary incubation, the slide was washed twice with TBSTT and twice with TBS as described above. Secondary Cy3-conjugated anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA) were diluted to 0.15 µg/ml in binding buffer and incubated to the slide for 3 h on a shaking table. The slide was washed twice with TBSTT and twice with TBS as described above,

quickly rinsed three times in de-ionized water, and dried. The slide was subsequently scanned at 2-µm resolution using a NimbleGen MS200 scanner (Roche NimbleGen Inc.).

The scan images were aligned and peptide feature mean fluorescence values were exported using the NimbleScan2 software (Roche NimbleGen Inc.). Before further analysis, confirmed false-positive signals caused by dirt on the arrays were removed.

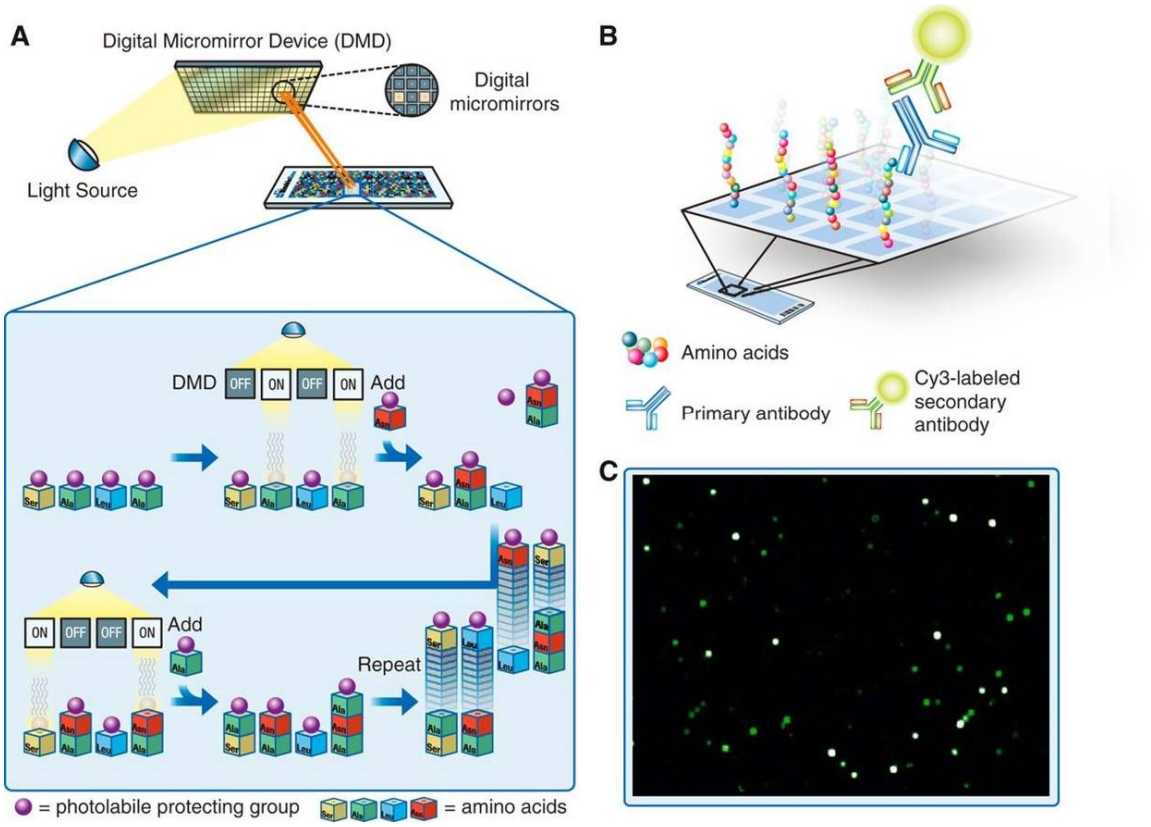

Figure 7. Schematic representation of the whole-proteome microarray that was performed for the identification of cross-reactive targets of anti-p200 antibodies. (A) Depiction of peptide library synthesis by means of selective activation of square features on the array through light reflected by digital micromirrors. (B) Schematic picture of incubation of the peptide array with the primary antibody and fluorophore-labeled secondary antibody. (C) A scan image of a part of the peptide array in which the bright spots correspond to peptide features bound by antibodies (Image from: Forsström B *et al*., *Mol Cell Proteomics.* 2014;13:1585-97)

3.4 Synthetic peptides and proteins

Five different synthetic peptides were used in this study and their sequences are illustrated below:

- p200: LEKDEREQLRILGEKEAKLAQQSQALQELISELDRRCHSS
- pGAK: PEIIDLYSNFPI
- pTG: SQYPGSYSDFST
- biotinylated pGAK biotin-aminohexanoic acid-YRTPEIIDLYSNFPI
- biotinylated pTG biotin-aminohexanoic acid-GQLSQYPGSYSDFST

Briefly, human p200 peptide corresponding to amino acid 200-239 of the human Ro52 protein and containing the main CHB related epitope was included and was purchased from Thermo BioSciences, Ulm, Germany. PGAK and pTG have the same protein sequence of the peptides used in the whole proteome microarray. Biotinylated pGAK and biotinylated pTG contain the same protein sequence of pGAK and pTG, respectively, and are conjugated to a biotin molecule through a linker aminohexanoic acid at the N-terminal molecule. These peptides were purchased from TAG Copenhagen A/S (Frederiksberg, Denmark). Purity was confirmed to be >90% by high-performance liquid chromatography and mass spectrometry.

3.5 Confirmation of cross-reactivity to peptides

3.5.1 Competitive ELISA

Competitive ELISA with a pre-incubation step was performed to investigate the cross-reactivity of anti-Ro52 monoclonal antibodies to the selected peptides. High-binding 96-well plates (Nunc, Odense, Denmark) were coated with 1 µg per well of

recombinant full-length Ro52, p200 or maltose binding protein (MaBP) and diluted in carbonate buffer, pH 9.6. When Ro52 or MaBP were the antigens, plates were coated overnight at +4°C; when p200, coating was performed overnight at room temperature and for at least 24 hours at 4°C before use. Plates were blocked with phosphate buffered saline (PBS)/0.05% Tween/5% milk powder for 1 hour. Monoclonal antibodies (mAbs) and sera were tested at a dilution of 0.1µg/ml and 1:1,000, respectively, in PBS/0.05% Tween/1% milk powder. Bound antibodies were detected by affinity-purified, alkaline phosphatase (AP)-conjugated anti-mouse or anti-human IgG antibodies (Dakopatts, Glostrup, Denmark). Phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.8, were used as substrate and incubated for 30 minutes for detection of IgG. The absorbance was measured at 405 nm. Between each step, plates were washed four times with wash buffer (0.25M NaCl, 0.05% Tween/1 liter of de-ionized water). All steps were performed at room temperature except coating, which was performed at 4°C. Sera from healthy subjects lacking anti-Ro52 antibodies and mAb8, not specific for p200 region, were used as controls, and showed an optical density (OD) of <0.1 in all readouts. All mAbs and sera were tested for antibodies to the MaBP fusion partner of the recombinant Ro52, showing OD values <0.1 in all cases. All mAbs and sera were run in duplicate.

Cross-reactivity of mAbs and sera to the peptides was analysed in anti-p200 and anti-Ro52 competitive ELISA with a pre-incubation step. In this phase diluted mAbs or sera were incubated with serial dilution of p200, pGAK, pTG, and MaBP in concentrations ranging from 0.025-0.1 mg/ml for 1h at room temperature prior to analysis in ELISA as described above. Incubation with only PBS/0.05% Tween/1% milk powder was also performed.

3.5.2 Biotinylated-peptides ELISA

Pierce™ Streptavidin Coated High Capacity 96-wells plates (Thermo Fisher Scientific, Waltham, Massachusetts, United States) were coated overnight at room temperature with 1µg per well of biotinylated peptides diluted in Tris-buffered saline (25mM Tris, 150mM NaCl; pH 7.2)/0.05% Tween/0.1% BSA. Monoclonal antibodies (mAbs) and sera were tested at a dilution of 5µg/ml and 1:300, respectively, in Tris-buffered saline/0.05% Tween/0.1% BSA and plates were incubated at room temperature for 2h. The experiment, then, proceeded as described above for anti-p200 and anti-Ro52 ELISA.

3.5.3 Anti-Thyroglobulin ELISA

The DIASTAT anti-Thyroglobulin (Tg) ELISA kit (Eurodiagnostica, Malmö, Sweden) was used to analyse the cross-reactivity to Tg of mAbs and sera according to the manufacturer's instructions with slight modifications. Monoclonal antibodies (mAbs) and sera were tested at a dilution of 5µg/ml and 1:100, respectively, in diluent buffer and plates were incubated for 1h at room temperature. Unbound antibodies were washed away with wash buffer (5 times). The experiment was then performed as described above for anti-p200 and anti-Ro52 ELISA

Cross-reactivity of mAbs and sera to the peptides were analysed in anti-Tg competitive ELISA with a pre-incubation step. In this step diluted mAbs or sera were incubated with serial dilution of p200, pTG, Tg and MaBP in concentrations ranging from 0.1-0.2 mg/ml for 1h at room temperature prior to analysis in ELISA as described above. Incubation with only diluent buffer was also performed.

3.6 Study of cross-reactivity to Cyclin G-associated kinase (*GAK*)

3.6.1 Transfection

HEK293T cells were cultured in 75cm^2 flask (Sarstedt AG & Co, Nümbrecht, Germany) with Dulbecco's Modified Eagle Medium (DMEM, Sigma), supplemented with 5% Fetal calf serum (FCS), 2mM L-glutamine, 500U/ml penicillin, 0.01mg/ml streptomycin, 1mM Hepes (Sigma) and 10mM Sodium Pyruvate (Sigma). Sixteen hours before transfection, 4 x 10^6 cells were seeded per well in 6-wells plates (Sarstedt). Transfection was performed in duplicates with the following plasmids: pGAK-tGFP containing human *GAK* (Origene Technologies©, Rockville, Maryland, United States), pGFP-Ro52 with Ro52, pGFP-DNAJC6 containing DNAJC6 (NM_001256864). All the genes represented human isoforms with full-length, recombinant sequence tagged with GFP at the N-terminal, except GAK, which was fused with the isoform turboGFP (tGFP) at the C-terminal. Cells were transfected with also plasmid containing only GFP (pGFP). One µg of plasmids was transfected 100µl of pure DMEM and 3µl X-tremeGENE™ 9 DNA transfection reagent per each well. Negative control transfection was also performed by adding the transfection mix without DNA. Twenty-four hours after transfection, fluorescence emission was checked with a fluorescence microscope to check the efficacy of transfection. Cells were, then, washed with PBS and brought in suspension, centrifuged for 5' at 500 x g at room temperature to pellet them.

3.6.2 Protein sample preparation

After centrifugation, pellets containing cells were lysed with 100µl of lysis buffer constituted by Cell lytic M cell Lysis reagent (Sigma) and 1% of Halt Protease and Phosphatase inhibitor cocktail 100x (Thermo Fisher). The mixture was kept for 15' in agitation and then centrifuged for 15' at 15 x 10^3 rpm to pellet cellular debris. All these steps were performed at +4°C. Supernatant containing protein was saved and protein concentration was measured using DC protein Assay (Bio-Rad Laboratories AB, Hercules, California, U.S.A.) following the manufacture's recommendations and using a standard curve composed of 6 serial dilutions in PBS of BSA in known concentration ranging from 2mg/ml to 0.125mg/ml. Briefly, 25µl of activated reagent A and 200µl of reagent B were added to 5µl of 1:10 pre-diluted samples or standards or PBS only in duplicate to 96-well plates. Absorbance was read at 650nm after 15' of incubation. Proteins were, then, denaturated by boiling for 5' in 5% sodium dodecyl sulfate (SDS) sample buffer.

3.6.3 SDS-page and Western-blot

Twenty µg of proteins from each transfection experiment lysate were loaded on 4%-12% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Proteins were transferred by electrophoresis onto nitrocellulose membranes and blocked for 1 hour with PBS/0.05% Tween/5% milk powder at room temperature and in agitation. Thereafter, filters were incubated overnight at +4°C with Ab31, patient sera, diluted to 1µg/ml or 1:500, respectively, in PBS/0.05% Tween/1% milk powder. Mouse monoclonal anti-tGFP (Origene) and rabbit anti-GFP (Life Technologies) diluted to 1µg/ml and to 2µg/ml respectively, were used to detected the fusion protein GFP. To detect bound antibodies, membranes were incubated for 1 hour at room temperature with anti-human, anti-mouse or anti-rabbit IgG

antibodies HRP-conjuated (Dakopatts), diluted 1:2,000 in PBS/Tween/1% milk powder. As loading control detection of the housekeeping protein β-actin was revealed with a mouse anti-β-actin HRP-conjugated antibody diluted 1:20,000 in PBS/Tween/1% milk powder. Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, United Kingdom) was used as substrate to reveal bands in correspondence of bound antibodies. Membranes were washed in PBS/0.05% Tween between each step and all incubations were done in agitation.

3.7 Statistical analysis

Comparison of antibodies values between anti-Ro52-positive and anti-Ro52-negative population was performed with the non-parametric Mann-Whitney U-test. Fisher's exact test was used to compare the frequency of antibody positivities between the two groups. All the analysis were done using GraphPad Prism V.5.01 (GraphPad Software, San Diego California, USA). A p-value <0.05 was considered significant.

3.8 Bioinformatic tools

Clustal Omega (University College of Dublin, Dublin, Ireland, http://www.ebi.ac.uk/Tools/msa/clustalo/,) is a multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. This tool was used to align the sequence of the cross-reactive peptides.

The Basic Local Alignment Search Tool (BLAST), (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi) is an algorithm that finds regions of local similarity between sequences. The program compares nucleotide or protein

sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. Specifically, BLASTp, was used in this study to analyze the sequence similarity between two proteins.

The Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do) is a crystallographic database for the three-dimensional structural data of large biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray crystallography, NMR spectroscopy, are freely accessible on the Internet (156). This tool was used to get the protein structure of GAK (PDB ID: 4C57) and to check where the epitope sequence is localized.

4. Results

4.1 Cross-reactive peptides revealed by the whole proteome microarray

To identify cross-reactive targets of anti-Ro52/p200 antibodies a screening on 2.1 million 12-mer peptides covering the human proteome using the monoclonal antibody Ab31 was undertaken. In the whole proteome microarray, anti-p200 monoclonal antibodies bound significantly to 17 peptides. The sequence of each peptide with the corresponding bar of intensity of cross-reactivity of Ab31 is depicted in Figure 8.

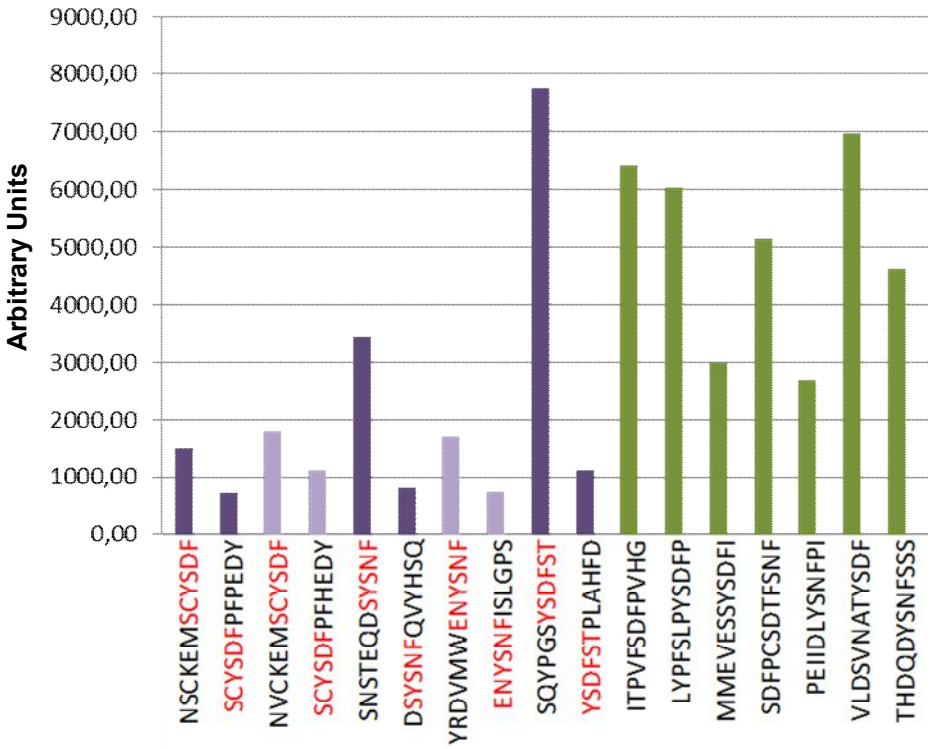

Figure 8. Graph representing the intensity of binding of monoclonal Ab31 to the 12-mer peptides. Purple bars correspond to pairs of six amino acid-overlapped peptides. Green bars correspond to sequences that are solely bound.

Aligning the amino acid sequence of the cross-reactive peptides to the human protein databases with blastp it emerged that they were contained in 12 different proteins. In particular, 7 peptide sequences were found uniquely in 7 proteins, while the remaining 10 ones were mapped in 5 proteins, as the monoclonal Ab31 bound pairs of adjacent and overlapped by 6 amino acids peptides (Table 3). Each pair of overlapped cross-reactive peptides allowed identification of a cluster of minimal epitopes of cross-reactivity shown by Ab31 binding these amino acid sequences:

- "SCYSDF": "Ser-Cys-Tyr-Ser-Asp-Phe"
- "DSYSNF": "Asp-Ser-Tyr-Ser-Asn-Phe"
- "ENYSNF": "Glu-Asn-Tyr-Ser-Asn-Phe"
- "YSDFST": "Tyr-Ser-Asp-Phe-Ser-Thr"

PROBE_SEQUENCE	Minimal epitope	Protein
NSCKEMSCYSDF	SCYSDF	FMO1
SCYSDFPFPEDY	SCYSDF	FMO1
NVCKEMSCYSDF	SCYSDF	FMO4
SCYSDFPFHEDY	SCYSDF	FMO4
SNSTEQDSYSNF	DSYSNF	C3orf63
DSYSNFQVYHSQ	DSYSNF	C3orf63
YRDVMWENYSNF	ENYSNF	ZFP14
ENYSNFISLGPS	ENYSNF	ZFP14
SQYPGSYSDFST	YSDFST	TG
YSDFSTPLAHFD	YSDFST	TG
ITPVFSDFPVHG		NPC1
LYPFSLPYSDFP		MRPL4
MMEVESSYSDFI		PKIG
SDFPCSDTFSNF		LPIN1
PEIIDLYSNFPI		GAK
VLDSVNATYSDF		CDC20B
THDQDYSNFSSS		SLC39A12

Table 3. Table showing the protein sequence of the cross-reactive peptides and in which protein they are included. The first 10 sequences correspond to paired overlapped peptides that share a minimal epitope.

In an attempt to investigate if the cross-reactive sequences had a conserved motif and to refine the cross-reactive epitope, multiple alignment of the 17 sequences was performed with Clustal Omega. The alignment revealed that all these peptides shared the “YSDF” (Tyr-Ser-Asp-Phe) or “YSNF” (Tyr-Ser-Asn-Phe) amino acidic sequences (Figure 9).

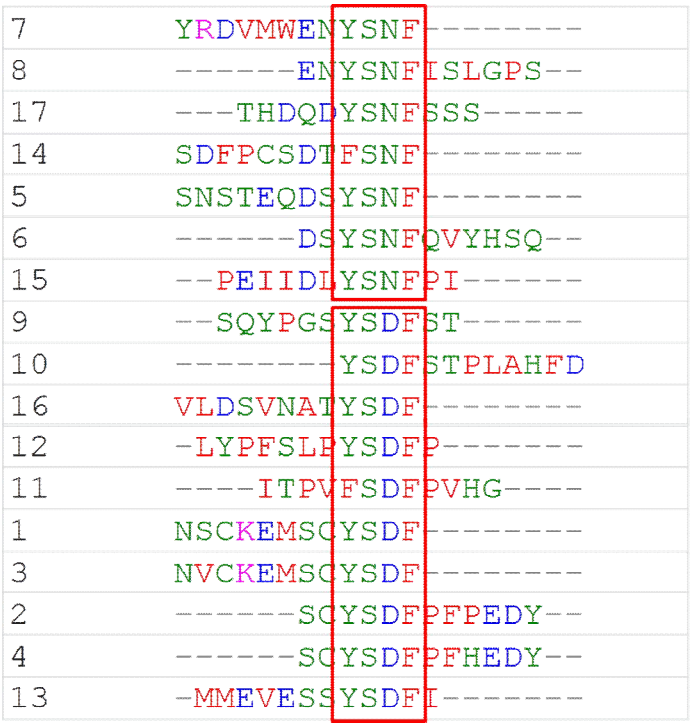

Figure 9. Representation of the multiple alignment of the cross-reactive peptides. Encircled in red are the two epitope sequences “YSNF” and “YSDF” shared by all the peptides.

4.2 Cross-reactivity to Thyroglobulin

Among the cross-reactive peptides, the one generating the strongest positive signal of binding by Ab31 was contained within Thyroglobulin (Tg). Notably, hypothyroidism has been associated with CHB (152). In order to investigate and confirm cross-reactivity to this target, ELISA coated with a biotinylated Tg peptide (pTG) was performed. When running monoclonal antibodies towards different regions of Ro52, it was found that Ab11 and Ab31, specific for the p200 region,

bound biotinylated pTG 3.1 - 2.7-fold stronger than Ab8, not p200 specific (Figure 10).

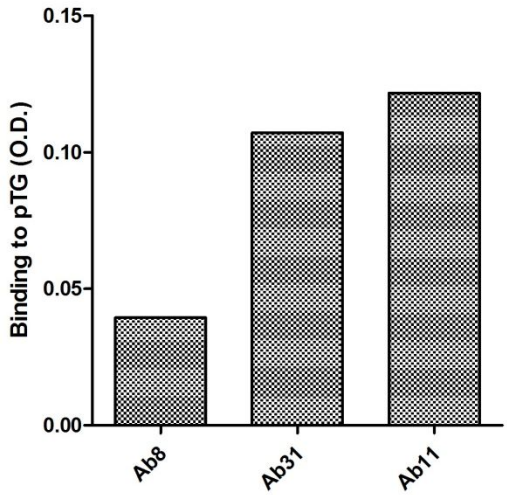

Figure 10. Graph illustrating the binding of monoclonal antibodies anti-Ro52 Ab8, Ab31 and Ab11 to biotinylated pTG.

After monoclonal antibodies, cross-reactivity to pTG was investigated also in sera from mothers who gave birth to babies with CHB. A cut-off value for a positive signal in the assay for binding to biotinylated pTG was established as the 95° percentile in sera from 40 anti-SSA/Ro and anti-SSB/La-negative women without any diagnosis of rheumatic disease. According to this, 24.4% of sera of mothers with CHB children were positive for binding to pTG, while only 5% of controls were, revealing a significant difference of frequency of positivities ($p=0.017$) and median (0.064 vs 0.023, $p<0.0001$) between the two populations (Figure 11 A and B).

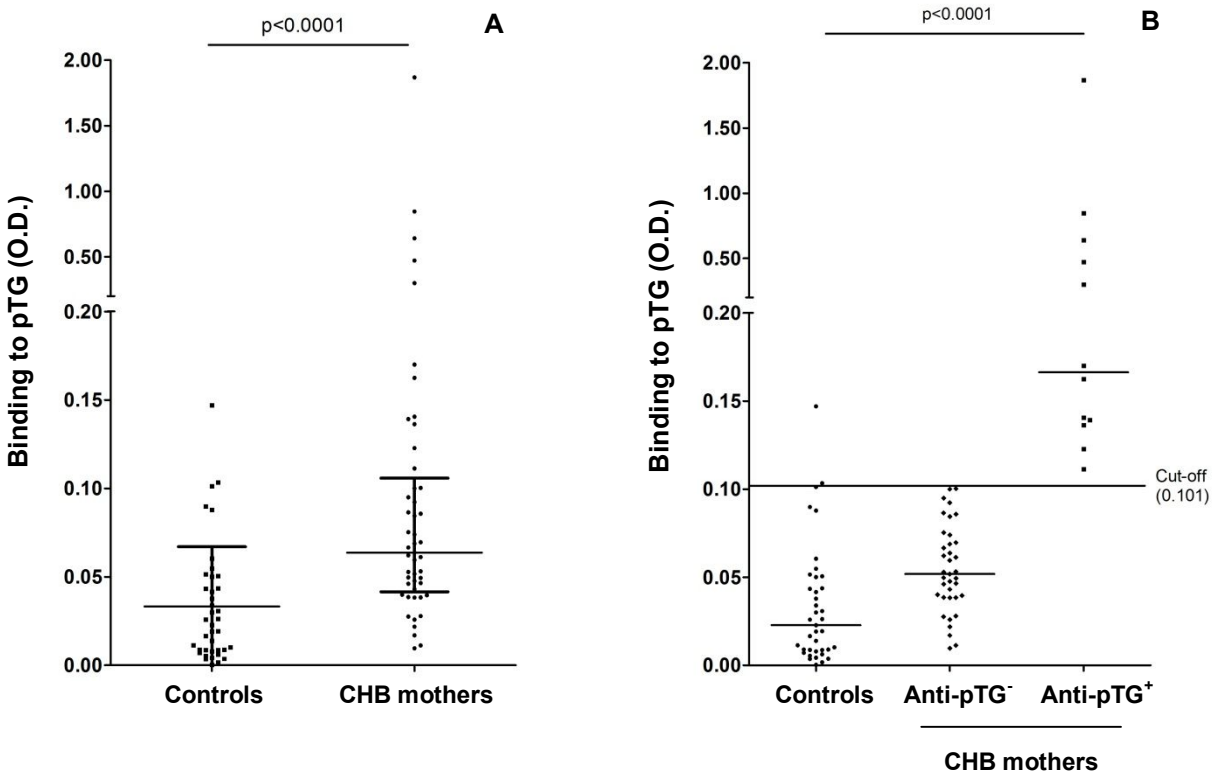

Figure 11. A: graph illustrating the distribution of value of cross-reactivity to pTG of the two population. B: representation of distribution of value of cross-reactivity to pTG with division of patients positive and negative for cross-reactivity to pTG.

After confirming the binding to pTG at a peptide level, we wanted to investigate if anti-p200 can bind also to the whole TG antigen. In order to do this, monoclonal anti-Ro52 antibodies and anti-p200-positive sera were analysed in anti-TG ELISA. The monoclonals Ab31 and Ab11, specific for the p200 region, bound TG and did so a 3.7 and 10-fold stronger than Ab8, which is not p200 specific. In contrast, monoclonal antibodies towards the intracellular antigens IRF-1 and IRF-5 were used as controls and did not bind TG (Figure 12 A). Five anti-p200 positive sera from mothers with CHB children which exhibited the highest cross-reactivity to biotinylated pTG, showed different reactivity to TG: from low (sera number 1, 3, 4), to high binding to TG (sera 2, 5) (Figure 12 B), indicating that the binding to the pTG epitope is not merely a reflection of TG autoantibodies.

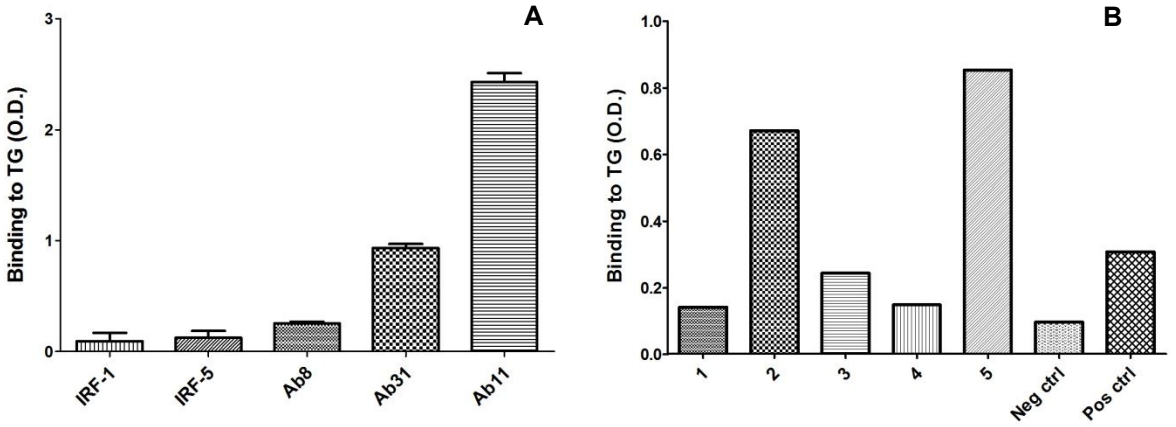

Figure 12. Graphs illustrating the binding of monoclonal antibodies (A) and sera (B) to TG.

To further investigate the specificity of cross-reactivity of anti-p200 antibodies to pTG, we performed competition experiments based on pre-incubation of monoclonal antibodies with pTG followed by testing their binding to TG and p200 in ELISA. For this experiment, Ab11 was chosen as it was the one showing highest reactivity to biotinylated pTG (Figure 10). Incubation of Ab11 with pTG decreased the binding to TG by up to 53.5% in a concentration-dependent manner, compared with the pre-incubation with buffer only. Incubating Ab11 with the specific 0.1mg/ml and 0.2mg/ml of p200 instead reduced the binding to 10% and 0.5%, respectively (Figure 13). Incubation of Ab11 with pTG also decreased the binding to p200 with up to 14% in a concentration-dependent manner, compared to the pre-incubation with only buffer (Figure 13). In all the experiments, incubation with MaBP, used as a negative control, did not inhibit the binding of antibodies to the target in ELISA.

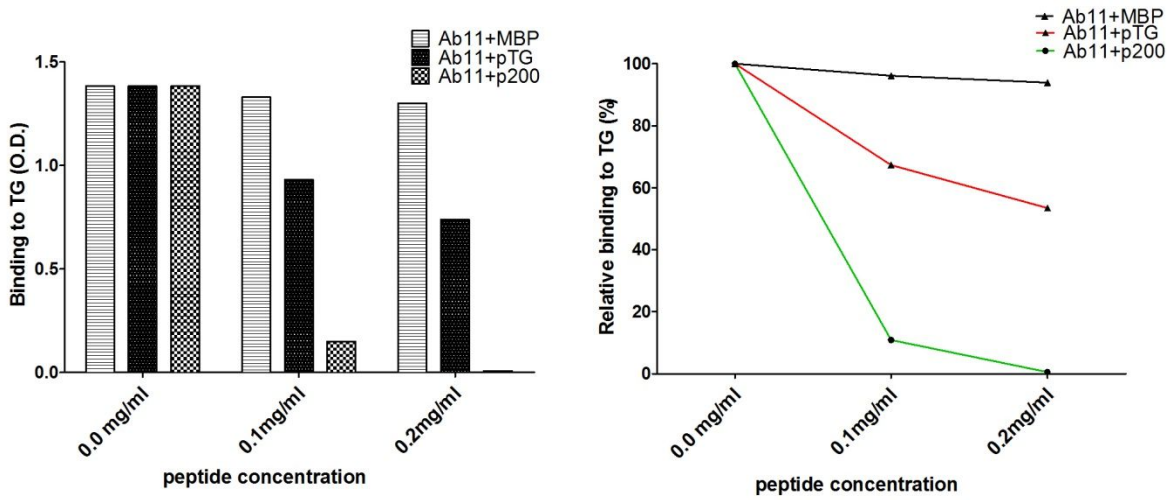

Figure 13. Graphs illustrating the binding of Ab11 to TG after incubation with different concentration of p200, pTG and MaBP.

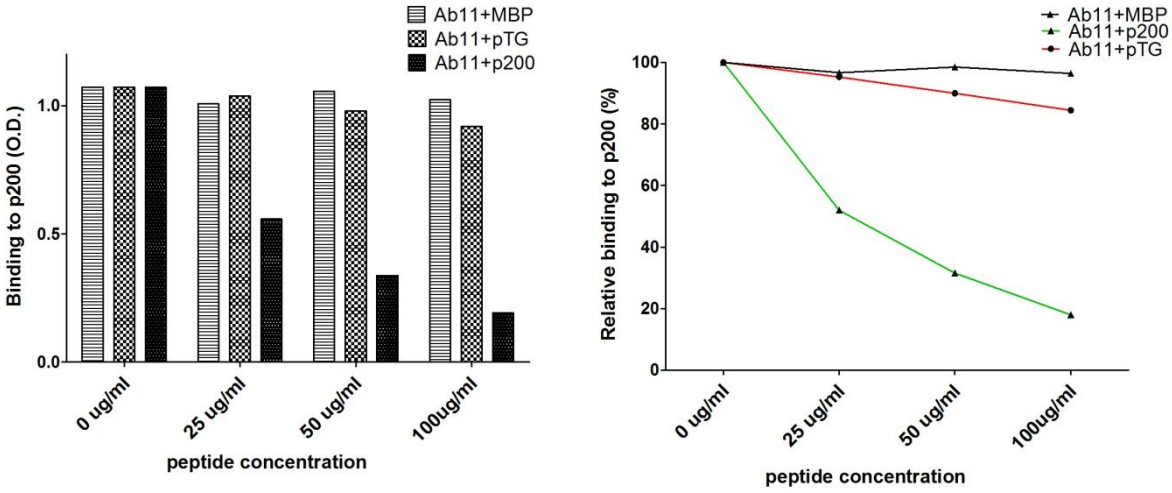

Figure 14. Graphs illustrating the binding of Ab11 to p200 after incubation with different concentration of p200, pTG and MaBP.

In order to assess if reactivity to TG shown by anti-p200-positive sera (Figure 12B) was due to presence of anti-TG antibodies and to investigate the specificity of cross-reactivity to TG, competition experiments with a pre-incubation step, as for monoclonal antibodies, were performed. Pre-incubation of sera with pTG reduced the binding to TG up to 30% in a concentration-dependent manner (Figure 15 A). Specificity of cross-reactivity to TG by anti-p200 antibodies was revealed when sera, incubated with p200, showed a decreased binding to TG up to 47% in a

concentration-dependent manner (Figure 15 B). These data, together, indicate that reactivity to TG was due partly to the presence of anti-TG antibodies, but also to cross-reactivity of anti-p200 antibodies.

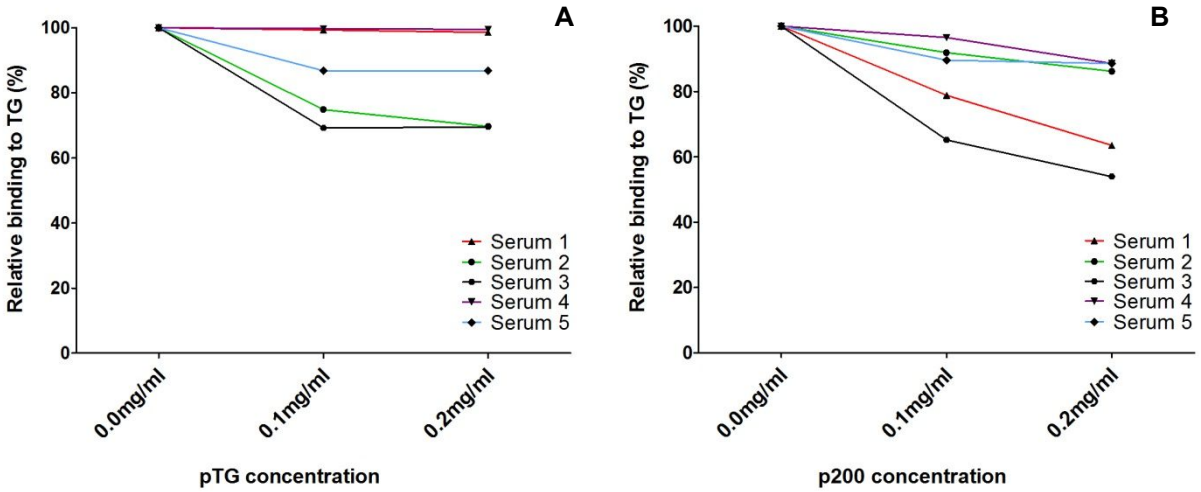

Figure 15. Graphs illustrating the binding of sera to TG after incubation with different concentration of, pTG (A) and p200 (B).

4.3 Cross-reactivity to GAK

4.3.1 Analysis at peptide level

Among the peptides bound by the monoclonal Ab31 in the whole proteome microarray, we wanted to investigate the cross-reactivity to the peptide included in GAK/Auxilin2 (*Cyclin G Associated Kinase*). In fact, this gene shares highly similarity to DNAJC6/Auxilin (*DnaJ heat shock protein family (Hsp40) member C6*), which has been recently indicated as a novel fetal susceptibility gene for CHB as well as functionally associated to CHB (149). In an attempt to confirm cross-reactivity to the peptide GAK (pGAK), ELISA coated with biotinylated pGAK was performed. When running monoclonal antibodies towards different regions of Ro52, it was found that Ab31 and Ab11, specific for p200 region, bound

biotinylated pGAK 7.3 – 7 fold stronger than Ab8, which is not p200 specific (Figure 16).

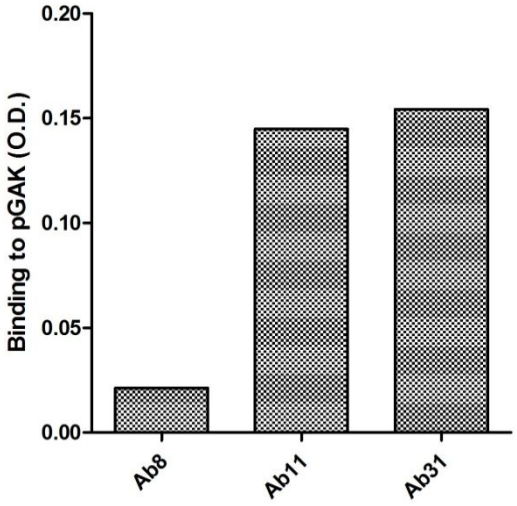

Figure 16. Graph illustrating the binding of monoclonal antibodies anti-Ro52 Ab8, Ab31 and Ab11 to biotinylated pGAK.

As for pTG, cross-reactivity to pGAK was investigated also in sera. The cut-off of reactivity to pGAK was found to be 0.132 O.D. According to this, 22.4% of sera of mothers with CHB children were positive for cross-reactivity to pGAK, while only 5% of controls were, revealing a significant difference of frequency of positivities ($p=0.032$) and median (0.081 vs 0.038, $p=0.0014$) between the two populations (Figure 17 A and B).

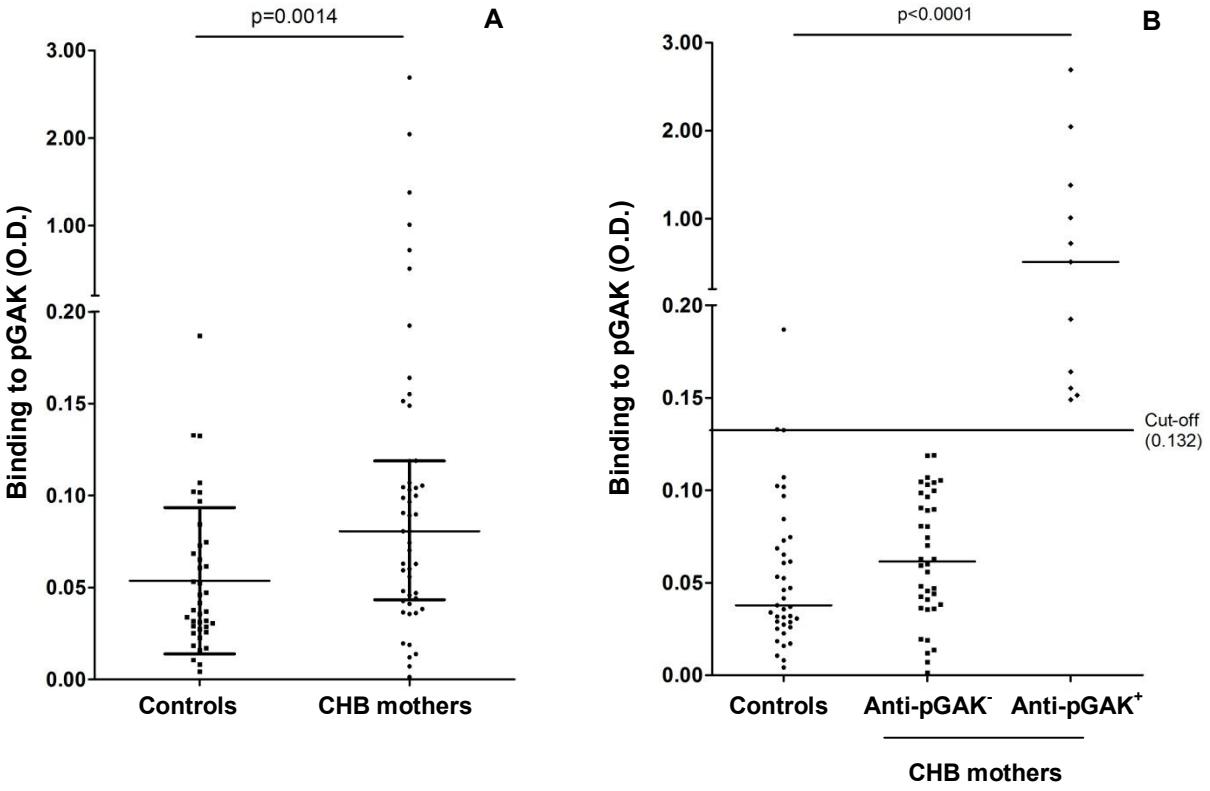

Figure 17. A: graph illustrating the distribution of value of cross-reactivity to pGAK of the two population. B: representation of distribution of value of cross-reactivity to pGAK with division of CHB mothers into two sub-population, according to positive and negative reactivity to pGAK.

To further investigate the specificity of cross-reactivity of anti-p200 antibodies to pGAK, competition experiments based on pre-incubation of monoclonal antibodies with pGAK followed by testing their binding to p200 and to Ro52 in ELISA were performed. For this experiment, Ab31 was chosen as it was the one showing the highest reactivity to biotinylated pGAK (Figure 16). Incubation of Ab31 with pGAK decreased the binding to p200 up to 21% in a concentration-dependent manner, compared to the pre-incubation with only buffer (Figure 18). Incubating Ab31 with pGAK reduced the binding to Ro52 up to 43%, compared to the pre-incubation with only buffer (Figure 19). In all the experiments, incubation with MaBP, considered as negative control, did not inhibit the binding of antibodies to the target in ELISA.

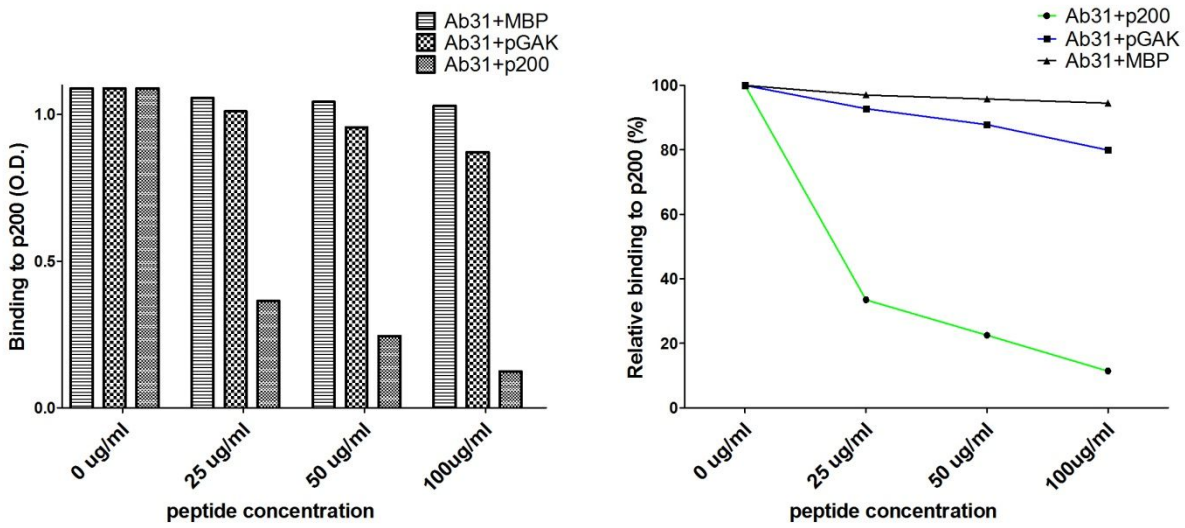

Figure 18. Graphs illustrating the binding of Ab31 to p200 after incubation with different concentration of p200, pGAK and MaBP.

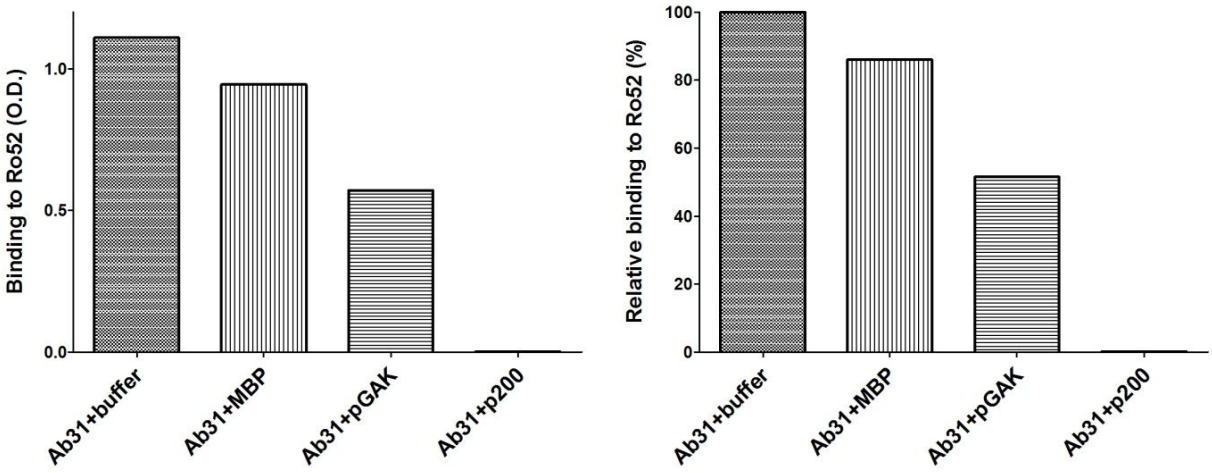

Figure 19. Graphs illustrating the binding of Ab31 to p200 after incubation with different concentration of p200, pGAK and MaBP.

Incubation of anti-p200-positive sera from mothers with CHB children with pGAK, instead, did not affect the binding to p200 in ELISA. However, incubation of the same sera with p200 as positive control, reduced the reactivity to p200 in ELISA only to 30% - 50%, indicating that the amount of antibodies present in the sera was still too high to be inhibited by pGAK.

4.3.2 Cross-reactivity at whole antigen level

After confirming the cross-reactivity to GAK at the peptide level, we wanted to examine if anti-p200 antibodies can also bind detectably to the whole GAK antigen, or rather if the specific epitope of cross-reactivity is exposed for binding of antibodies. First, analysis of the cross-reactive "YSNF" epitope was performed on the published 3D structure of the GAK protein based on the X-ray cristallography structure (157). This analysis revealed that the cross-reactive epitope is located in a flexible activation segment in the external side of the protein (Figure 20).

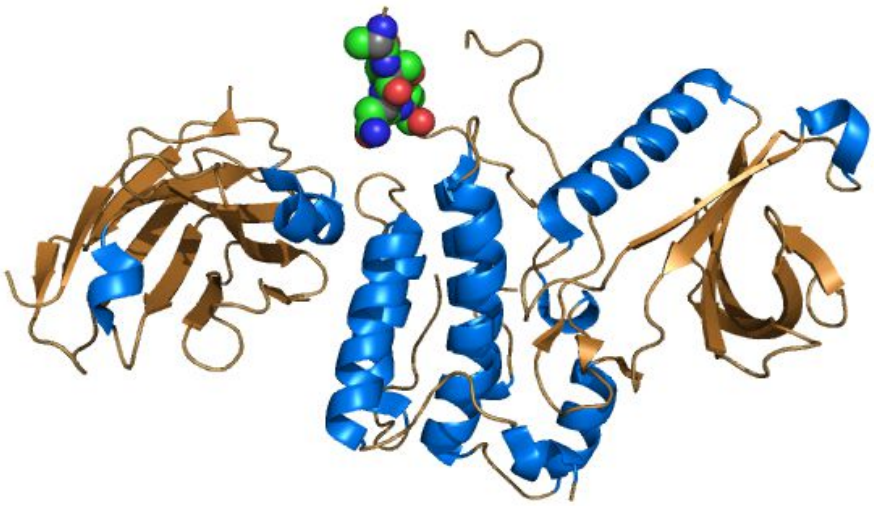

Figure 20. Three-dimensional structure of GAK, with representation of the cross-reactive epitope "YSNF" in circles.

In order to study if anti-p200 antibodies can cross-react to the whole GAK protein, overexpression of a cloned plasmid encoding recombinant tGFP-tagged GAK in HEK293T cells with following Western blot of protein lysates was performed. On blotted membranes, Ab31 bound to recombinant GAK, revealing a band at 169KDa, at the same level of the band recognized by anti-tGFP antibodies, directed to the fusion protein tGFP. This band appeared only in cells transfected with GAK, and not in non-transfected cells or control vector tranfected cells. Ab11, on the other hand, did not bind GAK in GAK-transfected cells, but rather revealed

unspecific bands at 150-160KDa in transfected and untransfected cells. As positive controls, Ab11 and Ab31 bound to the recombinant eGFP-Ro52 (81KDa) (Figure 21).

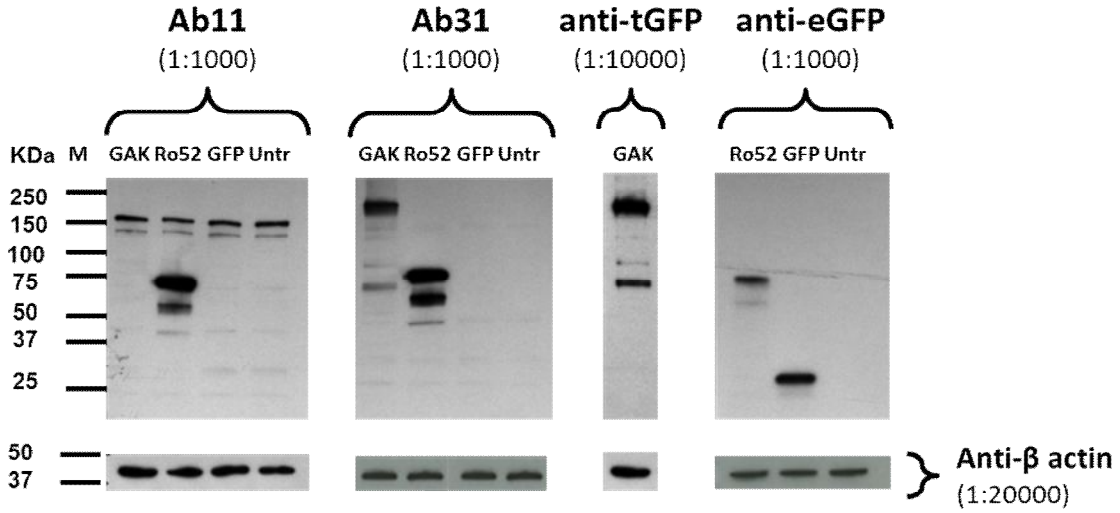

Figure 21. Western Blots to study cross-reactivity of Ab11 and Ab31 to recombinant GAK. On the left ladder with correspondent molecular weight; Braces indicates with which antibody and relative concentration the membranes were incubated. On the top of membranes loading order of cell lysates, with the correspondent overexpressed gene is reported.

In order to study the specificity of cross-reactivity of Ab31 to GAK, two Western blot membranes with GAK overexpressed lysates, previously incubated with Ab31 and anti-tGFP (Figure 22 A), were stripped of bound antibodies (Figure 22 B), and then incubated with the reciprocal antibodies (Figure 22 C). In this way it was seen that Ab31 and tGFP bound to recombinant GAK-GFP at the same molecular weight (169kDa).

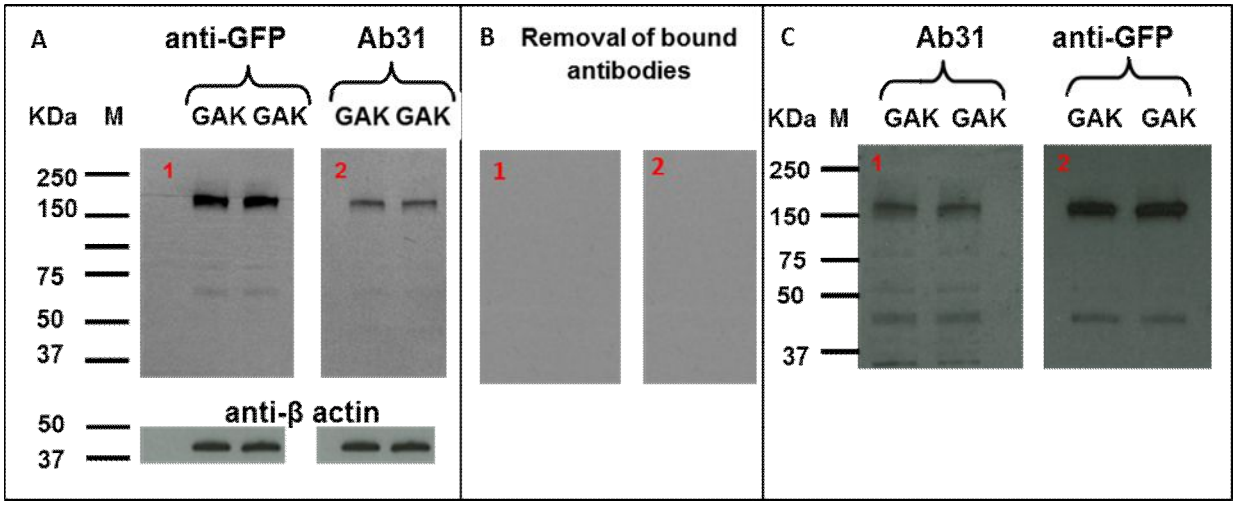

Figure 22. Western Blots to study specificity of cross-reactivity of Ab31 to recombinant GAK. On the left ladder with correspondent molecular weight of the bands. A: membrane 1 was incubated with anti-tGFP and membrane 2 with Ab31. B: bound antibodies were removed from membranes. C: Membrane 1 was incubated with Ab31 and membrane 2 with anti-tGFP.

Other than monoclonal antibodies p200-specific, binding to the whole GAK protein was also analyzed in 5 sera that showed the highest reactivity to pGAK in ELISA. After exposure three out of five sera bound to GAK with different strenght, revealing a band at the same level as the one revealed by the anti-tGFP antibody (Figure 23).

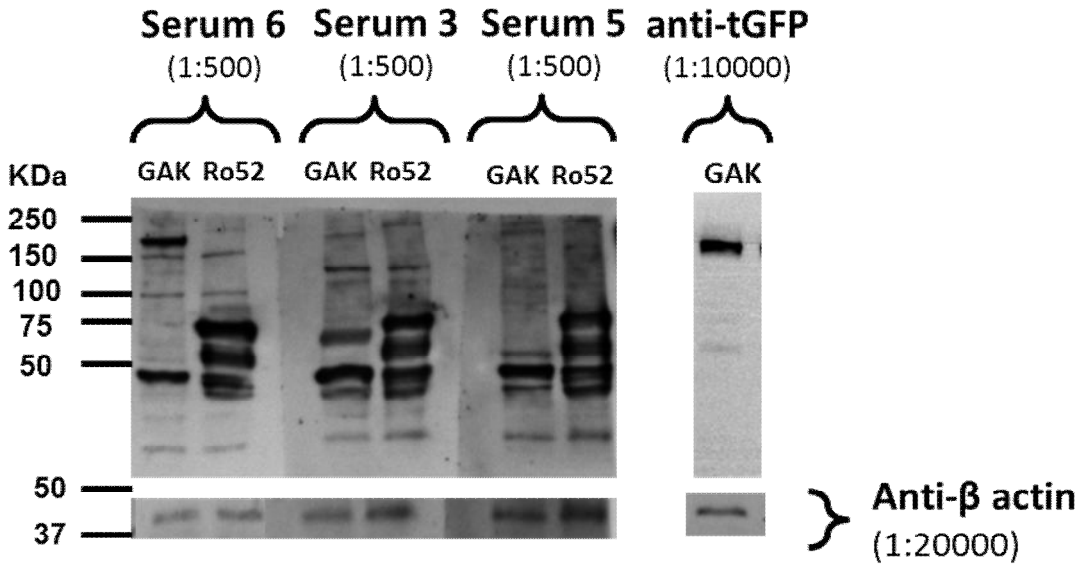

Figure 23. Western Blots to study specificity of cross-reactivity of sera to recombinant GAK.

4.3.3 Differential cross-reactivity to GAK and Auxilin

After confirming that the whole GAK antigen can be bound both by p200-specific monoclonal antibodies and by anti-p200-positive sera, we focused on the similarity of GAK with Auxilin. In particular we wanted to investigate if the cross-reactive epitope is present also in Auxilin and if the protein can be bound by anti-p200 antibodies. Aligning the two protein sequences it was observed that the two proteins have 58% of sequence homology but that the "YSNF" epitope is present only in GAK, in a protein kinase domain that is missing in Auxilin (Figure 24).

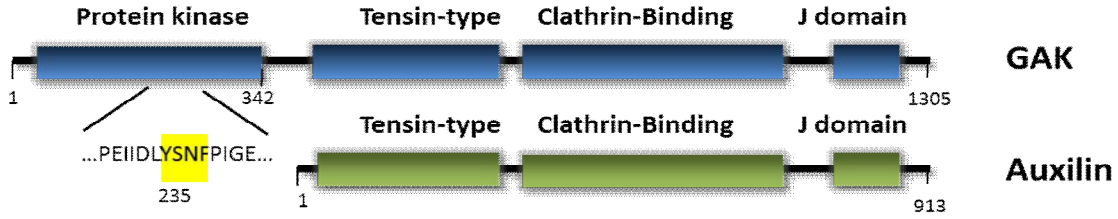

Figure 24. Representation of domain structure of GAK and Auxilin with focus on position of the cross reactive epitope, underlined in yellow.

According to the result of the protein alignment, the monoclonal antibody Ab31 did only bind to GAK in Western blot and not to Auxilin, indicating a specific cross-reactivity towards this protein (Figure 25).

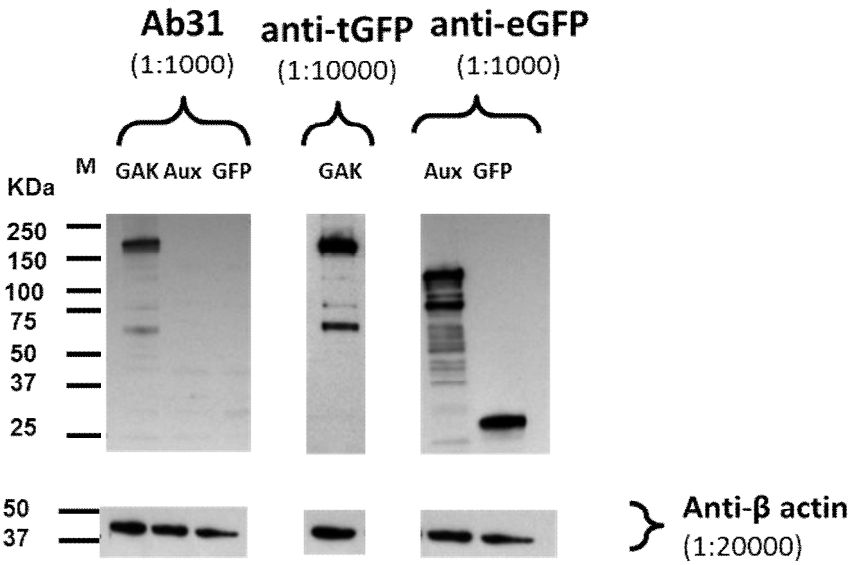

Figure 25. Western Blots to study the possible cross-reactivity to Auxilin.

5. Discussion

Association of anti-Ro/SSA and anti-La/SSB antibodies with CHB is well established (19-21), but the pathogenic mechanism of this disease is far from being completely elucidated (139). The intracellular localization of Ro52, Ro60 and La proteins and the lack of evidence that maternal antibodies can cross the sarcolemma of a normal cardiomyocyte and bind to the specific antigens are the biggest obstacle in understanding the molecular mechanism leading to CHB. Based on these fact, it has been proposed that anti-Ro52 antibodies may mediate their pathogenic effect on fetal hearts by cross-reacting with other target/targets available in the extracellular space. So far, several cross-reactive targets have been proposed, from the cardiac 5-HT4 serotoninergic receptor to L-Type and T-Type calcium channels and α-Enolase (123, 124, 130-133, 136). Even though each of the suggested cross-reactive target is supported by experimental findings, contradictory data from other studies have also emerged with regard to the same proteins (125, 126, 135, 138). Notably, none of them was confirmed to be a main cross-reactive target recognized by most of the sera analyzed from mothers with CHB-children. Furthermore, cross-reactivity of the subset of anti-Ro52 antibodies specific for p200 region, the major antigenic part of Ro52 molecule, has not been deeply investigated yet.

In this context a screening based on 12-mer peptides covering the whole proteome was undertaken using a monoclonal antibody specific for p200 (Ab31). Basing the screening on 12-mer peptides enabled antibodies to recognize only linear epitopes, that depend only on the primary sequence and are not based on the tertiary structure of the proteins. Including an overlap in the peptides by 6

amino acids also increased the chance for the antibodies to bind the targets and reduced the risk of the loss of epitopes at the extremities of one sequence. With this array, 17 peptides were found to be cross-reactive with Ab31. Interestingly, 2 repeated linear motifs were shared among these peptides, suggestive of consituting the epitope of cross-reactivity recognized by anti-p200 antibodies. The amino acid sequence of the two epitopes is similar between each other, “YSDF” (N term-Tyr-Ser-Asp-Phe-C term) and “YSNF” (N term-Tyr-Ser-Asn-Phe-C term). The only difference in the motif is the presence in the third position of an acid aspartic (Asp, D) or an asparagine (Asn, N), which diverge only by the presence of an amine group in the latter amino acid (Figure 26).

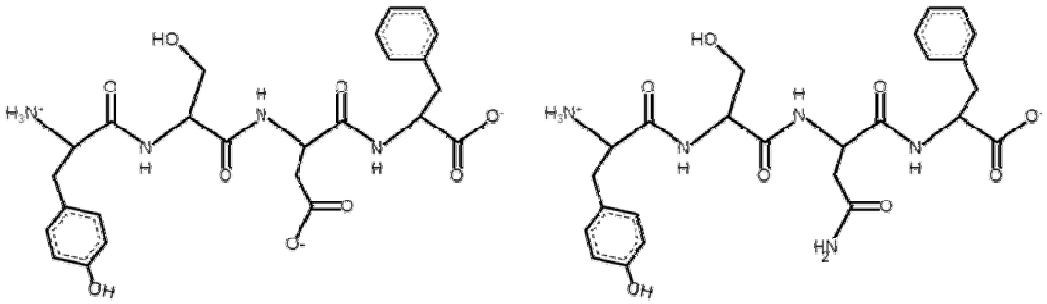

Figure 26. Primary protein structure of the two cross-reactive epitopes.

Among the cross-reactive targets, thyroglobulin was thought to be interesting from the pathogenic point of view, considering that hypothyroidism was previously revealed to be a risk factor for CHB development in anti-Ro/SSA-positive pregnancies (152) and anti-TG antibodies were found in 33% of mothers with CHB children (153). In the current study, reactivity to pTG was confirmed for the monoclonal antibodies to p200 (Ab11 and Ab31), and found to be positive in 24.4% of anti-p200-positive sera from mothers having children with CHB. In addition to reactivity at the peptide level, reactivity was also seen to the whole TG protein with Ab11 and in sera. Finally, competition experiments based on pre-

incubation of sera with pTG or p200 followed by testing their binding to TG in ELISA showed that reactivity to TG was due to both presence of anti-TG antibodies and specific cross-reactivity of anti-p200 to pTG. According to the previous findings where anti-TG antibodies were significantly more prevalent in CHB mothers than in women with primary SS (152), we here found significantly higher prevalence of reactivity to pTG in CHB mothers compared to the control group. As the p200 antibodies were able to bind the whole TG protein, it is likely that the cross-reactive epitope is located on the exterior of the molecule. Unfortunately, the three-dimensional structure of this protein is not known and we cannot confirm the exact spatial location of the epitope.

TG is a glycoprotein synthesized in thyrocytes (158) and plays an important role in the synthesis of thyroid hormones T3 and T4 (159). After synthesis, TG is transported and stored in the follicular lumen of the thyrocyte (160), where the tyrosine residues of TG undergo iodination to produce mono- (MIT) and di-iodotyrosines (DIT) catalyzed by thyroid peroxidase (161) and hydrogen peroxide (162). Subsequent coupling of these iodotyrosines produces T3 and T4 (163, 164). TG is pinocytosed into the thyroid cell (165) and undergoes proteolysis by lysosomes to release T3 and T4 (166), which are then secreted into the bloodstream (167). TG was also reported to be expressed in Follicular Dendritic Cells in the context of Thyroid Mucosa-Associated Lymphoid Tissue (MALT) lymphoma (168). Immunogenicity of TG is long known (169-171), and indeed it is the major autoantigen in both Hashimoto's and Grave's diseases (172). In this context, it would be interesting to find the connection between the higher prevalence of anti-TG antibodies in women with CHB pregnancy and cross-reactivity to TG of anti-p200 antibodies. Considering the limited expression of TG

to thyroid gland and to MALT lymphoma and that the etiology of anti-TG antibodies is not known, more studies are needed to unravel their biological involvement in the pathogenesis of CHB.

Other than TG, among the cross-reactive targets of the whole proteome microarray, cross-reactivity of anti-p200 antibodies was further investigated for the peptide included in GAK. The interest for this target arose because GAK shares 58% of sequence homology with Auxilin, recently reported as a novel fetal susceptibility gene genetically and functionally associated to CHB (149). In this study, binding to pGAK was confirmed with monoclonal antibodies binding p200 (Ab11 and Ab31) and found to be positive in 22.4% of anti-p200-positive sera from mothers having children with CHB. Analysis of the three-dimensional protein structure revealed that the cross-reactive epitope was located on the external side of the molecule and, hence, most likely accessible to be bound by antibodies. This was confirmed with western blot demonstrating that the monoclonal antibody Ab31 and sera from mothers with children affected from CHB bound to the whole GAK protein, but not Ab11. Further experiments would be needed to test if the reactivity to GAK seen in sera is generated by anti-p200 antibodies or by the presence of antibodies primarily specific for GAK. A possible approach would be the purification of anti-p200 antibodies from sera and analysis of their binding to GAK and/or the binding to p200 of the purified immunoglobulins after incubation with pGAK. Despite the high similarity between GAK and Auxilin, binding of anti-p200 antibodies was specific for GAK. This is not surprising as the cross-reactive epitope is not present in the latter. Cyclin G associated kinase (GAK) was first identified in experiments investigating proteins associated with cyclin G, a protein involved in cell cycle regulation (173). GAK (also known as Auxillin 2) is a 169 kDa

serine/threonine protein kinase that belongs to the numb-associated kinase (NAK) family. GAK is a key regulator of clathrin-mediated trafficking in both the endocytic and secretory pathways. It recruits clathrin and clathrin adaptor protein complex 2 (AP-2) to the plasma membrane (174) and stimulates its binding to cargo proteins and enhances cargo recruitment, vesicle assembly, and efficient internalization (174-177). Moreover, GAK regulates endocytosis of receptors mediated by alternate clathrin adaptors (174) and is implicated in later steps of endocytosis, including regulation of clathrin-coated vesicles uncoating, which enables recycling of clathrin back to the cell surface (174, 176). GAK also plays an important role in regulating clathrin-mediated sorting events in the trans-Golgi network (174, 176). GAK is an important regulator of epidermal growth factor receptor (EGFR); it is known to promote EGF uptake (174) and may also function in receptor signaling (178). GAK has a central role in development, as $GAK^{-/-}$ mice died early in gestation, and also in adult viability, as conditional knock out of the gene in adult mice caused death of these animals (179). GAK is expressed ubiquitously with peaks of expression in testis, heart and skeletal muscle (180) and bears a strong homology (58%) to the protein Auxilin, but it has an additional serine/threonine kinase domain at the N-terminal, where the cross-reactive epitope is located. Auxilin is a brain- and heart-specific cofactor of heat shock cognate 70 (Hsc70) and plays a role in uncoating clathrin-coated vesicles by Hsc70 (181-183).

Meisgen and colleagues have recently found that Auxilin is a novel fetal susceptibility gene for CHB (149). In particular, the risk genotype is associated to a lowered expression of Auxilin in the fetal heart and deletion of the gene in mice caused calcium disturbance in cardiomyocytes and disregulation of calcium channel expression on the surface of the cells.

It is to note, though, that GAK is an intracellular protein, with localization to the nuclear membrane, to the filamentous network of the endoplasmic reticulum and associated to focal adhesion, (180, 184). The relevance of reactivity to GAK would need to be further investigated. It might be speculated that, in fetuses with the risk genotype for Auxilin, a prolonged calcium disturbance and an impaired clathrin-dependent traffic of vesicles might lead to exposure of GAK to the surface, where it would be available for binding by the maternal anti-Ro52 antibodies.

Since GAK and Auxilin are involved in the same cellular pathway of clathrin-mediated trafficking and Auxilin has already been functionally associated to CHB, it would be interesting to further investigate their simultaneous effect in the development of CHB. Correlation of the profile of reactivity to GAK in sera with the corresponding genotype of Auxilin could be done in anti-p200-positive women with CHB children. If a positive binding to GAK corresponds to the presence of the risk genotype for Auxilin in the same patients, this would support the association of an impaired clathrin-dependent traffic of vesicles with the development of the disease.

It is worth to note that the two targets analysed in this study, TG and GAK, were recognized differently by the two monoclonal anti p200 antibodies used. At peptide level, both antibodies Ab11 and Ab31 cross-reacted similarly. At the whole antigen level however, Ab11 bound TG more than 2-fold stronger than Ab31 did (Figure 12A), while only Ab31 was able to bind GAK. This difference can be due to the different techniques used to determine the binding to the two proteins, ELISA for TG and Western blot for GAK. However, it might be that the differential behaviour of the two monoclonal antibodies is target-specific. If this is confirmed, we can suggest that anti-p200 antibodies might have different targets of cross-reactivity, depending on which part of the p200 region they are specific for. If this concept is

extended to the whole specificities of anti-Ro52, anti-Ro60 and anti-La antibodies, we can speculate that several cross-reactive targets could exist and vary among each patients sera. According to this hypothesis, each cross-reactive target could account for a subset of patients. Consistently, in our study, positive reactivity to pTG and to pGAK was seen, respectively, for 24.4 and 22.4% of sera and also in the previous studies the putative cross-reactive targets haven't been recognized by 100% of sera analysed, leading to an intriguing scenario of several cross-reactive targets involved in the pathogenesis of the CHB. The recently developed platforms for profiling of autoantibody repertoires based on high-density protein microarray of recombinant proteins fragments (185, 186) could be an useful tool for determination of cross-reactivity profiles of different sera in order to identify common cross-reactivity targets or pathways involved in the pathogenesis of CHB.

In conclusion, in this study with a whole proteomic microarray 17 peptides were found to be significantly cross-reactive with a monoclonal antibody p200-specific (Ab31) and two linear motifs were shared ("YSDF" and "YSNF). Among the targets, cross-reactivity was further studied for TG and GAK, for which reactivity was shown either at peptide and at the whole protein level with anti-p200 monoclonal antibodies and with sera from mothers whose children have CHB. We propose that identification of reactivity to GAK or TG in sera of women with anti-p200 antibodies may represent a subset of patients at risk to have a fetus developing CHB during pregnancy. Additional studies are needed to confirm the functional relevance of cross-reactivity to these proteins with the development of CHB and to better assess their association with the clinical outcome and severity of the disease.

PART II

FAMILY-BASED STUDY ON HLA ASSOCIATIONS WITH CHB

6. Materials and methods

6.1 Study population

Patients and their unaffected first-degree relatives were selected from the Swedish cohort (90) and from collaborating European study sites in Finland, Norway (Bergen) and Italy (Padua and Rome). All patients included in the study were diagnosed with third-degree CHB. In total 119 Swedish, 38 Finnish, 2 Norwegian and 14 Italian families, comprising 636 individuals (160 mothers, 136 fathers, 173 cases and 167 unaffected siblings) were included. Detailed composition of families per Country is outlined in Table 4. For all families, maternal serum was available and was tested positive for anti-Ro52 antibodies (n=160). Further autoantibody profiling revealed n=127 (79.4%) mothers with anti-Ro60 and n=79 (49.4%) with anti-La antibodies. Amongst the 160 mothers 72 had a rheumatic diagnosis (24 with SLE, 37 with Sjögren's syndrome, 9 with undifferentiated connective tissue disease and 2 with other rheumatic disease), whereas for 88 there were no information available or no diagnosis of autoimmune disease.

The study was approved by the Regional Ethical Committees in Stockholm, Helsinki, Bergen, Padua and Rome. Informed consent was given from all individuals (or their parents if individuals were <18 years) enrolled in the study.

	Cohort				
n	Swedish	Norwegian	Finnish	Italian	Total
n Families	119	2	38	14	173
n Cases	119	2	38	14	173
n Fathers	91	1	31	13	136
n Mothers	109	1	37	13	160
n Unaffected Siblings	128	4	29	6	167
Total	447	8	135	46	636

Table 4. Description of composition and Country of origin of the families under investigation.

6.2 DNA preparation

Erythrocytes were lysed in EDTA blood samples by adding Red Blood Cell Lysis Buffer (160 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 50 mmol/L EDTA), and leucocytes collected by centrifugation at 1200 x g for 20 min at +4°C. Leucocytes were solubilized with 0.75% (w/v) SDS/ddH_2O, and proteolytic digestion performed by adding 10 mg/mL Proteinase K (Invitrogen, Stockholm, Sweden) followed by overnight incubation at 37°C. Saturated NaCl (6M at a 1:3 ratio) was added, and precipitated proteins and lipid fractions spun down at 6000 x g for 10 min at room temperature. DNA was precipitated with 99% ethanol and dissolved in 1 x Tris-EDTA buffer at 37°C. Photometric analysis of DNA concentration and purity was performed. DNA working solutions were diluted to a final concentration of 50 µg/mL and stored at -80°C.

6.3 HLA Imputation

Low-resolution genotyping of DNA samples of the studied population was performed using the Illumina Human OmniExpress 950K chip. Briefly, after amplification of the whole genome, specific allele detection was performed in two steps, in which, BeadChip probes hybridized with single strand DNA in correspondence of a particular SNP and polymerase-dependent single base extension with labeled nucleotide gave specificity of the allele of the SNP (Figure 27).

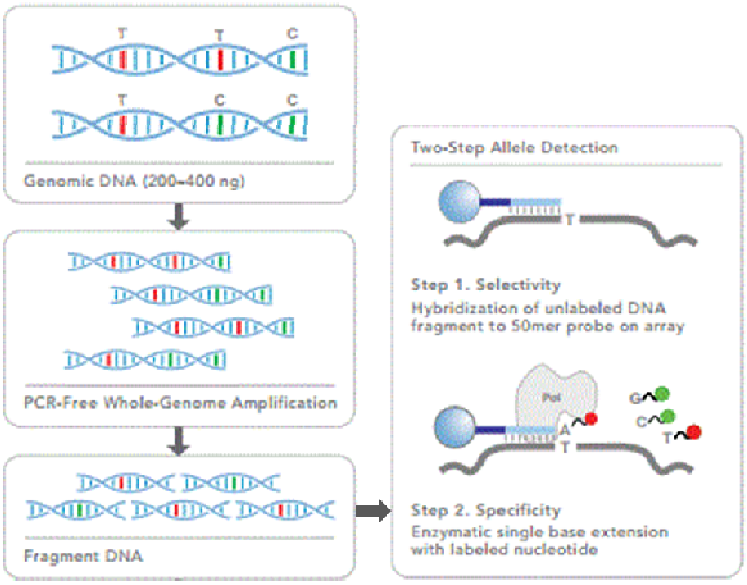

Figure 27. Workflow of the DNA genotyping performed with Illumina Human OmniExpress 950K chip.

HLA-A, -B, -Cw, -DPA1, -DPB1, -DQA1, -DQB1, -DRB1, -DRB3, -DRB4 and -DRB5 genotyping was performed by imputation based on 5,636 tag SNPs from the chromosome 6 using the HLA*IMP:02 software (https://oxfordhla.well.ox.ac.uk/hla/), and an updated software version including Swedish reference genotypes. After quality control, alleles with >0.98 imputation quality measures were included in the analysis. Unaffected siblings' genotypes were used to infer parental alleles when such information was missing. High-resolution genotyping of the HLA alleles significantly associated to CHB was, then, performed by DNA sequencing in order to identify specific sub-allele variants.

6.4 Statistical analysis

Statistical analyses in families were performed with the pedigree disequilibrium test (PDT) using Unphased (Version 3.0.13.) (187) selecting the "Full model" and "Test individual haplotypes" options for calculating transmission disequilibrium to cases, odds ratios (OR) and 95% confidence intervals (CI). P-values <0.05 were

considered significant and were further tested for statistical significance using Bonferroni correction. P-values denote deviation from 50% Mendelian transmission, whilst OR and CI calculations are based on risk allele frequencies in relation to all other allele frequencies as a reference and rely on the Unphased output of ratio between the estimated counts of a particular allele transmitted to cases and estimated number of the other alleles of the same gene transmitted to affected individuals. In particular, an OR>1 confers to a certain allele susceptibility to the disease outcome, whereas, if the OR<1 the allele is protective.

Parental transmission frequency calculations were carried out using the parent-of-origin tool in PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).

7. Results

7.1 Low resolution typed-HLA alleles associated with CHB

In the HLA class I region, genetic analyses with Unphased and PLINK identified HLA-Cw*06 as a protective allele associated with CHB (p=0.003, OR=0.22, 95% CI 0.07 to 0.67, table 5). Notably, maternal HLA-Cw*06 alleles were significantly less transmitted to affected children (p=0.006) compared to paternal alleles (non-significant), indicating that maternal regulatory effects in addition to the Cw*06 allele transmission may contribute to the observed association. As for the HLA-B gene locus, it was found that HLA-B*50 significantly associated with CHB (p=0.046, table 5), however this finding was not verified after Bonferroni correction. Regarding HLA class II, HLA-DRB1*13 was associated with protection from CHB (p=0.007, OR=0.47 95% CI 0.24 to 0.91, table 6). Interestingly, HLA-DRB1*13 parental transmissions were skewed with significantly fewer paternal transmission of the alleles to index cases (p=0.009).

For the HLA-DQ genes, associations with CHB were found for both gene loci, namely HLA-DQA1 and DQB1. HLA-DQA1*01 and HLA-DQB1*06 were found to have a protective effect for CHB development (p=0.0016, OR=0.52, 95% CI 0.28 to 0.95 and p=0.0036, OR=0.48, 95% CI 0.28 to 0.81, respectively, table 6), while HLA-DQA1*04 transmissions were associated with susceptibility (p=0.025, OR=1.25, 95% CI 0.56 to 2.79, table 6). Of note, paternal HLA-DQB1*06 allele transmissions were increased (p=0.038) while maternal transmissions did not deviate from the expected 0.5 transmission frequency, therefore suggesting an additional paternal influence in the reported protective effect.

None of the HLA-A, -DPA1, -DPB1, -DRB3, -DRB4, or -DRB5 alleles were significantly associated with CHB development (Supplementary Table 1).

Gene*allele	*P*-value	Transmission frequency to cases	OR	95% CI
HLA-B				
*07	0.362	0.44		
*08	0.849	0.52		
*13	0.414	0.43		
*14	0.257	0.29		
*15	0.547	0.53		
*18	0.819	0.43		
*27	0.655	0.43		
*35	0.106	0.63		
*39	1	0.45		
*40	0.317	0.58		
*44	0.090	0.61		
***50**	**0.046**	**0**	**0**	**0**
*51	0.549	0.46		
*55	0.739	0.56		
*57	0.059	0.17		
Remaining alleles §	0.131	0.34		
HLA-Cw				
*01	1	0.53		
*02	0.655	0.43		
*03	0.323	0.56		
*04	0.052	0.63		
*05	0.056	0.61		
***06**	**0.003**	**0.22**	**0.22**	**(0.07-0.67)**
*07	0.369	0.48		
*08	0.096	0.22		
*12	0.366	0.67		
*14	1	0.56		
*15	0.617	0.44		
*16	0.564	0.46		
*17	0.655	0.60		
*18	0.317	0		

Table 5. HLA-B and HLA-Cw allele association to CHB with corresponding p-value and parental transmission frequency to cases. OR and 95% CI are reported only for alleles with p-value<0.05. § Alleles *37, *38, *41, *42, *45, *47, *48, *49, *52, *56 and *58 displayed low individual prevalence and were clustered together

Gene*allele	*P*-value	Transmission frequency to cases	OR	95% CI
HLA-DQA1				
***01**	**0.016**	**0.42**	**0.52**	**(0.28-0.95)**
*02	0.058	0.41		
*03	0.074	0.64		
***04**	**0.025**	**0.63**	**1.25**	**(0.56-2.79)**
*05	0.237	0.56		
*06	0.157	0		
HLA-DQB1				
*02	1	0.50		
*03	0.108	0.59		
*04	0.058	0.60		
*05	0.786	0.52		
***06**	**0.004**	**0.39**	**0.48**	**(0.28-0.81)**
HLA-DRB1				
*01	0.578	0.52		
*03	0.472	0.52		
*04	0.248	0.58	1.25	(0.66-2.37)
***07**	**0.037**	**0.39**	**0.55**	**(0.27-1.14)**
*08	0.077	0.60		
*09	0.103	0.83		
*10	1	1		
*11	0.450	0.64		
*12	0.366	0.58		
***13**	**0.007**	**0.29**	**0.47**	**(0.24-0.91)**
*14	0.478	0.63		
*15	0.251	0.44		
*16	0.480	0.38		

Table 6. HLA-DQA1, -DQB1 and -DRB1 allele association to CHB with corresponding p-value and parental transmission frequency to cases. OR and 95% CI are reported only for alleles with p-value<0.05.

7.2 High resolution typed-HLA allele variants associated with CHB

To further investigate if particular HLA class II sub-alleles are associated with CHB, high resolution HLA-typing using the imputation method was performed. For the HLA-DQA1*01, we found DQA1*01:02 and DQA1*01:03 sub alleles protectively associated (p=0.043, OR=0.67, 95% CI 0.43 to 1.04 and p=0.019, OR=0.37, 95% CI 0.15 to 0.91, respectively, table 7) while HLA-DQA1*04:01 associated with susceptibility for CHB (p=0.025, OR=1.60, 95% CI 0.82 to 3.10, table 7).

As for the HLA-DQB1*06 allele, we found variants DQB1*06:03 and DQB1*06:04 associated with CHB in a protective manner (p=0.033, OR=0.41, 95% CI 0.15 to 1.09 and p=0.033, OR=0.43, 95% CI 0.16 to 1.14, table 7).

Variant HLA-DRB1*13:01 was protectively associated with CHB development (p=0.0495, OR=0.45, 95% CI 0.17 to 1.23, table 7), while HLA-DRB1*13:02 and HLA-DRB1*13:03 became not significant (table 7). However, such analysis could not be performed for the rest of the significantly associated alleles since only HLA-DRB1*07:01 and HLA-Cw*06:02 variants were found within the study population.

Gene*allele	*P*-value	Transmission Frequency to cases	OR	95% CI
HLA-DQA1				
*01:01	0.537			
***01:02**	**0.043**	**0.41**	**0.67**	**(0.43-1.04)**
***01:03**	**0.019**	**0.28**	**0.37**	**(0.15-0.91)**
*02	0.058			
*03	0.074			
***04:01**	**0.025**	**0.63**	**1.60**	**(0.82-3.1)**
*05	0.237			
*06	0.157			
HLA-DQB1				
*02	1			
*03:01	0.276			
*03:02	0.182			
*03:03	0.808			
*04:02	0.058			
*05	0.786			
*06:02	0.192			
***06:03**	**0.033**	**0.27**	**0.41**	**(0.15-1.09)**
***06:04**	**0.033**	**0.28**	**0.43**	**(0.16-1.14)**
*06:rest	0.655			
HLA-DRB1				
*01	0.578			
*03	0.473			
*04	0.248			
***07:01**	**0.037**	**0.39**	**0.55**	**(0.27-1.14)**
*08	0.077			
*09	0.102			
*10	1			
*11	0.450			
*12	0.366			
***13:01**	**0.0495**	**0.30**	**0.45**	**(0.17-1.23)**
*13:02	0.117	0.31	0.53	(0.22-1.25)
*13:03	0.317	0.25	0.23	(0.02-2.44)
*14	0.480			
*15	0.251			
*16	0.480			

Table 7. HLA-DQA1, -DQB1 and -DRB1 sub-allele variants association to CHB with corresponding p-value and parental transmission frequency to cases. OR and 95% CI are reported only for alleles with p-value<0.05.

7.3 Analysis of DRB1-DQA1-DQB1 haplotype in association with CHB

Exploring the relationship of specific DRB1-DQA1-DQB1 haplotypes with the CHB family cohort under investigation, we performed a DRB1-DQA1-DQB1 haplotype association analysis with CHB development. In line with the single allele associations, we found the DRB1-DQA1-DQB1 13-01:03-06:03 haplotype significantly associated with protection from CHB development (p=0.025, OR=0.38, 95% CI 0.11 to 1.30, Supplementary Table 2). Moreover, haplotype DRB1-DQA1-DQB1 08-04:01-04:02 is significantly associated with CHB susceptibility (p=0.022, OR=1.55, 95% CI 0.58 to 4.20, Supplementary Table 2).

8. Discussion

Transplacental transport of maternal anti-Ro52 autoantibodies is critical for CHB development. However, the low recurrence rate of CHB in subsequent gestations despite persistent maternal autoantibodies has turned researchers towards investigating the contribution of fetal factors in disease outcome. The strong link between the MHC locus and autoantibody mediated diseases provided the conceptual basis for a series of studies attempting to find genetic associations between CHB and specific alleles in the fetal MHC locus (143, 188). Emerging GWAS data (147) identified several polymorphisms within the MHC locus of children with cardiac manifestations of neonatal lupus associated with CHB development, substantiating influence of the fetal MHC locus in disease outcome. Additional in vivo findings further supported this notion by demonstrating that fetal MHC genetic constitution was a determining factor of CHB in a rat model (114). Meisgen et al recently identified novel susceptibility and protection conferring fetal alleles in the context of a transmission disequilibrium study in families with 3rd degree CHB cases (148).

Herein, replication of these findings was sought in a cohort encompassing the 86 Swedish families participating in the study by Meisgen et al. with the addition of 33 Swedish, 2 Norwegian, 38 Finnish and 14 Italian families. Enrollment of families from different European countries would also help overcome limitations associated with regional effects (eg. environmental factors, population stratification) that could stochastically be mirrored in the calculated transmission frequencies and ensure that our findings reflect true genetic associations rather than casual findings

biased by regional confounders. Furthermore, additional associations with CHB outcome in the extended MHC locus complex were explored.

In the genetic analyses, we corroborated previous findings that HLA-A allele transmission does not seem to play any significant role in disease outcome. In concordance with studies documenting a dominant effect of the HLA-Cw locus in CHB occurrence (144, 148), the HLA-Cw*06 allele was found to have a pronounced protective effect on disease outcome since allele transmission to affected children is significantly reduced. This finding appears in line with reports of the previous study. Notably, inclusion of more families added power to that finding as indicated by an even lower p-value in the present study (0.003 versus 0.022 in the previous one).

Numerous reports have linked specific HLA-DRB1 alleles with a variety of autoantibody induced diseases. Accordingly, we confirmed the strong protective effect conferred by DRB1*13 transmission. In line with this, decreased prevalence of DRB1*13 has been described in the context of several autoimmune diseases including anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis (189) and a subtype of myasthenia gravis (190), as well as part of a protective haplotype for type 1 diabetes in an Iranian population (191). High resolution typing revealed that the variant conferring protection for CHB outcome was DRB1*13:01, whilst subtypes DRB1*13:02 and*13:03 were below the level of significance. On the contrary, HLA-DRB1*04 association with CHB was not confirmed in the expanded European cohort. This is potentially due to the fact that the addition of families with a different ancestry eliminated a population stratification effect that yielded the observed statistical significance in our previous study.

The present study further revealed a panel of novel genetic associations between HLA alleles and fetal susceptibility to CHB. Among them, HLA-B*50 allele transmission was observed to convey protection, however, low number of informative pedigrees for this specific allele (5 unaffected individuals, 0 cases) in conjunction with a marginally significant p-value decreased the power of this observation and does not allow for safe conclusions. Of note, HLA-B*50 has been additionally reported as a protection-conferring allele for Henoch-Schonlein purpura which is a common form of childhood vasculitis (192).

Analysis of the MHC class II genetic region provided additional CHB associated alleles. HLA-DQA1*01 allele transmission was significantly decreased in children with CHB. High resolution typing singled out variants DQA1*01:02 and *01:03 contributing to the observed protective effect. Only a few genetic analyses have associated these variants with autoimmune disease outcome so far. Interestingly, DQA1*01:02 was also under-represented in celiac disease children (193), suggesting a protective function of the variant in autoimmunity. Contrarly, allele DQA1*04:01 was more frequently transmitted to CHB affected fetuses. The only association observed within the DQB1 gene locus is the joint protection conferring effect of DQB1*06:03 and *06:04 allele variants. It is noteworthy that a similar protective effect is conveyed by these two allele variants also in type I diabetes mellitus in Arab populations (194). Similarly, HLA-DRB1*07 transmission frequency was also found to be significantly decreased in affected children (HLA-DRB1*07:01 was the only observed variant in our cohort). Consistent with this observation HLA-DRB1*07 has also been reported to be significantly less frequent in patients with Grave's disease (195) and multiple sclerosis in a meta-analysis study (196).

The association of specific DRB1-DQA1-DQB1 haplotypes with NLE (197) and several other autoimmune diseases (198-202) are long reported in the literature. This haplotype transmission was also investigated in this study and two DRB1-DQA1-DQB1 haplotypes were identified to be significantly associated with CHB development. In particular, transmission of the haplotype DRB1*08-DQA1*04:01-DQB1*04:02 was markedly increased in CHB patients predominantly reflecting the strong susceptibility conferring influence of DQA1*04:01 allele variant. On the contrary, DRB1*13-DQA1*01:03-DQB1*06:03 haplotype transmission was significantly decreased in CHB patients. Intriguingly, this haplotype was also demonstrated to convey protection in the context of Hashimoto thyroiditis (203). Coherently, all three alleles of this particular haplotype were found to confer protection in this study.

Finally, our analysis revealed a parent-of-origin effect on transmission of CHB associated alleles. More specifically, maternal Cw*06 alleles were transmitted less frequently to affected children. Inversely, DQB1*06 and DRB1*07 displayed a markedly lower paternal transmission percentage than the respective maternal. Parent-of-origin influence has been suggested in the context of type 1 diabetes (204) and, more recently, for CHB, both in the human disease and in an animal model of the condition (114, 148). Such phenomena are considered to add an additional layer of epigenetic regulation in disease development.

According to this findings, it would be tempting to envision a condition whereby MHC molecules encoded by these genes bind and present specific epitopes that promote the resolution of inflammatory phenomena *in utero* that would otherwise lead to irreversible 3rd degree block, as reviewed by Ambrosi et al. (139). We can alternatively hypothesize that the products of such protective alleles essentially

present antigenic epitopes that are not recognized by maternal pathogenic autoantibodies. Consistent with this hypothesis is the observation that haplotype DRB1*13-DQA1*01:03-DQB1*06:03 comprises genes encoding both α and β DQ polypeptidic chains, thereby influencing both subunits responsible for HLA-DQ molecules. However, in order to strengthen such claims the molecular pathways of specific antigen presentation need to be further elucidated.

Taking into consideration that CHB can develop as fast as a single week following a normal echocardiographic examination, parental and/or fetal genotyping in anti-Ro52-positive pregancies might be a useful tool to assess the risk of CHB in the fetuses in order to plan a proper management of the pregnancy, especially between the susceptible weeks 18^{th} to 24^{th} of gestation.

9. Supplementary data

Gene*allele	*P*-value	Transmission Frequency to cases	OR	95% CI
HLA-A				
*01	1	0.48	1	(1.00-1.00)
*02	1	0.53	1.05	(0.64-1.73)
*03	0.816	0.45	0.965	(0.54-1.74)
*11	0.480	0.58	1.3	(0.59-2.86)
*23	0.083	0.75	1.071e+008	(1.071e+008-1.071e+008)
*24	0.480	0.25	0.814	(0.37-1.78)
*25	0.564	0.75	1.88	(0.16-21.82)
*26	0.467	0.42	0.811	(0.28-2.31)
*29	0.317	0.33	0.502	(0.12-2.08)
*30	1	0.56	1.077	(0.25-4.67)
*31	0.564	0.42	0.733	(0.22-2.44)
*32	0.439	0.60	1.513	(0.51-4.50)
*33	1	0.25	0.878	(0.05-15.51)
*68	0.602	0.53	1.234	(0.56-2.72)
HLA-DPA1				
*01	0.906		1	(1.00-1.00)
*02	0.906		1.03	(0.65-1.64)
HLA-DPB1				
*01	0.208		1	(1-1)
*02	0.777		1.21	(0.55-2.65)
*03	0.800		1.24	(0.59-2.59)
*04	0.652		1.35	(0.74-2.46)
*05	0.170		2.76	(0.76-9.93)
*09	0.157		0	0
*10	0.178		5.00	(0.53-47.33)
*11	0.564		3.10	(0.20-47.07)
*13	0.655		1.58	(0.20-12.65)
*14	0.706		1.04	(0.21-5.11)
*15	0.564		2.36	(0.21-27.18)
*16	0.564		3.25	(0.22-48.78)
*17	0.564		2.45	(0.21-28.15)
*19	1		1.35	(0.08-23.02)
*21 & *26	0.157		0	0
HLA-DRB3				
*01	0.710		1	(1-1)
*02	0.345		1.33	(0.73-2.41)
*03	0.239		0.67	(0.29-1.54)

N/A ¥	0.801	1.08	(0.74-1.58)
HLA-DRB4			
*01	1	1	(1-1)
N/A ¥	1	1	(0.66-1.53)
HLA-DRB5			
*01	0.210	1	(1-1)
*02	0.157	0.42	(0.08-2.16)
N/A ¥	0.096	1.37	(0.87-2.17)

Supplementary Table 1. HLA-A, DPA1, -DPB1, -DRB3, -DRB4 and –DRB5 allele association to CHB with corresponding p-value, parental transmission frequency to cases, OR and 95% CI. To note, none of these alleles are reached significance.

DRB1-DQA1-DQB1 Haplotype	*P*-value	OR	95% CI
01-01:01-05	0.578		
03-04-04:02	0.317		
03-05-02	0.531		
04-03-03:01	0.439		
04-03-03:02	0.237		
04-03-04:02	0.157		
07-02-02	0.131		
07-02-03:02	1		
07-02-03:03	0.058		
08-04:01-04:02	**0.022**	**1.55**	**(0.58-4.20)**
08-06-03:01	0.317		
08-06-04:02	0.317		
09-03-03:03	0.103		
10-01:01-05	1		
11-01:03-03:01	0.317		
11-05-03:01	0.336		
12-05-03:01	0.366		
13-01:02-06:03	0.317		
13-01:02-06:04	0.074		
13-01:02-06:rest	0.564		
13-01:03-06:02	1		
13-01:03-06:03	**0.025**	**0.38**	**(0.11-1.3)**
13-01:03-06:04	0.157		
13-05-03:01	0.564		
14-01:01-05	0.480		
15-01:02-05	0.564		
15-01:02-06:02	0.232		
15-01:02-06:03	0.317		
15-01:03-03:02	0.317		
15-01:03-06:rest	1		
15-03-05	0.317		
16-01:02-05	0.480		

Supplementary Table 2. DRB1-DQA1-DQB1 haplotype association analysis with CHB development. Each combination of alleles analyzed is reported with the correspondent p-value and OR and 95% CI if the haplotype reached significance.

10. Conclusions

The molecular mechanisms underlying CHB are yet unclear. Several genetic and enviromental risk factors are emerging as associated with the disease, making it difficult to generate an overall picture considering the complexity of this autoimmune disease. With the current thesis, we focused, firstly, on understanding more about the pathogenic mechanism leading to CHB by the identification of cross-reactive targets of anti-Ro52 antibodies. Secondly, we investigated genetic associations in the MHC locus to CHB in an European cohort of families in which children with CHB were born.

In the first part of the thesis, we found two linear epitopes shared by 17 cross-reactive peptides, and we confirmed cross-reactivity of anti-Ro52/p200 antibodies to GAK and TG. Notably, hypotyroidism in anti-Ro52-positive women has been associated with the development of CHB (152). Our findings suggest that there is a link between reactivity to TG in anti-p200-positive sera and hypotyroidism with CHB. In order to understand this connection clearly, more studies are necessary. Secondly, GAK, the second cross-reactive target, shares high homology with DNAJC6/Auxilin, which has recently been indicated as a novel fetal susceptibility gene for CHB as well as being functionally associated with CHB (149). Both these two proteins are involved in the clathrin-dependent trafficing of vesicles in the cell, revealing that this pathway might be involved in the development of the disease.

In the second part of the thesis, our genetic analysis identified HLA-Cw*06, -DRB1*13, -DQA1*01 and -DQB1*06 as protective alleles for CHB, while HLA-DQA1*04 transmission was associated with susceptibility to the disease. These alleles encode specific isoforms of the MHC receptors expressed intracellularly

(-Cw*06), or on the surface of dendritic cells, macrophages as well as B- and T-lymphocytes (-DRB1*13, -DQA1*01, -DQB1*06 and -DQA1*04), which are responsible for presenting specific epitopes for activation or resolution of an immune response.

Taking the findings of both studies into consideration, one may visualize a situation where the fetal HLA genes modulate the inflammatory response following the exposure to anti-Ro/SSA antibodies. In particular, MHC molecules encoded by the protective alleles could bind and present specific epitopes that promote the resolution of inflammation that would otherwise lead to irreversible atrioventricular block, as reviewed by Ambrosi et al. (139). Alternatively, the products of such protective alleles could present antigenic epitopes that are not recognized by maternal pathogenic autoantibodies. Fetal MHC proteins that confer susceptibility, on the other hand, could have a specific conformation of the epitope recognition site that enables the binding of sequences that are cross-reactive with maternal anti-Ro52 antibodies, allowing the initiation of an immune response which may lead to CHB. According to this hypothesis, cross-reactivity of maternal anti-Ro52 antibodies would be correlated with the fetal MHC protective or susceptibility-conferring allele repertoire. A recent study provided molecular evidence of cross-reactivity of anti-citrullinated antibody (ACPA) to citrullinated vinculin epitopes presented by the predisposing HLA-DQ5, -DQ7.3 and -DQ8 haplotypes in RA (205), showing an interplay between HLA haplotype and a cross-reactive autoimmune response.

In conclusion, CHB is emerging as a complex autoimmune disease where maternal anti-Ro/SSA and/or anti-La/SSB antibodies are necessary but not sufficient for the onset of the disease. CHB may develop in fetuses with a specific

MHC genetic background, potenitally favorable for the production and exposition of epitopes that are cross-reactive with anti-Ro52 antibodies. Enviromental and maternal factors, like age, hypothyroidism, low vitamin D levels, gestational susceptibility weeks occurring during the winter season as well as infections during pregnancy may interplay in the development of the condition and affect the clinical outcome and the severity of the disease in the fetus (Figure 28).

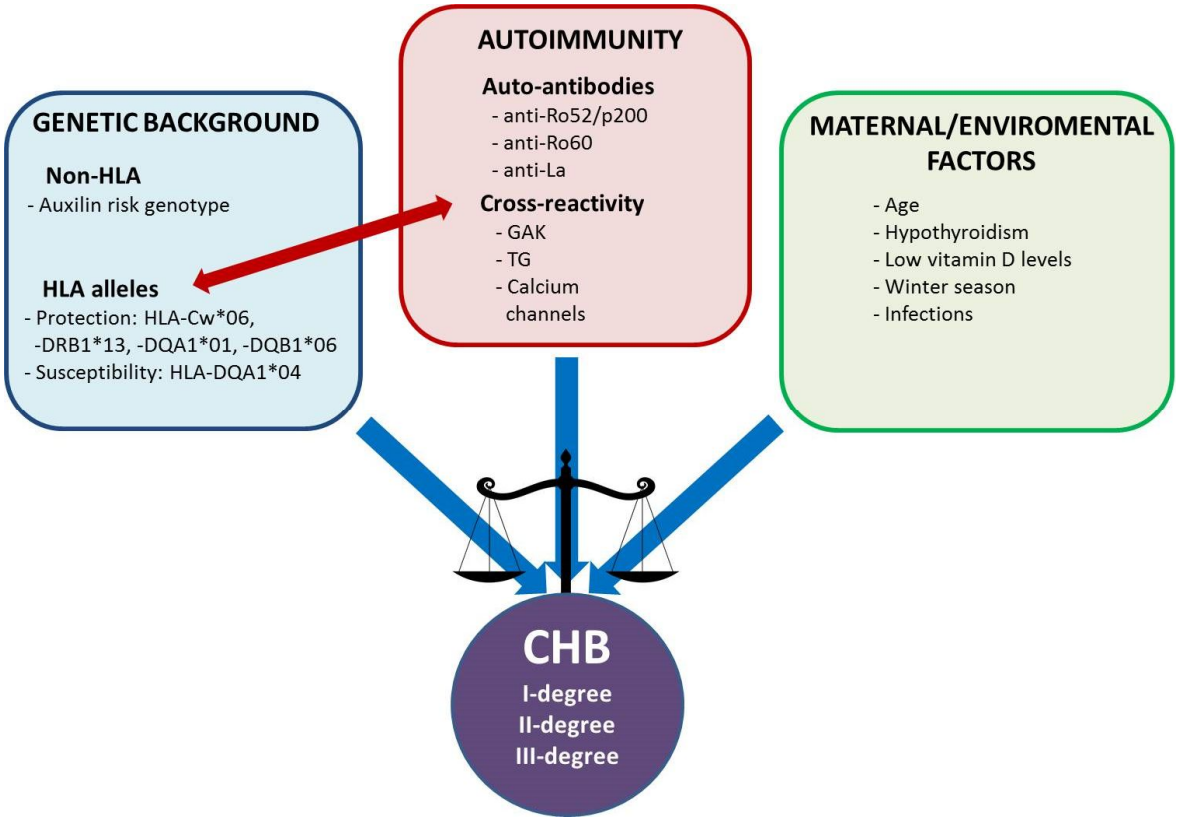

Figure 28. Schematic pictures of the factors involved in the development of CHB.

11. References

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12. Acknowledgments

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A particular thanks to my tutor prof. Amelia Ruffatti for supervising me and teaching the proper approach to research during this period and for being a big example of patience and perseverance. A special thank also for taking the contacts with prof. Marie Wahren-Herlenius and allowing me to go abroad for a long period during my PhD.

A special thank to prof. Marie Wahren-Herlenius for accepting me in your group in the friendly environment at CMM in Karolinska Institutet and for supervising me during my research. Thanks for being an example of the highest efficiency, time management, multitasking and energy for working. I am deeply grateful also for all the precious suggestion and motivation I got in every meeting and for the professional support for my PhD thesis. It was a big pleasure for me working in your group, I learnt a lot, increasing my professional experience day by day and I'm happy that my working experience will continue there.

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A special greeting to the guys of room 63, Elena, Anna, Paola, Nicola, Erika, Francesca, Mariangela and Roberto for all the happy and funny moments I had with them at work, at congresses and at various dinners.

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13. Curriculum Vitae

Curriculum Vitae Europass

Informazioni personali

Cognome/Nome	**LAURO MENEGHEL**
Indirizzo	via Braida I, 4 Eraclea (VE)
Telefono	0421 316509 Cell: +39 3295482808 +46 700959443
E-mail	lauro.meneghel@gmail.com
Cittadinanza	Italiana
Data di nascita	04/02/1988
Sesso	maschile

Esperienza lavorativa e formativa

Data	Dal Gennaio 2013 – in corso
	Dottorato di Ricerca in Scienze mediche, cliniche e sperimentali Indirizzo: Scienze Reumatologiche Ciclo: XXVIII
Attività di ricerca svolta	- Validazione del Chemiluminescence Immunoassay per la determinazione degli anticorpi anticardiolipina, anti-β2 glicoproteina I di classe IgG/IgM tramite il confronto con un metodo ELISA home made. - Identificazione di nuovi markers predittivi di sindrome da anticorpi antifosfolipidi ad alto rischio; in particolare degli anticorpi anticardiolipina e anti-β2 glicoproteina I di classe IgA, degli anticorpii anti-fosfatidilserina/protrombina e degli anticorpi anti-dominio I della β2 glicoproteina I di classe IgG.
Supervisore	Prof.ssa Amelia Ruffatti
Organizzazione erogatrice dell'istruzione e formazione	Università degli Studi di Padova
Data	Dal 02/06/2014 al 18/12/2015
	Visiting PhD student presso il Karolinska Institutet

Attività di ricerca svolta	- Identificazione dei target di cross-reattività degli anticorpi anti-Ro52/p200 associati alla patogenesi del *congenital heart block* (CHB) - Studio multicentirico Europeo di associazione genica del locus Human Leucocyte Antigen (HLA) con il CHB per l'identificazione di alleli che conferiscono suscettibilità o protezione alla malattia, - Studio di espressione genica in organi di topo wildtype di una famiglia genica associata alla Sindrome di Sjögren
Supervisore	Prof.ssa Marie-Wahren Herlenius
Organizzazione erogatrice dell'istruzione e formazione	Karolinska Institutet (Stoccolma, Svezia)
Data	Ottobre 2011- Ottobre 2012 Internato di tesi presso il laboratorio di Genetica Umana, Dipartimento di Biologia, Complesso interdipartimentale "A. Vallisneri" presso l'Università degli Studi di Padova
Attività di ricerca svolta	- Ricerca di mutazioni alla base della malattia Paraparesi Spastica Ereditaria mediante la tecnica di *Exome sequencing*
Supervisore	Prof.ssa Maria Luisa Mostacciuolo
Organizzazione erogatrice dell'istruzione e formazione	Università degli Studi di Padova

Principali tecniche/competenze professionali possedute	- Estrazione di DNA da sangue umano e da linee cellulari - Amplificazione di DNA mediante PCR - Elettroforesi su gel di agarosio - Screening di mutazioni in DNA mediante RFLP (Restriction Fragment Length Polimorphism), PCR tetra-primer ARMS e cromatogramma da sequenziamento Sanger - Enzyme-Linked Immunosorbent Assay (ELISA) - Chemiluminescent Immunoassay (CLIA) - Purificazione di proteine in particolare di β2GPI su colonna di Eparina - Elettroforesi di proteine (SDS-PAGE) - Tecniche di clonaggio con plasmidi di espressione genica - Trasformazione di batteri chimico-/elettro-competenti con plasmidi - Purificazione di plasmidi clonati da batteri - Colture di linee cellulari HEK293T e Raji - Trasfezione transiente di cellule eucariotiche con plasmidi clonati - Microscopio ottico e a fluorescenza - Isolamento di organi da topi - Isolamento di RNA e proteine da tessuto - Quantificazione di DNA, RNA e proteine estratti - PCR real time con SYBR green e TaqMan - Competenze bioinformatiche: analisi di dati da *exome sequencing*, analisi di dati da studi di associazione genica e di analisi eQTL - Consultazione di database bioinformatici quali: PubMed, UCSC Genome Browser, dbSNP, ExomeVarianServer, OMIM, Human Genome Variant Server, BLAST, ExPASy, PDB, Clustal Omega. - Esperienza nell'impiego del pacchetto Microsoft Office e di software per analisi statistica di dati scientifici: GraphPad Prism.

Istruzione e formazione

Data	Da Gennaio 2013 – in corso
	Dottorando in Scienze mediche, cliniche e sperimentali Indirizzo: Scienze Reumatologiche Ciclo: XXVIII
Supervisore	Prof.ssa Amelia Ruffatti
Organizzazione erogatrice dell'istruzione e formazione	Università degli Studi di Padova
Data	Dicembre 2012
Titolo della qualifica rilasciata	Abilitazione all'esercizio della professione di Biologo (Sezione A)
Organizzazione erogatrice dell'istruzione e formazione	Università degli Studi di Modena e Reggio Emilia
Data	09/10/2012

Titolo della qualifica rilasciata	Laurea Magistrale in Biologia Sanitaria (D.M. 270/2004)
Titolo della Tesi di Laurea	"Exome sequencing come nuovo approccio per la ricerca di mutazioni in una famiglia con Paraparesi complicata". Relatore: Prof.ssa Maria Luisa Mostacciuolo, co-relatore: Dott. Giovanni Vazza.
Votazione riportata	110/110
Organizzazione erogatrice dell'istruzione e formazione	Università degli Studi di Padova
Data	27/09/2010
Titolo della qualifica rilasciata	Laurea triennale in Biologia Molecolare
Titolo della Tesi di Laurea	Tesi compilativa dal titolo: "Screening su scala genomica dei fattori cellulari necessari alla replicazione di HIV mediante utilizzo della tecnica di RNA interference". Relatore: Dott.ssa Arianna Calistri
Votazione riportata	97/110
Organizzazione erogatrice dell'istruzione e formazione	Università degli Studi di Padova
Data	Luglio 2007
Titolo della qualifica rilasciata	Diploma di maturità Scientifica presso il Liceo "Galileo Galilei" di San Donà di Piave (VE)
Votazione riportata	78 / 100
Livello nella classificazione nazionale o internazionale	Diploma di Scuola Superiore

Pubblicazioni Scientifiche

Articoli

Meneghel L, Ruffatti A, Gavasso S, Tonello M, Mattia E, Spiezia L, Campello E, Hoxha A, Fedrigo M, Punzi L, Simioni P. Performance of a chemiluninescent immunoassay on detection of anticardiolipin and anti-β2 Glycoprotein I antibodies. Comparison with a home-made ELISA method. Clin Chem Lab Med. 2015 Jun;53(7):1083-9.

Meneghel L,Ruffatti A, Gavasso S, Tonello M, Mattia E, Spiezia L, Tormene D, Hoxha A, Fedrigo M, Punzi L, Simioni P. Detection of IgG anti-Domain I Beta2 Glycoprotein I antibodies by chemiluminescence immunoassay in primary antiphospholipid syndrome. Clin Chim Acta. 2015 Jun 15;446:201-5.

Mattia E**,** Ruffatti A, **Meneghel L**, Tonello M, Faggian D, Hoxha A, Fedrigo M, Punzi L, Plebani M. A contribution to detection of anticardiolipin and anti-β2Glycoprotein I antibodies: comparison between a home-made ELISA and a fluorescence enzyme immunoassay. Clin Chim Acta. 2015 Jun 15;446:93-6.

Hoxha A, Ruffatti A, Mattia E, **Meneghel L**, Tonello M, Salvan E, Pengo V, Punzi L. Relationship between antiphosphatidylserine/prothrombin and conventional antiphospholipid antibodies in primary antiphospholipid syndrome. Clin Chem Lab Med. 2015 Jul;53(8):1265-70.

Mattia E, Ruffatti A, Tonello M, **Meneghel L**, Robecchi B, Pittoni M, Gallo N, Salvan E, Teghil V, Punzi L, Plebani M. IgA anticardiolipin and IgA anti-β2 glycoprotein I antibody positivity determined by fluorescence enzyme immunoassay in primary antiphospholipid syndrome. Clin Chem Lab Med. 2014 Sep;52(9):1329-33.

Abstracts di Congressi

L. Mentlein, G. E.. Thorlacius, J. I. Ramirez, **L. Meneghel**, S. Brauner, A. Espinosa, M. Wahren-Herlenius. Characterizing the FAM167 gene family. **Poster** presentato al **Keystone symposia** on Systems Immunology 2016. Big Sky, Montana USA.

L. Meneghel, C. Mattsson, P.Nilsson, M. Wahren-Herlenius. Screening for cross-reactivite targets of anti-Ro52 antibodies in congenital heart block. **Poster** presentato al **KiiM retreat** 2015, Stoccolma, Svezia.

G. E. Thorlacius, J. I. Ramirez, L. Mentlein, **L. Meneghel**, S. Brauner, A. Espinosa, M. Wahren-Herlenius. Characterization of the Sjögren's syndrome susceptibility gene Fam167a. **Poster** presentato al **KiiM retreat** 2015, Stoccolma, Svezia.

L. Mentlein, G.E. Thorlacius, **L. Meneghel**, A. Espinosa, M. Wahren-Herlenius. Deciphering the function of the uncharacterized FAM167B gene. **Poster** presentato al **KiiM retreat** 2015, Stoccolma, Svezia.

L. Meneghel, A. Ruffatti, S. Gavasso, M. Tonello, E. Mattia, L. Spiezia, D. Tormene, A. Hoxha M. Fedrigo, P. Simioni. Detection of IgG anti-Domain I Beta2 Glycoprotein I antibodies by chemiluminescence immunoassay in primary antiphospholipid syndrome. **Poster** presentato all'**EULAR 2015**.

L. Meneghel, A. Ruffatti, S. Gavasso, M. Tonello, E. Mattia, A. Hoxha, M. Fedrigo, P.Simioni, L. Punzi. Performance di un test immunoenzimatico basato sulla chemiluminescenza nel rilevamento degli anticorpi anticardiolipina e anti β2 glicoproteina I. Confronto con un ELISA home made. Reumatismo2014;66(N.Spec.3): 475. **Poster** presentato durante il **51° Congresso della Società Italiana Reumatologia 2014**

L. Meneghel, A. Ruffatti, S. Gavasso, M. Tonello, E. Mattia, A. Hoxha, M. Fedrigo, P.Simioni, L. Punzi. Clinical value in primary antiphospholipid syndrome of IgG anti-domain I β2 glycoprotein I antibodies detected by chemiluminescent immunoassay. Reumatismo2014;66(N.Spec.3):212-213. **Comunicazione orale** durante il **51° Congresso della Società Italiana Reumatologia 2014.**

E. Mattia, A. Ruffatti, **L. Meneghel**, M. Tonello, D. Faggian, A. Hoxha, M. Fedrigo, M. Plebani, L. Punzi. Confronto tra fluorescence enzyme immunoassay ed ELISA nella determinazione degli anticorpi anti cardiolipina e anti β2 glicoproteina I. Reumatismo2014;66(N.Spec.3): 475-476. **Poster** presentato durante il **51° Congresso della Società Italiana Reumatologia 2014**

A. Hoxha, A. Calligaro, M. Favaro, E. Mattia, **L. Meneghel**, M. Tonello, M. Facchinetti, A. Ruffatti, L. Punzi. Relationship between antiphosphatidylserine/prothrombin antibodies and conventional antiphospholipid antibodies in primart antiphospholipid syndrome. Ann Rheum Dis 2014;**73**:Suppl 2 190. Poster presentato durante Eular 2014.

L. Meneghel, E. Mattia**,** M.Tonello, N. Gallo, B. Robecchi, V. Teghil, M. Plebani, L. Punzi, A. Ruffatti. Clinical relevance in Primary Antiphospholipid Syndrome of IgA anticardiolipin and IgA anti-β2Glycoprotein I antibodies tested by Fluorescence Enzyme Immunoassay. **Comunicazione** durante il **9° Congresso Internazionale sull'autoimmunità. Nizza, 2014**

E. Mattia, **L. Meneghel**, B. Robecchi, M. Tonello, D. Faggian, M. Pittoni, M. Plebani, A. Ruffatti, L. Punzi. L'isotipo A degli anticorpi anti-cardiolipina e anti-β2 Glicoproteina I nella sindrome da anticorpi antifosfolipidi primaria. **Poster** presentato durante il **50° Congresso Nazionale della Società Italiana di Reumatologia 2013**

A. Hoxha, E. Mattia**, L. Meneghel**, M. Tonello, M. Facchinetti, E. Salvan, A. Ruffatti, L. Punzi. Relationship between antiphosphatidylserine/prothrombin antibodies and conventional antiphospholipid antibodies in primart antiphospholipid syndrome. Reumatismo 2013;65(N.Spec.3):442. **Poster** presentato durante il **50° Congresso della Società Italiana Reumatologia 2013**

A. Hoxha, E. Mattia**, L. Meneghel**, M Tonello, M Facchinetti, E. Salvan, V Pengo, A. Ruffatti. Relationship between antiphosphatidylserine/prothrombin antibodies and conventional antiphospholipid antibodies in primary antiphospholipid syndrome. **Poster** presentato durante il **"9th Meeting of the European Forum on Antiphospholipid Antibodies" Cracovia, 2013**

E. Mattia, **L. Meneghel**, B. Robecchi, M. Tonello, D. Faggian, M. Pittoni, M. Plebani, A. Ruffatti. Value of IgA anticardiolipin and anti-β2Glycoprotein I antibodies in Primary Antiphospholipid Syndrome. **Poster** presentato durante il **"9th Meeting of the European Forum on Antiphospholipid Antibodies" Cracovia, 2013**

Partecipazione a Corsi/Meeting/Congressi

15-16 Ottobre 2015	Partecipazione al KiiM (Karolinska Inflammation and Immunology network) retreat", con contributo scientifico (poster), presso il Karolinska Institutet, Stoccolma, Svezia
07 Dicembre 2014	Partecipazione alla Nobel Lecture in occasione del la premiazione del premio Nobel per la Medicina e la Fisiologia presso il Karolinska Institutet, Stoccolma, Svezia
26 – 29 Novembre 2014	Partecipazione con contributo scientifico (poster e presentazione orale) al 51° Congresso Nazionale della Società Italiana di Reumatologia, Rimini"
1 – 2 Settembre 2014	Partecipazione al Nobel Symposium "Renaissance in diagnosis of monogenic diseases", Nobel Forum, Karolinska Institutet, Stoccolma, Svezia.
26 Agosto 2014	Partecipazione all'Inflammation Day 2014, Karolinska Univesity Hospital, Stoccolma, Svezia
26 – 30 Marzo 2014	Partecipazione con contributo scientifico (comunicazione orale) al 9th International Congress on Autoimmunity, Nizza, Francia.
14 – 15 Marzo 2014	Partecipazione al Joint Meeting Padova – Strasburgo, Palazzo Bo, Università degli Studi di Padova, Padova
Gennaio – Giugno 2014	Partecipazione ai "Meetings di Reumatologia 2013-2014. XXXVIII edizione"
Gennaio – Marzo 2014	Corso di Inglese Accademico (30 ore) di livello B2/C1 per dottorandi presso l'Università degli Studi di Padova, Padova.
Ottobre – Dicembre 2013	STEPS 2013 Seminars Towards Enterprise for PhD Students organizzato dalla Confindustria di Padova
9 – 10 Dicembre 2013	Partecipazione al IV Symposium Autoinflammatory Days, Palazzo Bo e Aula Morgagni, Università degli Studi di Padova, Padova
27 – 30 Novembre 2013	Partecipazione con contributo scientifico (poster) al 50° Congresso Nazionale della Società Italiana di Reumatologia. Napoli
23 – 27 Settembre 2013	Partecipazione alla Summer School delle Scuole di Dottorato dell'Area Medica dell'Università degli Studi di Padova
16 – 18 Maggio 2013	Partecipazione con contributo scientifico (poster) al 9th Meeting of the European Forum on Antiphospholipid Antibodies. Cracovia, Polonia

Collaborazioni con altri gruppi di ricerca

- Medicina di Laboratorio dell'Azienda Ospedaliera di Padova (prof. M. Plebani)
- Laboratorio di Coagulazione dell'Azienda Ospedaliera di Padova (prof. P. Simioni)
- Laboratorio di Patologia Cardiovascolare dell'Università di Padova (prof. V. Pengo)

Competenze comunicative

Buona competenza comunicativa posseduta, anche in inglese

Possiedo buone competenze comunicative acquisite durante le comunicazioni orali nei congressi e durante il periodo di ricerca presso il Karolinska Institutet in cui ho dovuto partecipare attivamente con presentazioni a frequenti lab meeting e Journal club.

Competenze organizzative e gestionali

Indipendente nel portare avanti il mio progetto
Flessibile, responsabile e sistematico
Capace di lavorare in gruppo
Capace di lavorare sotto condizioni di stress
Strutturato, organizzato ed ordinato nel mio lavoro
Preciso e meticoloso, attento al dettaglio

Madre lingua **Italiano**

Autovalutazione	Comprensione		Parlato		Scritto
Livello europeo (*)	Ascolto	Lettura	Interazione orale	Produzione orale	
Inglese	C1	C1	C2	C1	C1
Spagnolo	B1	A2	A2	A1	A1
Svedese	A1	A1	A1	A1	A1

(*) Quadro comune europeo di riferimento per le lingue

Patente automobilistica (Patente B)

14. Attivita' svolte nel triennio di dottorato

I anno

Durante il primo anno di dottorato la mia attività di ricerca è cominciata con una collaborazione con la mia collega dott.ssa Elena Mattia nel progetto di confronto tra le performance di un saggio immunoenzimatico basato sulla chemiluminescenza (CLIA) e quella di un ELISA *home made* per la determinazione degli anticorpi anticardiolipina (aCL) IgG/IgM e anti-β2 Glicoproteina I (anti-β2GPI) IgG/IgM in una coorte di pazienti affetti da APS primaria ed in un gruppo di pazienti sieronegativi per valutarne il valore diagnostico. Concomitantemente a tale attività, d'accordo con la mia tutor, prof.ssa Amelia Ruffatti, sono stati presi contatti con la prof.ssa Marie Wahren-Herlenius del Karolinska Institutet (KI, Stoccolma, Svezia) per un mio soggiorno presso il suo laboratorio da effettuare nella seconda metà del dottorato per lavorare su un progetto di ricerca riguardante la suscettibilità genetica associata al *congenital heart block*. (CHB). Per tale collaborazione ho individuato le famiglie in cui si sono nati bambini con CHB. Dopo l'arruolamento, campioni di siero e di DNA, inseme alle informazioni cliniche, sono stati inviati al laboratorio della prof.ssa Wahren-Herlenius.

Nell'arco di quest'anno ho partecipato ai "Meetings di Reumatologia 2013. XXXVII edizione" organizzati dal coordinatore di indirizzo; al "9th Meeting of the European Forum on Antiphospholipid Antibodies" a Cracovia (Polonia) con contributo scientifico (poster), alla "Summer school" scuole/corsi di dottorato di ricerca dell'area medica, allo STEPS 2013 Seminars Towards Enterprise for PhD Students organizzato dalla Confindustria di Padova, al 50° Congresso Nazionale SIR con contributo scientifico a Napoli (poster) e al 4th Symposium "Autoinflammatory day".

II anno

Nel corso del secondo anno, ho continuato raccolta di campioni di siero e di DNA dalle famiglie con figli affetti da CHB. Il 2 Giugno 2014 ho iniziato la mia attività di ricerca presso il KI. Qui, ho iniziato un progetto rivolto all'identificazione di target di cross-reattività degli anticorpi anti-Ro52/p200 associati al CHB attraverso uno screening su base proteomica attraverso un anticorpo monoclonale specifico per la regione p200. Una volta identificati gli epitopi di cross-reattività, ulteriori esperimenti hanno confermato cross-reattività con anticorpi monoclonali e con sieri di pazienti verso le proteine Tiroglobulina e GAK (Cyclin G-Associated Kinase). Nell'arco di quest'anno ho partecipato ad un corso di Inglese Accademico di 30 ore di livello B2-C1 per dottorandi presso l'Università degli Studi di Padova. Ho partecipato, inoltre, ai "Meetings di Reumatologia 2014. XXXVIII edizione"

organizzati dal coordinatore di indirizzo; al Convegno "Joint Meeting Padova – Strasburgo", Palazzo Bo al "9th International Congress on Autoimmunity" a Nizza (Francia) con contributo scientifico (comunicazione orale), al 51° Congresso Nazionale SIR con contributo scientifico a Rimini (poster e comunicazione orale). In Svezia ho partecipato all'Inflammation Day 2014" e al Nobel Symposium "Renaissance in diagnosis of monogenic diseases" presso il KI a Stoccolma (Svezia). Ho partecipato inoltre alla Nobel lecture dei vincitori del premio Nobel per la Medicina e Fisiologia presso il Karolinska Institutet a Stoccolma (Svezia).

III anno

Nel corso di quest'anno ho continuato la permanenza presso il KI, portando avanti la ricerca riguardante l'identificazione dei target di cross-reattività degli anticorpi anti-Ro52/p200. Contemporaneamente, ho iniziato un secondo progetto multi-centrico volto a studiare l'associazione genetica del locus *Human Leucocyte Antigen* (HLA) con il CHB. L'analisi genetica è stata condotta in una coorte di famiglie Europee in cui sono nati bambini con CHB con lo scopo di identificare quali alleli del locus HLA sono trasmessi dai genitori ai figli con CHB con una frequenza che si discosta significativamente dalla frequenza del 50% in accordo alle leggi di Mendel sulla segregazione indipendente dei caratteri. Oltre a tale progetto, ho partecipando, collaborando ad uno studio di caratterizzazione funzionale di una famiglia genica associata geneticamente alla Sindrome di Sjogren. In particolare, mi sono occupato dello studio di espressione genica di tali geni in un set di 14 organi provenienti da 10 topi *wildtype*. Tale lavoro, però, non è parte della mia tesi.

Nel corso di quest'anno ho partecipato al "KiiM (Karolinska Inflammation and Immunology network) retreat", Stoccolma (Svezia) con contributo scientifico (poster).

Il 18 Dicembre 2015 ho concluso il mio soggiorno di ricerca presso il KI e sono tornato a Padova.