New insight into
the pathogenesis and treatment of
autoimmune congenital heart block
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ABSTRACT

Background. Congenital atrioventricular block (CHB) is the most frequent manifestation of neonatal lupus which is attributed to anti-Ro/SSA and/or anti-La/SSB-mediated inflammation and subsequent fibrosis of the atrioventricular node. Despite the observation of a direct role for maternal antibodies in inducing CHB both in vitro and in vivo studies, the mechanisms involved remain unclear. Currently no therapy is found to be effective in the treatment of 2nd and 3rd degree CHB induced by maternal antibodies; also because the rarity of the disease does not allow for controlled clinical trials.

Objectives. To explore the role of anti-Ro52 antibodies in the pathogenesis of CHB by identifying of the epitope specificity of anti-p200 antibodies. To study in an animal model the effect on heart conduction system of the different doses of injected human IgG antibodies from a CHB patient. To evaluate the clinical efficacy and safety of a combined therapy protocol utilizing plasmapheresis, intravenous immunoglobulin (IVIG) and betamethasone and the effect of this treatment on anti-Ro52, anti-p200 and anti-La antibody levels.

Materials and Methods. Laboratory methods: ELISA assay, circular dichroism spectroscopy and antibody purification. Procedure: antibody transfer by intra-peritoneally injection of purified IgG. Instrumental methods: fetal ecocardiography and electrocardiogram recordings. Treatment strategy: Six consecutive women diagnosed CHB underwent a combination therapy protocol composed of weekly plasmapheresis, fortnightly 1 g/Kg IVIG and daily Betamethasone (4 mg/day) throughout pregnancy. IVIG (1 g/Kg) treatment in the neonates was begun at birth and administered every fifteen days until passive maternal antibodies became undetectable. Statistical analysis: Wilcoxon, Mann-Whitney U, two way ANOVA and Spearman’s tests; p<0.05 was considered as significant.
**Results and Conclusion.** Part I: The epitope specificity of anti-p200 antibodies was the position 233. We suggest that this antibody specificity might be a tool to identify high risk pregnancies for CHB. The results might contribute to identify antibody specificity closely associated with development of CHB and to provide the possibility to explore the role of this antibody in the pathogenesis of CHB.

Part II: We developed an animal model for CHB by a simple technique of passive transfer of human IgG from a patient with a child with CHB. High levels (4 mg) of anti-SSA/Ro and anti-SSB/La antibodies induce bradycardia, atrioventricular time prolongation and a decreased cardiac performance. Whereas, low levels (2 mg) of these antibodies determine a decrease in cardiac performance.

Part III: The obtained results from these case series are: a) the efficacy in treating 2\textsuperscript{nd} degree CHB, and b) the safety of plasmapheresis and IVIG therapies. The low number of treated cases, the relatively short follow-up (mean 22.7 months ± 10.4 SD, range 12-42 months) and the high cost of the procedure can all be considered limits.

Part IV: Plasmapheresis reduces the levels of anti-Ro52, anti-p200 and anti-La antibodies. However, a decrease of antibody amounts in patients with the highest anti-Ro52 antibody levels has not been demonstrated.
RIASSUNTO

Premesse. Il blocco atrioventricolare congenito (CHB) è la manifestazione più frequente del lupus neonatale. Esso è causato dal passaggio transplacentare degli anticorpi anti-Ro/SSA e/o anti-La/SSB con successiva infiammazione e fibrosi del nodo atrioventricolare. Nonostante la dimostrazione di un ruolo diretto degli anticorpi materni nell'indurre CHB sia in vitro che in vivo, i meccanismi coinvolti rimangono poco chiari. Inoltre finora nessuna terapia è risultata efficace nel trattamento del CHB sia di secondo che terzo grado, anche perché la rarità della malattia non consente studi clinici controllati.

Obiettivi. Studiare il ruolo degli anticorpi anti-Ro52 nella patogenesi del CHB attraverso la definizione della specificità epitopica degli anticorpi anti-p200. Sviluppare un modello animale per studiare l'effetto sul sistema di conduzione cardiaco degli anticorpi IgG purificati da una paziente con CHB fetale. Infine valutare l'efficacia clinica e la sicurezza di un protocollo di terapia combinato comprendente l'impiego di plasmaferesi, immunoglobuline per via endovenosa (IVIG) e betametasone, nonché l'effetto di questo trattamento su i livelli degli anticorpi anti-Ro52, anti-p200 e anti-La.

Materiali e Metodi. Metodi di laboratorio: ELISA, spettroscopia con dicroismo circolare e purificazione di IgG umane. Procedura sperimentale: trasferimento per via intra-peritoneale degli anticorpi mediante l'iniezione di IgG purificate da una madre con CHB. Metodi strumentali: ecocardiografia fetale ed elettrocardiografia dopo la nascita. Strategia di trattamento: Sei donne diagnosticate con CHB sono state trattate durante la gravidanza con un protocollo di terapia di associazione composta da plasmaferesi settimanale, IVIG 1g/kg a cadenza quindicinale e Betametasone giornaliero (4 mg). Inoltre, IVIG (1 g/kg) sono state somministrate ai neonati ogni quindici giorni subito dopo la nascita fino alla negativizzazione degli anticorpi materni passivi. Analisi statistica: Wilcoxon, Mann-Whitney U, ANOVA e Spearman tests. Il valore di p<0,05 è stato considerato significativo.
**Risultati e Conclusioni. Parte I:** La specificità epitopica dell'anticorpo anti-p200 è stata localizzata nella posizione 233. Tale epitopo potrebbe essere utile per identificare la specificità anticorpale strettamente associata allo sviluppo del CHB e costituire quindi uno strumento per individuare le gravidanze ad alto rischio per CHB. Inoltre potrebbe contribuire a chiarire il ruolo di questo anticorpo nella patogenesi del CHB.

**Parte II:** Abbiamo sviluppato un modello animale del CHB con una semplice tecnica di trasferimento passivo di IgG umane purificate da un paziente con CHB fetale. Gli alti livelli (4 mg) degli anticorpi anti-Ro/SSA ed anti-La/SSB hanno indotto bradicardia, prolungamento dell'intervallo PR e una depressione delle funzione cardiaca nel animale. Invece, i bassi livelli (2 mg) hanno determinato solo una diminuzione della funzione cardiaca. In questo modo è stato dimostrato la patogenicità degli anticorpi anti-Ro/SSA ed anti-La/SSB sul sistema di conduzione cardiaco.

**Parte III:** I risultati ottenuti da queste serie di casi sono i seguenti: a) l'efficacia nel trattamento del CHB di 2° grado, e b) la sicurezza della plasmaferesi e delle IVIG. Tuttavia il basso numero di casi trattati, e il follow-up relativamente breve (media 22,7 mesi ± 10,4 DS, range 12-42 mesi), come anche l'alto costo della procedura possono essere considerati limiti.

**Parte IV:** La plasmaferesi riduce i livelli degli anticorpi anti-Ro52, anti-p200 e anti-La. Tuttavia, un decremento significativo anticorpale non è stato dimostrato nelle pazienti con livelli molto alti di anticorpi anti-Ro52.
1. INTRODUCTION

1.1 Neonatal lupus syndrome
Neonatal lupus syndrome (NLS), is a passively acquired autoimmune disease caused by the transplacental transfer of maternal autoantibodies (anti-SSA/Ro and anti-SSB/La) into the fetal circulation (1). The woman may have Sjögren syndrome (SS), systemic lupus erythematosus (SLE) or undifferentiated connective tissue disease (UCTD), but may be also asymptomatic. NLS is characterized by cutaneous rash, cytopenias, cholestatic hepatitis and congenital heart block (CHB) (2). The NLS symptoms are transient, except CHB, and disappear when the maternal antibody levels have decreased in the neonate system at approximately 6-8 months (3).

1.2 Congenital heart block
CHB is the most common and severe manifestations of NLS which develops in the fetal heart in the absence of any major structural malformation and in the presence of anti-SSA/Ro and anti-SSB/La maternal autoantibodies (4,5). CHB is considered congenital if diagnosed in utero or during the first 27 days of life (6). It is hypothesized that CHB progress gradually from a first-degree atrio-ventricular block (AVB) to a second-degree AVB and finally this could progress to the irreversible third-degree AVB (7). A third-degree AVB is a potentially lethal condition associated with high rate of fetal morbidity and mortality (2). However, antibodies and complement deposits together with signs of fibrosis and calcification are found in the conduction fetal system as well as in the entire myocardium (8-10), suggesting the potential involvement of maternal autoantibodies in other cardiac manifestations of CHB, such as myocardial inflammation, dilated cardiomyopathy, endocardial fibroelastosis, sinus bradycardia, and QTc prolongation rather than AVB (11-14).
1.2.1 History

Morquio was the first to describe, more than a century ago the rare defect of third-degree heart block in an infant (15). Subsequently, in 1928, Aylward reported the cases of heart block in 2 children whose mothers “suffered from Mikulicz’s Disease” (16). This clinical association begun strengthens by the 1970s, with reports of several children with heart block whose mothers had autoimmune diseases (17-19) but only in 1983 was observed that sera from mothers whose children have heart block contain antibodies to SSA/Ro ribonucleoproteins (20, 21). Further clinical refinement demonstrated that is the heart block in an otherwise normal structurally heart, identified in utero, not in childhood, which is associated with maternal autoantibodies (22). Hence, CHB, a lifelong disability previously of interest only to cardiologist, became an important model of passively acquired autoimmunity. However, still now after five decades, this rare disease is underdiagnosed and the mothers, most of the time do not receive the adequate counseling.

1.2.2 Epidemiology

CHB developed in the presence of maternal autoantibodies associated with the rheumatic diseases such as SS, SLE or UCTD, but the mother of an affected child may also be asymptomatic. CHB is a rare disease with an incidence of 1:15,000-1:20,000 of cases for year (23) which is most commonly developed during weeks 18 to 24 of pregnancy, even rather it can be manifested until 30 weeks of gestation. CHB occur only in 1–2% of anti-SSA and/or anti-SSB positive primigravid women (24) but carries a recurrence rate around 20%, in subsequent pregnancies despite the persistence of the autoantibodies (25). The development of third-degree CHB requires insertion of a pacemaker after birth. Despite pacemaker placement, ~10% of these children develop cardiomyopathy (26). Moreover, the cardiomyopathy due to inflammatory immune damage causes 40–60% of infant morbidity and 20–30% of mortality, mostly fetal and neonatal deaths (27). Long-term
follow-up of children with CHB to the age of 10 years has shown mortality rates of 20–30% and morbidity rates of ~35%, with an 80% cumulative probability of survival at 3 years of age (26). Recently, it has been described an association of the development of CHB with maternal age and the seasons (4).

1.3 Autoantibodies associated with congenital heart block

The relationship between maternal antibodies against the SSA/Ro and SSB/La antigen and CHB is well established, and the antigen contains several components to which rheumatic patients develop autoantibodies.

1.3.1 The SSA/Ro and SSB/La antigens

The SSA/Ro antigen consists of a 52 kDa protein (Ro52) and a non-homologous 60 kDa protein (Ro60) (Figure 1).

Figure 1. A) SSA/Ro and SSB/LA antigens; B) Fluoroscopic pattern on HEp-2000 cell of 60 kD anti-SSA/Ro

Ro52 belongs to the tri-parted motif (TRIM) family of proteins (28), characterized by a RING domain (29), a B-box type 2 motif (30) and a coiled-coil region (Figure 2).
Several studies have shown that TRIM family members act as an E3 ubiquitin ligases involved in the ubiquitination process (31-34). Ubiquitination process (Figure 3) is a post-translational modification mechanism of the proteins by ubiquitins, small regulatory proteins found in all eukaryotic cells from plants to mammals, leading either to proteolysis in the proteasome, internalization from membranes or functional alteration (35-37). The protein ubiquitination is a multi-step process that involve three enzymes; first, ubiquitin is activated by ubiquitin-activating enzyme (E1), then is transferred to a ubiquitin-conjugating enzyme (E2) followed by a ubiquitin ligase (E3) which interacts directly with E2 enzyme and its substrate and mediates the transfer of ubiquitin from E2 to a target protein (38).

The process can be repeated until a short chain is formed, with three or more ubiquitin molecules usually targeting the protein to the proteasome. In the proteasome,
polyubiquinated proteins are degraded. The role of Ro52 as an E3 ligase in the ubiquitination process is revealed only recently (31, 32). Ro52 called also TRIM 21 mediates ubiquitination of several members of the interferon regulatory factor transcription factor family, thereby regulating cytokine production (39-42). RING-dependent autoubiquitination of Ro52 was demonstrated in vivo and in vitro, and overexpression of Ro52 in stably transfected B cells leads to decreased proliferation and increased sensitivity to CD40-mediated cell death, suggesting a biological role for Ro52 in cell growth and/or apoptosis (31).

Ro60 is complexed with small non-coding cytoplasmic (Y) RNAs. It has been demonstrated that the protein binds misfolded, defective, non-coding RNAs that are eventually degraded (43-45), suggesting a function in a quality control pathway in which incorrectly folded and otherwise imperfect non-coding RNAs are targeting for decay (46, 47). The SSB/La antigen (Figure 1) is a single 48 kDa nuclear phosphoprotein that binds many newly transcribed non-coding RNAs, including Y RNAs (47). There is accumulating evidence that La is necessary for correct folding of many small RNAs, suggesting an RNA polymerase III transcript maturation role for this protein (48).

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

Several studies aimed to identify antibody specificities associated with CHB, have been performed. Although the data vary among the different studies, depending on the method used for the detection of the antibodies and the criteria chosen to enroll the patients, most of them demonstrate that the risk is higher in women in whom the anti-Ro activity is targeted to the 52 kDa component of the antigen rather than to the 60 kDa component (49-51), especially in those women in whom the antibody response is directed to an immunodominant region against the amino acids 200–239 (p200) of the 52 kDa protein (50, 52). In a prospective study in which Doppler echocardiography was performed weekly
during susceptibility weeks 18–24 in Ro52-positive women, mothers with higher anti-p200 levels had fetuses with significantly longer AV time intervals (50). The p200 peptide encompasses a functional domain, a leucine-zipper structure. Association with autoantibodies specific for a functional domain is not a unique feature for congenital heart block. In the case of heart block binding of anti-p200 autoantibodies might possibly mediate inhibition or decrease the functional activity of the antigen, as is evident for autoantibodies against RNA polymerase I inhibiting RNA synthesis, or against DNA topoisomerase I causing relaxation of super-coiled DNA (53). Furthermore, is demonstrated that cardiac conduction disturbances are associated with moderate to high levels of anti-Ro antibodies (50, 54). So, both the fine specificity and the levels of maternal anti-Ro52 antibodies are of importance in predicting fetal outcome.

Antibodies to Ro60 and La have been suggested to have a minor role in predicting the fetal clinical outcome in anti-Ro and anti-La antibody–positive mothers (50, 55, 56), although an association also between these autoantibodies and the incidences of congenital heart block has been demonstrated (55, 57). 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 (55). Although congenital heart block may develop independently of maternal antibodies against Ro60 and La, their presence, might be able to amplify the immunological response after onset in affected fetuses (58).

1.3.3 Other autoantibodies

Given the low risk for the development of CHB in anti-SSA/Ro and or anti-SSB/La positive mothers other antibodies directed to several other targets then Ro and La proteins, have been suggested to be associated with development of CHB. Antibodies against neonatal heart muscarinic acetylcholine receptor (mACHR) have been demonstrated to have a role in the disease supported by in vitro studies when is reported the binding, and biological
effects of these antibodies in CHB (59, 60). Calreticulin, a calcium binding, multifunctional protein, involved in calcium storage has been suggested a target in the disease initiation, and antibodies to calreticulin have been demonstrated in sera from mothers with affected children (61). Another suggested antibody specificity associated with congenital heart block is antibodies to p57, which was identified in a child with the disease (62). Antibodies to a cleavage product of α-fodrin has, in addition to being identified as an organ-specific antibody in SS, been suggested as an additional serologic marker in congenital heart block (63). Moreover, the role of antibodies to α-enolase in the development of CHB has been investigated, but a relationship was not founded (64).

1.4 Etiopathogenesis
Direct evidence for a pathogenic role of maternal anti-SSA/Ro and/or anti-SSB/La antibodies and especially anti-Ro52 antibodies in CHB comes from experimental studies of heart block, both in vitro and in vivo. The presence of anti-SSA/Ro antibodies in the cardiac tissue of fetuses dying of CHB, together with deposition of complement, fibrosis, and calcification, was demonstrated in the early 1980s by several groups (8-10, 18) providing the first link between maternal antibodies and pathogenesis of heart block by placing the antibodies at the site of injury.

1.4.1 Experimental models of congenital heart block
In vitro studies demonstrated a direct pathogenic role of antibodies from mothers of children with CHB, as perfusion with maternal IgG containing anti-SSA/Ro and anti-SSB/La antibodies of rat or human hearts isolated with the Langendorff technique, induced bradycardia and complete AVB within 15 minutes (65, 66). Affinity-purified anti-Ro52 antibodies induced the same effects, showing the individual pathogenic potential of anti-
Ro52 antibodies. Similar results were obtained in Langendorff - perfused rabbit hearts exposed to anti-Ro/La antibodies purified from mothers of children with CHB (67, 68). *In vivo* studies demonstrated the pathogenicity of anti-SSA/Ro and anti-SSB/La antibodies based on animal models of active immunization of females before gestation or passive transfer of antibodies.

**A. Immunization-based models of CHB**

Active models of CHB, in which female mice, rats, or rabbits are immunized with a particular antigen before gestation, have made possible to investigate the pathogenic potential of specific antibodies toward Ro52, Ro60, or La separately. Miranda-Carus et al (69) showed that immunization of BALB/c mice with Ro60 or La led to the development of first-degree AVB in 19% and 7% of the offspring, respectively, and similar results were observed in C3H/HEJ mice by Suzuki et al (70). In the study by Miranda-Carus (69), immunization of BALB/c mice with the human or mouse Ro52 protein induced first-degree AVB in only 9% of the offspring; instead Boutjdir et al (66) demonstrated a 25% incidence in the same model. Both studies reported higher degrees of AVB but a lower frequency, 3.5% to 10% of AVB II/III. Immunization of rats with the human Ro52 protein led to the development of first-degree AVB in 10% to 45% of the pups, depending on the strain (71). Immunization of rabbits with the human Ro52 protein also induced first-degree AVB in 12% of the offspring, moreover the authors reported a large number of neonatal deaths, which might have been related to higher degree blocks; however, no histological evaluation of neonatal hearts was performed to support this hypothesis (72). Salomonsson et al (52) showed that immunization of DA (Dark Agouti) rats with the Ro52/p200 peptide led to the development of first-degree AVB in 20% (10/52) of the offspring. Immunization of mice with a recombinant Ro52β protein (Ro52 isoform lacking exon 4) induced first-degree AVB in 12% and AV block II/III in about 6% of the pups (72); however, the relevance of
these findings is uncertain as the endogenous protein has never been detected in humans or rodents, despite a report of Ro52β mRNA expression in the fetal human heart (73).

B. Antibody transfer-based models of CHB

In a study by Mazel and colleagues (74), transfer of affinity-purified anti-SSA/Ro and anti-SSB/La antibodies from two mothers of children with CHB into pregnant female BALB/c mice induced first-degree AV block in 47% to 90% of the offspring, depending on the day of gestation at which the injection was performed. Sinus bradycardia was also observed, albeit in a somewhat smaller proportion of pups. However, the use of a mixture of anti-Ro and anti-La antibodies in this study did not allow the precise identification of the antibody specificity contributing to the development of heart block. Ambrosi et al (75) in attempt to find the specificity of anti-Ro52 antibodies inducing heart block established a heart block model based on the transfer of anti-Ro52 monoclonal antibodies during gestation. Using this model, the authors observed that anti-Ro52 antibodies specific for the p200 part of the protein induced AVB in the offspring but that antibodies targeting other domains of Ro52 did not (75). In addition, anti-Ro52-p200 antibodies were shown to disturb calcium homeostasis in cultured neonatal cardiomyocytes, supporting a pathogenic role for anti-Ro52 p200 antibodies in CHB (52). It is worth noting that first-degree AV block developed in 100% of the rat pups exposed to the antip200 monoclonal antibodies in utero but that only 19% of pups born to females immunized with the Ro52-p200 peptide were shown to develop AV block in a previous study. This was interpreted as a sign of the importance of the specificity and level of the antibodies in induction of CHB, as the specificities and levels induced by immunization were variable, while the monoclonal transfer model exposed all pups to the same specificity - and at the same concentration.
1.4.2 Proposed hypothesis for CHB

Despite the observation of a direct role for maternal antibodies in inducing CHB both in vitro and in vivo, the mechanisms involved remain unclear. Based on the facts that there is no convincing evidence that maternal antibodies can cross the sarcolemma of a normal cardiomyocyte and bind to the Ro52, Ro60, and La proteins, which are the major targets of maternal autoantibodies associated with CHB; two categories of mechanisms, not mutually exclusive and each supported by experimental data, have been put forward. The first is the abnormal surface expression of intracellular Ro-La antigens, and the second is the cross-reactivity of maternal antibodies with targets other than Ro-La antigens. To explain the pathogenesis of CHB based on these two categories of mechanisms, three major hypotheses have been proposed: the apoptosis hypothesis [cell-surface expression of the Ro-La antigen] (76), the serotoninergic hypothesis (77) and the Ca channel hypothesis [cross-reactivity of maternal antibodies with cell-surface receptors and Ca channel proteins, respectively] (78).

A. Apoptosis hypothesis

The apoptosis hypothesis proposes abnormal (opsonization) surface expression of intracellular Ro-La antigens that become accessible to circulating maternal antibodies on the surface of the cardiac myocytes. It is suggested that opsonization converts the physiologic process of apoptosis to circumstances in which an inflammatory component is evoked (79). Miranda et al., (69, 79) demonstrated that induction of apoptosis results in surface translocation of Ro-La antigens in human fetal cardiac myocytes. To examine whether antibodies reactive to Ro-La antigen system indeed bind to the surface of human fetal myocytes and to assess the consequences of this surface bindings, the authors used biotinylation of cell-surface proteins, scanning electron microscopy of immune gold labelled cells and examined the consequences of the release of the inflammatory cytokine (79). They confirmed that induction of apoptosis results in surface accessibility of all Ro-La
antigens for recognition by tumor necrosis factor (TNF) from macrophages co-cultured with apoptotic fetal cardiac myocytes. It was concluded that opsonized apoptotic fetal cardiac myocytes promote an inflammatory response by macrophages causing damage to surrounding conducting tissue. Clancy et al. (80) demonstrated that healthy fetal cardiocytes are involved in physiologic clearance of apoptotic cells and that surface binding by anti-SSA/Ro and anti-SSB/La antibodies inhibits the uptake of apoptotic cells by the healthy cardiocytes. The result is accumulation of apoptotic cells promoting further inflammation and subsequent scarring. It is, however, difficult to correlate the earlier findings to AV conduction abnormalities seen in CHB because the experiments were performed in ventricular myocytes, not in conduction system myocytes such as AV node myocytes. However, evidence for the subcellular translocation of La autoantigen during physiologic apoptosis in the fetal mouse conducting system has been provided (81). Other experimental evidence has been proposed to account for the translocation of Ro/La to the cell surface, including viral infection (82), UV light and interferon (IFN) treatment (83).

B. Serotoninergic hypothesis

Accessibility of intracellular target antigens to circulating maternal antibodies remains a challenge. This lead to the consideration of alternative hypotheses such as cross-reaction between one or any of the Ro/La components and a cell-surface cardiac receptor and/or channel. Indeed, support for this second hypothesis is the report by Eftekhar et al. (77) that antibodies reactive with the serotoninergic 5-hydroxytryptamine (5-HT4 receptor), cloned from human adult atrium, also bind 52-kD Ro. Moreover, affinity-purified 5-HT4 antibodies antagonized the serotonin-induced L-type Ca channel activation in human atrial cells (77). The finding that functional beta-adrenoceptors in experimental animals appear late in the gestation stage, makes this hypothesis more intriguing, because 5-HT4 receptors could functionally replace the beta-adrenergic receptor during development. Two peptides in the C terminus of 52-kD Ro, aa365–382 and aa380–396, were identified that
shared some similarity with the 5-HT4 receptor. The former was recognized by sera from mothers of children with neonatal lupus and it was this 52-kD Ro peptide that was reported to be cross-reactive with antibodies to peptide aa165–185, derived from the second extracellular loop of the 5-HT4 receptor (77). These findings are of particular importance, because over 75% of serum from mothers whose children have CHB contains antibodies to 52-kD Ro. Given the intriguing possibility that antibodies to the 5-HT4 receptor might represent the hitherto elusive reactivity that could directly contribute to AVB, Buyon et al (84) tried to determine the prevalence of anti-5-HT4 antibodies in mothers whose children have CHB. Although, the Authors (84) demonstrated mRNA expression of the 5-HT4 receptor in the human fetal atrium the electrophysiologic studies established that human fetal atrial cells express functional 5-HT4 receptors they failed to find reactivity to 5-HT4 receptors (84).

C. Ca channel hypothesis

The formulation of Ca channel hypothesis was driven by the fact that AV node electrogenesis is under the control of L-type Ca channel, which is responsible for conduction between the atria and the ventricle. Inhibition or blockade of this channel ultimately leads to AV block reminiscent of conduction abnormalities seen in CHB. The Ca channel hypothesis states that circulating maternal antibodies recognize L-type Ca channel pore-forming protein a1-subunit to which they bind and prevent Ca ions from entering the myocyte (78). Consequently, conduction of the SA impulse through the AV node to the ventricle will be hampered leading to delay in conduction or worst to complete AV block.

Two L-type Ca channels expressed in the heart are Cav1.2 (a1C) and Cav1.3 (a1D). Cav1.2 or a1C is ubiquitously expressed in the heart and essentially mediates cardiac excitation–contraction coupling. Cav1.3 or a1D, on the other hand, is restricted to the supraventricular tissue in the adult with the highest expression in the SA node and AV node.
Boutjdir et al (65, 72, 85-87) have demonstrated that IgG purified from mothers of children with CHB inhibit the ICa-L by 62%, 46.2% and 51% in ventricular myocytes, SA and AV nodal cells, respectively, meanwhile had no effect on the transient outward K current the delayed rectifier K current and the fast Na current indicating specificity for Ca channels. Experimental data supporting a possible cross-reactivity of maternal anti-Ro/La antibodies with the α1C and α1D calcium channel subunits have been provided by the same group (85-88). In particularly has been shown that the Ca channel α1D subunit is expressed in human fetal hearts (87). Furthermore, has been demonstrated that a fraction of sera from mothers of children with congenital heart block react to the extracellular loop of the calcium channel α1D subunit and that these maternal antibodies can inhibit α1D calcium currents in vitro (88). A chronic effect of maternal antibodies on the fetal heart has been proposed to be mediated by binding of antibodies to calcium channels and subsequent internalization and degradation, leading to not only inefficient signal conduction but also insufficient excitation-contraction coupling and reduction of cardiac contractile function (72). In support of this hypothesis, mouse pups transgenic for the L-type calcium channel subunit Cav1.2 were found to develop AV block and sinus bradycardia at a lower frequency than non-transgenic littermates following in utero exposure to anti-Ro/La antibodies in an immunization model (89, 90). 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, nonetheless at a low frequency, and the occurrence of AV block is increased following immunization of females with the Ro and La protein before gestation (88). Although this study does not prove that maternal anti-Ro/La antibodies directly cross-react with subunits of the L-type calcium maternal autoantibodies exert their pathogenic effects at least in part by affecting calcium homeostasis in the heart and disrupting the cardiac electric and contractile functions. In addition Salomonsson et al (52) demonstrated that
p200-specific anti-Ro52 antibodies dysregulated calcium oscillations of spontaneously beating cardiomyocytes in culture.

**Figure 3.** A two-phase model for the development of congenital heart block.(Ambrosi and Wahren-Herlenius. Arthritis Research & Therapy 2012).

Along with, these data support the hypothesis that maternal anti-Ro52 antibodies may cross-react to a fetal cardiac molecule involved in calcium regulation and initiate cardiac conduction disturbances, detected as first-degree AVB. It is possible that prolonged disruption of cardiac calcium homeostasis leads to increased apoptosis in the fetal heart (52) is then accompanied by exposure of the intracellular Ro and La proteins and subsequently the opsonized apoptotic debris engulfed by macrophage may lead to production of pro-inflammatory and pro-fibrotic cytokines which, together with antibody deposits and recruitment of complements component, will generate a sustained inflammatory reaction in the fetal heart, eventually leading to a permanent damage and complete CHB (Figure 3).
1.4.3 Maternal and fetal susceptibility factor for congenital heart block

Since 1980s the anti-SSA/Ro and anti-SSB/La have been associated with CHB. However, the incidence of CHB is estimated to be only 1-2% CHB of anti-SSA and/or anti-SSB positive primigravid women (24) and carries a recurrence rate around 20%, in subsequent pregnancies despite the persistence of the autoantibodies (25) suggesting that antibodies are necessary but not sufficient to provoke autoimmune-associated CHB and other maternal and/or fetal factors may be involved in the susceptibility to CHB.

There are several reports of dizygotic twins discordant for disease suggesting that there may be a genetic fetal factor involved in susceptibility to CHB (2, 25, 91-93). There are, also reports of discordant disease expression in monozygotic twins (94) and in ovodonation pregnancy (95) indicating that not only fetal genetics are responsible for induction of disease.

Genetic polymorphisms influencing fetal susceptibility to CHB in anti-SSA/Ro and/or anti-SSB/La-positive pregnancies were first investigated in a group of 40 children with CHB by using a candidate gene approach, focusing on two known polymorphisms of the genes encoding the pro-inflammatory and pro-fibrotic cytokines tumor necrosis factor-alpha (TNFα) and transforming growth factor-beta (TGFβ). The TGFβ polymorphism assessed was found significantly more frequently in children with CHB than in their unaffected siblings, whereas the TNFα polymorphism studied was found at an increased frequency in both affected and non-affected children in comparison with healthy controls (96). Recently, the same group (97) performing a genome-wide association study of individuals who have CHB and who were born to anti-SSA/Ro and/or anti-SSB/La-positive mothers demonstrated a significant association with polymorphisms in the HLA region and at the location 21q22 addressing a role of the identified SNPs in susceptibility to CHB. Recently, Strandberg and colleagues (71), using congenic rat strains and a Ro52 immunization model of heart block demonstrated an influence of both maternal and fetal MHC genes in
the development of CHB. The authors (71) showed that maternal MHC restricts generation of pathogenic anti-Ro52 antibodies and that the fetal MHC locus regulates susceptibility and determine the fetal disease outcome in anti-Ro52-positive pregnancies. Maternal and fetal factors other than genetic differences have also been suggested to contribute to the development of heart block. Although neither fetal gender nor maternal disease severity has been associated with CHB (98, 99) it has been proposed that maternal age may have an influence on the outcome of anti-Ro52-positive pregnancies (100). Ambrosi et al (4) in a population-based study, found that the risk for CHB increased with maternal age but was not influenced by parity. It is also possible that the increasing risk for heart block with increasing maternal age reflects the appearance or increased serum levels of anti-Ro/La autoantibodies in women over time. The authors (4) found, also, that the seasonal timing of the pregnancy influenced the outcome. There was an increased proportion of affected pregnancies among all pregnancies for which the susceptibility weeks (18 to 24 weeks of pregnancy) took place during the late winter season in Sweden. Whereas an association between the winter season, decreased sun exposure, and vitamin D levels comes readily to mind, other events linked to the winter season, such as viral infections, may also influence the development of heart block. Indeed, maternal infections occurring during pregnancy have been suggested to play a role in CHB, and a recent report by Tsang and colleagues (101) described cell surface exposure of the Ro antigen in fetal cardiomyocytes following cytomegalovirus infection. So, this clinical and experimental data suggest that CHB pathogenesis represents a complex interplay between immunologic, genetic, and environmental factors (Figure 4).
Figure 4. A model for the pathogenesis of CHB. Maternal MHC genes and maternal age determine the specificity of anti-SSA/Ro and anti-SSB/La antibodies. These specific antibodies in the presence of environmental factors and susceptibility fetal genes determine the initiate of immune process leading to inflammation, calcification and fibrosis. (Adapted from Ambrosi and Wahren-Herlenius. Arthritis Research & Therapy 2012).

1.5 Cardiac involvement in neonatal lupus

CHB was the first cardiac manifestation of NLS, described more than a century ago (15). Subsequently antibodies and complement deposits together with signs of fibrosis and calcification were described in the conduction fetal system as well as in the entire myocardium (8-10), suggesting the potential involvement of maternal autoantibodies in other cardiac manifestations of NLS (11-14).

1.5.1 Electrophysiological involvement

The conduction system in the human heart consists on sino-atrial node (SA node), the atrio-ventricular node (AV node), the His Bundle and branches and the Purkinje fiber (figure 5).

SA node situated in the right atrium is the principal pacemaker of the heart. The SA node generates the electrical impulse, which spreads rapidly over both atria, causing them to contract simultaneously. The AV node is the only electrical connection between atria and
ventricles. Its function is to transmit the electrical impulse from the atria to the ventricles, in order to contract the latter.

Figure 5. Conduction system in the human heart.

A. Atrio-ventricular block

The congenital atrio-ventricular block (AVB) is the most common and most recognized cardiovascular manifestation of NLS. An AVB is a block or delay in the signal conduction through the heart. It is hypothesized that CHB progresses gradually from a first-degree AVB to a second-degree AVB and finally this could progress to the irreversible third-degree AVB (7). In the first-degree AVB all the impulses are conducted from the atria to the ventricles, but there is a prolonged interval between the atrial and ventricular contraction (the AV-interval), beyond the upper limit of normal. The second-degree block refers to a failure to conduct some, but not all, atrial impulses to the ventricles. There are several types of second-degree block. In Mobitz type I (Wenckebach) second-degree AVB, there is a progressive lengthening of the AV conduction, until an isolated impulse is blocked. Mobitz type II second-degree AVB is characterized by a sudden block of an isolated impulse without prior lengthening of the AV conduction time. In 2:1 second-degree AVB, only every second atrial impulse is conducted to the ventricles. In third-degree AVB or complete AVB (CAVB) there is no AV conduction at all, and the atria and ventricles beat
independently. Ventricular rates in CAVB are typically between 50-70 beats per minute and atrial rates are usually normal (>120 beats per minute).

**B. Other arrhythmias and conduction abnormalities in NLS**

In addition to AVB, several other electrophysiological abnormalities have been reported in the fetus and infant with maternal autoimmu

ne-mediated cardiac disease. These abnormalities include both transient and persistent sinus node dysfunction, long QT interval, ventricular and atrial ectopy, ventricular and junctional tachycardia, and atrial flutter. Although several case reports and small case series have identified several arrhythmias and conduction abnormalities associated with AVB by foetal echocardiography, the frequency with which these rhythm and conduction disturbances occur has been appreciated best by foetal magnetocardiography (102). Recently, has suggested that fetal AVB is far more complex than previously appreciated with complex changing rhythms, variable atrio-ventricular conduction in second-degree AVB, abnormal QRS waveforms, co-existence of junctional and ventricular ectopy, and atrial and ventricular rate responsively in complete AVB (102).

**1.5.2 Myocardial involvement**

Although CHB is well tolerated in fetuses and neonates, has been shown that 15–20% of affected fetuses develop more diffuse myocardial disease before birth and others may clinically manifest myocardial dysfunction after birth despite adequate pacemaker therapy (12, 103-105). The echocardiographic appearance of more diffuse disease includes ventricular dilation and systolic dysfunction, myocardial hypertrophy, a non-compaction appearance to the fetal myocardium in some, and, most commonly, echogenicity of the endocardium confirmed in explanted hearts and at autopsy to represent endocardial fibroelastosis or EFE (Figure 6) (104, 105).
The EFE observed in some is patchy and may involve the crux of the heart, either ventricle (right more than left in the fetus and left more than right in the post-natal infant), chordal apparatus and papillary muscles and even one or both atria (13, 106). The true incidence of more diffuse myocardial disease remains unclear as the proportion of reported patients reflects only those with clinically recognized disease at the time of review. Some affected infants, for instance, evolve myocardial disease only later in life (12, 104). Moreover, the EFE detected echocardiographically often underestimates the degree of EFE based on the examination of corresponding pathological specimens (104). The observation of isolated EFE and cardiomyopathy, in the absence of conduction abnormalities has suggested that the more diffuse myocardial disease represents a separate manifestation of NLS (104). Histologically, has been shown maternal autoantibody induced EFE and cardiomyopathy to be associated with diffuse disarray of myocardial fibers with IgG deposition in all, IgM deposition and even T cell subset activation, the latter findings suggestive of a fetal immune response contributing to the disease process (104). Why more diffuse myocardial disease occurs in some but not all fetuses and infants with maternal autoimmune-
mediated CHB remains unclear, but variability in the fetal immune response may contribute (104, 107). In addition to myocardial disease, pericardial effusion without other signs of hydrops has been reported in some affected fetuses and could suggest the presence of pericarditis (108). Finally, recently has been reported an association between rupture of the AV valve tensor apparatus and maternal anti-Ro/SSA antibodies as a complication of fetal antibody-mediated cardiac inflammation (109).

1.5.3 Structural involvement

A spectrum of structural heart disease has been reported among fetuses and infants with maternal autoimmune-mediated cardiovascular disease. In children with maternal autoimmune-mediated CHB, structural congenital heart disease has been reported in 16–42% (92, 110). These lesions have included persistent ductus arteriosus most of which have required intervention, and atrial and ventricular septal defects. Of greater interest, semilunar and atrio-ventricular valve abnormalities have also been described in association with CHB, including stenosis, regurgitation and dysplasia without functional changes (92, 110, 111). Inflammation and fibrosis as well as haemodynamic changes could potentially contribute to the evolution of at least some of these lesions, as suggested in one case of acute chordal rupture with moderate mitral insufficiency in 7-week old infant with echocardiographic evidence of EFE involving left ventricular papillary muscles and chordae (111). However, the incidence of structural and even functional heart disease among infants of mothers with autoantibodies in the absence of CHB is still not certain.

1.6 Diagnosis and monitoring CHB

CHB is a gradually progressive condition (7) occurring during a critical time period and moreover has been observed the efficacy of fluorinated steroids in inhibiting the
progression of incomplete block (108, 112-115). Therefore, methods to detect first-degree CHB in early pregnancy have been developed.

1.6.1 Foetal electrocardiography
The fetal electrocardiography (ECG) is based on the acquisition of trans abdominal signals consist on individual ventricular depolarizations (QRS complexes). Notwithstanding, this technique does not allow identification of individual atrial depolarizations (P waves) (116-118) and therefore it is no possible to use as a surveillance technique at the moment. However, single studies (116, 117) have established reference ranges for cardiac time intervals reporting a higher success rate, though suggesting that fetal ECG might be the diagnostic tool of choice to detect prolonged PR intervals (119).

1.6.2 Foetal magnetocardiography
Fetal magnetocardiography has also been used to evaluate the PR intervals. This technique defines better than ECG, both atrial and ventricular depolarization’s (120, 121) and has provided important information regarding electrocardiographic time intervals, repolarization abnormalities (122) and complex rhythms in association with AVB (121), but it is expensive, requires a magnetically shielded room and is not suitable for routine surveillance of the fetuses.

1.6.3 Foetal echocardiography
Fetal echocardiography with M-mode and Doppler techniques is the gold standard method for prenatal diagnosis of fetal cardiac abnormalities. Using standard ultrasound echocardiographic equipment, atrial and ventricular depolarizations are identified indirectly by their mechanical [M-mode, Doppler tissue velocimetry (DTI)] or hemodynamic (Doppler) consequences. In M-mode, the systolic movements of an atrial wall and a ventricular free
wall or the aortic valve are detected. The limit of the M-mode recordings is that the onset and maximum of atrial and ventricular contraction is often not clearly defined, making it less suitable to diagnose first-degree AVB and even the second-degree AVB (123). Both experimental (124) and clinical (125) studies have demonstrated the superiority of the Doppler technique compared with the M-mode approach for measuring atrio-ventricular (AV) time intervals as a substitute for the electric PR interval in ECG. With Doppler technique, the PR interval can be measured recording signals from the mitral valve/left ventricular aortic outflow (MV-Ao) (123), the superior vena cava/ascending aorta (SVC-Ao) (124, 125) as well as a pulmonary vein and peripheral pulmonary artery (126). To note, all these Doppler techniques are angle dependent, though fetal position frequently decides which technique will give the best result. Therefore Sonesson et al (127) have also included recordings from the pulmonary trunk and ductus venosus when diagnosing foetuses with bradyarrhythmias. Doppler recordings of the ductus venosus are obtained in a midsagittal or transverse section as previously described (128). DTI is usually performed using an apical view of the atria and ventricles (4-chamber view) to obtain atrial and ventricular wall motion velocities for later off line analysis (113, 118). Most frequently, data recorded from the right ventricular free wall at the level of the tricuspid valve is used to identify the A’ wave as marker of atrial depolarization, and a point corresponding to the start of right ventricular isovolumetric contraction to denote ventricular activation (113, 118).

All the women positive for anti-SSA/Ro and or anti-SSB/La are followed with weekly echocardiography during the critically window of developing CHB and a PR interval>150 msec is considered pathologic. However, there is a clear need to identify an early marker of irreversible cardiac damage and progression to CAVB; there are not performed prospective controlled studies to answer this issue. In the multicenter PRIDE study (112) comprising 98 RoSSA antibody-positive pregnancies, the prolongation of the PR interval
was uncommon and did not precede more advanced block. The Authors (112) conclude that advanced block and cardiomyopathy can occur within 1 week of a normal echocardiogram without initial first-degree block. Moreover, echo densities and moderate/severe tricuspid regurgitation merit attention as early signs of injury. These findings were confirmed by Bergman et al (129) recently in single-centre study of 95 fetuses.

1.7 Treatment of CHB
The conventional therapy employed for CHB is fluorinated steroids, such as Betamethasone or Dexamethasone, which are only partially inactivated by the placental 11ß-hydroxysteroid-dehydrogenase complex, and thus have satisfactory bioavailability to the foetus. The rationale behind this treatment is to diminish the inflammatory component in the foetal conduction system and myocardium and, thus, to reduce cardiac injury. It has been demonstrated that steroid therapy could reverse the first degree AVB on a normal sinus rhythm (115, 130), but there is no evidence of its efficacy in reversing the 2nd degree AVB (112). Moreover it has been shown that the 3rd degree CHB is always irreversible (115), but steroid therapy may prevent the fetal complications such as heart failure, cardiomyopathy and hydrops related to.

The number of associated therapies, such as plasmapheresis or intravenous immunoglobulins (IVIG), is limited and not thoroughly investigated. Plasmapheresis (PF) (Figure 7) removes part of plasma of the patients by means of special separators and replaces it with a fluid replacement (4-5% albumine saline, plasma, or plasma-expanders). The rationale use of PF in CHB is the removal of pathogenetic maternal autoantibodies. PF lowers the levels of anti-Ro/SSA and anti-La/SSB antibodies in maternal blood inhibiting their transplacental transfer and preventing the damage they cause to the foetal heart. PF has until now been taken into consideration mainly to prevent
CHB and there are only few reports concerning its use in diagnosed mothers (108, 131-136). The procedure has, moreover, always been performed in conjunction with steroids.

**Figure 7.** Plasmapheresis technique.

IVIG is a unique immune-modulating therapy that has a wide range of effects on the immune system at multiple levels; nonetheless the exact mechanism of action is still unclear. Probably IVIG suppress harmful inflammation by a pleiotropic mechanism of action (137) that include: anti-idiotype regulation, inhibition of placental transport of maternal autoantibodies, accelerated clearance of anti-Ro/SSA and anti-La/SSB antibodies and modulation of inhibitory signals on macrophages with consequent reduction of the inflammatory response and fibrosis in the foetal heart. When employed as a preventive treatment for autoantibody-mediated CHB, it was incapable, at least at the dose and timing utilized, to prevent recurrence (138, 139). However, A few attempts have been made to treat women diagnosed with CHB utilizing IVIG in conjunction with or without steroids (136, 139-141). In all except one of these studies (136) the use of IVIG was not systematic throughout the pregnancies, but restricted to a single cycle. There were no signs of improvement (139) with the exception of a reversion from a 2\textsuperscript{nd}/3\textsuperscript{rd} degree block to a predominant sinus rhythm associated to an intermittent 2\textsuperscript{nd} degree block during one
pregnancy with a progression to a 2nd/3rd degree block at birth [140] or were limited to the resolution of echocardiographic signs of myocarditis (141). When IVIG were systematically employed in conjunction with plasmapheresis and betamethasone beginning from the time CHB was detected until birth, two cases of 2nd degree block reverted to normal atrioventricular conduction (136).
2. AIMS OF THE THESIS

To investigate the epitope specificity of antibodies to the p200 peptide (Part I):
To explore the role of anti-Ro52 antibodies in the pathogenesis of CHB by identifying the epitope specificity of human anti-Ro52-p200 antibodies using mutated human p200 peptides, rat-to-human p200 mutants and alanin scan p200 peptides.

To develop an animal model of heart block (Part II):
To study the effect on heart conduction of purified human IgG antibodies from a CHB patient mother in an animal model of CHB based on passive transfer of IgG.

To evaluate the efficacy and safety of a combined treatment protocol for CHB (Part III):
The objective was to evaluate the efficacy and safety of a combined therapy protocol utilizing weekly plasmapheresis, fortnightly IVIG and daily betamethasone in six cases of CHB during pregnancy and IVIG fortnightly in neonates after birth in order to improve outcomes in the treatment of this devastating disease.

To investigate the effect of the combined treatment protocol on antibody levels (Part IV):
This study aimed to evaluate the effect of the combine treatment protocol on anti-Ro52, anti-p200 and anti-La antibody levels.
Part I
EPITOPE SPECIFICITY OF ANTI-Ro52 ANTIBODIES

1. **Patient and methods**

1.1 **Study population**

Anti-SSA/Ro and/or anti-SSB/La-positive sera from 19 mothers of foetuses with CHB and 8 babies with CHB attending the Rheumatology Unit of University-Hospital of Padua were collected prospectively from 2001-2012 and stored at -80°C.

1.2 **Synthetic peptides**

Eighteen different synthesized peptides were used in this study (Figure 8). Peptide sequences are illustrated in Figure 9A, 10A, 11A, 13A and 14A. Briefly, human p200 peptide corresponding to amino acid 200-239 of the human Ro52 protein and containing the main CHB related epitope was included, as well as the corresponding rat and mouse p200 homologues. Further, human p197 corresponding to amino acid 197-232 and three truncated peptides representing N-term, mid and C-terminal parts of the human p200 peptide were used. Additionally, three peptides based on the rat p200 sequence with mutations of crucial amino acids to the residues present in the human sequence were included (LEK H8, LEK E18 and LEK E34), Figure 13. Finally, peptides pA233–pA239 representing an alanine scan with single amino acid residue mutations of the identified c-terminal epitope of p200 was assayed (Figure 14). N-term, mid, C-term and the LEK peptides were synthesized at the Department of Medical Biophysics, Linköping University, Linköping, Sweden. All other synthetic peptides were purchased from Thermo BioSciences, Ulm, Germany. Peptide purity was confirmed by high-performance liquid chromatography and mass spectrometry.
1.3 Detection of anti-peptide antibodies

Anti-peptide antibodies were detected with an in house ELISA as follows: High-binding 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 1 µg of peptide diluted in carbonate buffer, pH 9.6, per well. Plates were washed three times with PBS/0.05% Tween-20 (TPBS) and blocked with 200 µl of TPBS/5% milk powder per well for 1 h.
three washings with TPBS, the plates were incubated with patient sera diluted 1:500 in PBS/0.05% Tween-20/1% milk powder for 2 h. Plates were washed three times in PBS/0.05% Tween-20 and then incubated with AP-conjugated rabbit-antihuman IgG antibody (Dako). Plates were washed again three times, and phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer pH 9.8 was added. Absorbance was measured at 405 nm after 2 h. All steps were performed in room temperature except coating which was performed at +4°C.

The specificity and affinity of the antibody to the peptides were analyzed by ELISA in competition experiments, including a step of preincubation of diluted sera with peptides in concentrations ranging from 0.1-1 mg/ml for 1 h at 20°C prior to analysis in ELISA as described above.

1.4 Circular dichroism spectroscopy

Circular dichroism (CD) spectra of peptides were recorded with an AVIV 62DS spectrometer. All spectra were analysed at 25°C over the wavelength range 195–250 nm with a step size of 1 nm, a bandwidth of 1.5 nm, an average collection time of 2 s per point, an equilibration time of 1 min in a 0.1-cm cuvette. The CD spectra were averaged from four wavelengths scans and blanked against the used buffer (0.1% TFA buffer).

1.5 Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U-test. A p-value <0.05 was considered significant.
2. Results

2.1 Specificity of anti-Ro52 antibodies for p197 and p200 peptides

The human Ro52-p200 peptide has previously been described to contain the main epitope of CHB-related pathogenic antibodies. To identify the epitope/epitopes bound by sera from mothers of children with CHB within this 40 aa peptides several different strategies were used. First, reactivity to peptide p200 and peptide p197 with a high overlap but differing in the C- and N-terminal ends was analyzed (Figure 9A). Sera from mothers of babies with CHB (Figure 9B) and of the babies with CHB (Figure 9C) showed a significantly higher reactivity towards p200 than to p197. The difference in antibody binding to p200 and p197 could be explained by either sequence differences and thereby lack of amino acids forming the epitope or by structural differences in their secondary structure as was reported previously (143). Studying the secondary structure with CD was demonstrated that p200 has a substantially higher α-helical contribution than p197 (143).

2.2 Reactivity of anti-p200 antibodies to mutated p200 peptides

To understand whether the difference in binding of CHB sera to p200 and p197 peptides depended on sequence or folding differences three truncated peptides representing different parts of p200 were synthesized. N-term, mid and C-term peptides were derived from the p200 sequence, with the N-term peptide lacking 15 aa in the 3’ terminal of the peptide and 4 in the 5’ end, the mid peptide lacking 7 and 13 aa respectively in the 3’ and 5’ terminal of the peptide and the C-term peptide lacking 22 aa in the 5’ terminal of the peptide (Figure 10A). To investigate how anti-p200 antibodies from patient sera reacted with the mutated synthetic peptides, sera from mothers of children affected by CHB and babies with CHB were analyzed. The profile of reactivity was surprisingly similar. The mean value levels are reported in Figure 10B. Interestingly, the deletion of amino acids in
the C-terminal of p200 completely abolished the p200 reactivity, while the deletion of amino acids in the N-terminal only slightly decreased the p200 reactivity, thus supporting a conclusion that amino acids in the C-terminal end of the p200 peptide contain the dominant epitope.

\[
\begin{align*}
\text{A} & \quad \text{LQELEKDEREQLRILGEKEAKLAAQQSQALQELISEL} \\
\text{p197} & \quad \text{LEKDEREQLRILGEKEAKLAAQQSQALQELISELDRRCHSS} \\
\text{p200} & \\
\text{B} & \\
\text{C} &
\end{align*}
\]

**Figure 9.** (A) The p197 and p200 sequence. (B) The reactivity to p200 and p197 in the CHB mothers (n.19) and (C) the reactivity to p200 and p197 in the babies with CHB (n.8).
2.4 Reactivity of anti-p200 antibodies to p200 “rat-to-human” peptides

In the attempt to precisely define the epitope specificity, we took advantage of the fact that there is high homology between human, rat and mouse Ro52-p200 sequences (Figure 11A), but that while CHB sera bind well to human p200, they bind significantly less to rat and mouse p200, Figure 11B. Reasoning that peptide folding in p200 homologues was likely to be stable and similar to folding of the human p200 peptide, and that this folding would not be disturbed if mutations to amino acids occurring naturally in other species were introduced, we decided on a strategy for epitope identification introducing mutations going from rodent p200 sequence to human p200 sequence at amino acids we predicted would affect antigenicity.
Figure 11. (A) The h-p200, r-p200 and m-p200 peptides structure. (B) The p200 reactivity in different species expressed as the mean value levels of both mothers (n.19) and babies (n.8) sera tested against the different peptides.

We chose to start with the rat p200 peptide as the CHB serum binding was low, but the sequence more similar to human p200 than for the mouse p200, thus requiring fewer mutations for our purpose. The selection of amino acids to mutate was based on their chemical properties (charge, polarity or bulky side-chain), and their predicted position based on a helical wheel sketching assuming an alpha-helical fold of the peptide (Figure 12). Three mutated peptides rat-to-human p200 were generated and denoted LEK H8, LEK E18 and LEK E34. The peptides were identical to rat p200, with the exception that a set of selected amino acids in the N-terminal (LEK H8), mid (LEK E18) and C-terminal (LEK E34) were substituted for the respective amino acids of the human p200 peptide sequence (Figure 13A).
To investigate how anti-p200 antibodies from patient sera reacted with the mutated synthetic peptides, sera from mothers of children affected by CHB and babies with CHB were analyzed. The profile of reactivity was surprisingly similar, exemplified in Figure 13B, although levels in the individual sera varied. We observed that almost full anti-p200 reactivity was gained with the substitutions in the LEK E34 peptide, including a glutamic acid substituted for an aspartic acid in amino acid position 233 (Figure 13B) localized in C-terminal of the p200 peptide, while there was no gain of reactivity by the substitutions in the N-terminal or mid part of the peptide (Figure 13B).

Figure 12. (A) Model of a hypothetical dimer of the 200–239 stretch of Ro52. (B) Schematic helical wheel representation of the leucine zipper region of Ro52 [amino acid (aa) 198–240] oriented in a parallel homodimerization.
A

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<tr>
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<th>p200</th>
<th>LEKDEREQLRILGEKEA KL AQQ SQALQELIS ELDRR CHSS</th>
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<tr>
<td>LEK H8</td>
<td>LEKDEREQLRILGEKEAELAEKNQLQELISELRRSRS GS</td>
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<td>LEK E18</td>
<td>LEKDEREHLRLLGEKEAKLAQQ SQALQELISELRRSRS GS</td>
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<tr>
<td>LEK E34</td>
<td>LEKDEREHLRLLGEKEAELAEKNQ ALQELISELD RR CHSS</td>
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<td>r-p200</td>
<td>LEKDEREHLRLLGEKEAELAEKNQALQELISELRRSRS GS</td>
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**Figure 13.** (A) The p200, r-p200 and synthetic rat-to human p200 mutated peptides sequence. (B) Antibody reactivity in a single serum tested against the synthetic peptides.

Analysis by circular dichroism (CD) confirmed that introduction of the substitutions did not disrupt folding of the LEK peptides compared to the r-p200 peptide (Figure 14 e 15). These data clearly support the presence of a dominant epitope in the C-terminal of the p200 peptide.
Figure 14. Secondary structure analysis of the overlapping peptides p200 (A) and p197 (B), of r-p200 (C) and of the rat to human mutated peptides LEK H8 (D), LEK E18 (E) and LEK E34 (F).

Figure 15. Secondary structure analysis of the human p200 (hp200) and the p200 rat-to-human mutated peptides LEK E34 by circular dichroism spectroscopy (CD). The CD spectra of hp200 and LEK E34 are obtained at room temperature and pH 7.6.
Finally, to verify the epitope and pinpoint the crucial amino acid/s we performed an alanine scan of the C-terminal section of the human p200 peptide. The alanine scan included mutated peptides pA233–pA239 that were identical to p200, with the exception that one amino acid residue in each peptide (aa 233 in pA233, aa 234 in pA234, etc.) was substituted for an alanine in the C-terminal side of p-200 (Figure 16A). Interestingly, we observed that the substitution of the aspartic acid 233 in the pA233 peptide abolished the antibody binding (Figure 16B), while the other mutations did not affect the binding. This mutation at position 233 of the alanine scan (pA233) substitutes the same amino acid which was mutated in position 233 of the rat-to human mutated peptide (LEK E34). The aa is an aspartic acid and in the helical wheel representation of the leucine zipper it is localized on the outer surface of the modeled peptide homodimer (Figure 12A and B). Thus it is probably accessible for antibody binding, and we suggest it constitutes the crucial amino acid for binding of CHB sera to the p200 peptide.

2.5 Specificity for anti-p200 antibodies for LEK E34 peptide

To confirm the specificity of anti-p200 antibodies for the aspartic acid at aa position 233 we performed inhibition experiments based on preincubation and testing in ELISA. In these experiments, the anti-p200 reactivity was decreased by pre-incubation with soluble LEK E34 in a concentration-dependent manner (Figure 17) to a degree similar to pre-incubation with p200 while pre-incubation with r-p200 and PBS did not decrease the anti-p200 reactivity.
Figure 16. (A) The p200, p197 and synthetic mutated peptides structure. (B) Antibody reactivity expressed as the mean value levels of the sera of both mothers (n.19) and babies (n.8) sera tested against the different peptides.
Figure 17. Specificity of the LEK E34 antibody to p200 peptide. Sera diluted 1:2000 were pre-incubated with peptide or PBS for 60 min at room temperature before analysis in ELISA with human p200 peptide.

### 3. Discussion

CHB is a gradually progressive disease (7). So, we need a marker of high predictability to identify high-risk pregnancies and allow initiation of treatment at the critical stage to prevent irreversible CHB in the fetus. In the attempt to identify the epitope specificity of pathogenetic antibodies a set of mutated peptides were developed. As previously reported (50), we confirm that antibodies against the p200 peptide of Ro52 are associated with CHB, whereas almost no antibody response against the largely overlapping p197 peptide (aa 197–232) can be detected in sera from the mothers of affected children. The difference in binding might be explained by p200 carrying an epitope on the differing seven amino acids at the C-terminal although previous investigations could not distinguish between this possibility and that of structural disruption and differing folding between the peptides.

To investigate whether the difference in reactivity against the two different peptides was due to the non-overlapping part of p200 (aa 233–239), we generated 3 human p200 mutated peptides, N-term, mid and C-term and interestingly the deletion of amino acids in
the C-terminal end of p200 completely abolished the p200 reactivity, suggesting that aa 233-239 are important for antibody binding as binding occurred in this short and assuming unstructured peptide. To identify the amino acids contributing to the epitope we took advantage of the fact that the reactivity against rat and mouse p200 was low despite high homology. We generate 3 peptides representing human-to rat p200 mutations, LEK H8, LEK E18 and LEK E34. Anti-p200 reactivity was gained in the LEK E34 mutant which included a glutamic acid substitution for an aspartic acid in position 233 localized in the C-terminal of the p200 peptide, while there was no reactivity gain when amino acids were substituted in the N-terminal or mid sections. To pinpoint the amino acid/s contributing to the epitope we performed an alanine scan of this region by substituting one amino acid at a time for an alanine and interestingly the substitution of the aspartic acid in the position 233 (pA233 peptide) totally abolished the antibody binding. This mutation at position 233 of alanine scan (pA233) thus substitutes the same aa which was inserted in position 233 of rat-to human mutated peptide (LEK E34). Moreover, in competition experiment the pre-incubation of the sera with LEK E34 peptide block antibody reactivity to p200 to the same degree as the p200 peptide itself. Furthermore, investigating the secondary structure of the rat-to-human peptides by CD we show that all of LEK peptides increase the structured content, confirming that the mutations do not destroy the peptide structure. Further, the 233 aa is localized at the outer surface of the leucine zipper helical wheel modelled peptide homodimer, suggesting that is readily accessible for antibody binding.

4. Conclusion

We suggest that the aspartic acid at amino acid residue position 233 of the Ro52-p200 peptide is crucial in forming the main epitope of the Ro52-p200 peptide bound by CHB-related human Ro52 antibodies. This specificity might relate to the pathogenic process in CHB (addressed in Aim 2), and might be used as a tool to identify high risk pregnancies.
for CHB. However, it remains to investigate whether this epitope is specific for CHB-inducing sera, or whether it is also bound by sera from anti-SSA/Ro-positive patients without CHB. A difficulty in such analyses is the established low recurrence rate of 10-20%, demonstrating that pathogenic antibody may well be present without CHB development and making prediction based on serology alone difficult. Our results might still contribute to identification of an antibody specificity more closely associated with development of CHB than previously used markers, and provides the possibility to explore the role of this antibody in the pathogenesis of CHB and to generate possible drugs to prevent CHB. In fact, we could speculate on the possibility to generate a peptide, which binds to and absorb the circulating pathogenetic antibodies to prevent them from entering the fetal circulation.
Part II
DEVELOPMENT OF AN ANIMAL MODEL OF HEART BLOCK

1. Methods

1.1 Experimental animals

Dark Agouti (DA) rats (Charles-Rivers, Germany) were kept and bred in the Animal Department at the Center for Molecular Medicine at the Karolinska Institutet. All experimental protocols were approved by the Stockholm North Ethics Committee.

1.2 Purification of human IgG autoantibodies

Immunoglobulin fractions containing IgG from one mother with a child with CHB and from a healthy control donor were purified from plasma by protein A-Sepharose gel. Shortly, 20 ml of plasma from the patient and 20 ml from the healthy control were first centrifuged at 10,000 × g for 10 min to remove any precipitates, and then applied to HiTrap Protein A HP columns (GE Healthcare) in a chromatography system (ÄKTA, Sweden) to purify the IgG. PBS was used as binding buffer and the elution buffer was 0.1 M citric acid, pH 3.5. The eluate was collected in 1.5 ml fractions, and immediately neutralized by adding 150 µl of 1 M Tris-HCl, pH 9.0 per ml of fraction. Protein concentration was measured by the Bradford assay and retained Ro52 binding activity assessed by ELISA.

1.3 Experimental procedures

1.3.1 Antibody transfer

Female DA rats, 15 weeks old, were injected intra peritoneally (i.p.) with antibody or vehicle on day 7 after mating unless specified otherwise (Figure 18). The pregnant female rats were injected with either purified CHB-mother IgG (4 or 2 mg, n=3 and n=3, respectively), healthy donor IgG (4 or 2 mg, n=3 and n=3 respectively), CHB-inducing mouse monoclonal Ro52-antibody Ab31 (4 mg, n=2) (75) or vehicle (PBS, n=2).
1.3.2 Anesthesia procedure

Adult animals were anesthetized with Isoflurane (Baxter). First, the animals were put in a chamber ventilated with an Isoflurane concentration of 3% with air and O₂ pressure at 1.5 l/min for 2 minutes, and then kept on mask and ventilated with an Isoflurane concentration at 1-2% with air and O₂ pressure at 0.25 l/min.

1.3.3 Ultrasound recordings

Ultrasound (US) recordings were performed using an S2000 ultrasound system with a high density linear probe 18 MHz (Siemens medical solutions, Ultrasound Division, Mountain View, CA, USA) at 14 days of gestation (Figure 18), assessing the number of pregnancies and if the pups were alive or not. In live pups, Doppler recordings measuring the heart
rate, atrio-ventricular (AV) time, isovolumetric contraction time (ICT), isovolumetric relaxation time (IRT), ejection time (ET) and myocardial performance were performed.

1.3.4 ECG recordings
Three-lead electrocardiograms (ECGs) were recorded within 24 h of birth on conscious non-anesthesized pups using four microelectrodes attached to a body clip (Figure 18). ECGs were sampled for 5 s four times a minute, with a sampling rate of 1000 Hz. The ECGs were digitalized and analyzed with Pharmlab (AstraZeneca, Mölndal, Sweden). QRS complexes were averaged and used to calculate the PR interval. PR intervals were corrected for heart rate (HR) variation by expressing them as PR/√RR.

1.4 Blood collection from animals and ELISA assay
Blood samples were obtained from both mothers and pups. Blood specimens were centrifuged at 3000 rpm for 15 minutes and analyzed by ELISA for antibody levels as described above (Part I), excepted that rodent samples were incubated with an AP-conjugated rabbit-anti rat IgG antibody (Dako) or AP-conjugated rabbit-anti mouse IgG antibody (Dako). Antibody levels are expressed as OD values x100.

1.5 Statistical Analysis
Statistical analysis was performed using the Mann-Whitney U-test. A p-value <0.05 was considered significant.

2. Results
2.1 The echocardiography findings in rats fetuses
With the aim to investigate the effects of human Ro/La antibodies on the fetal heart to understand the pathogenesis of CHB, an animal model with assessment of fetal cardiac
function both during and after pregnancy was developed. Different doses of purified human IgG from a CHB-mother or healthy donor was administered i.p. to pregnant rats at GA d7. US and Doppler examinations were performed at approximately 14 days of gestation. Significant bradycardia and AV time prolongation were present in the group injected with 4 mg patient IgG compared to pups injected with 4 mg control IgG or PBS (Figure 19A and B). Moreover, the pups in the group injected with 4 mg patient IgG showed a significant prolongation of ICT and ET time compared to the group injected with 4 mg control IgG (Figure 19C and E). Furthermore, pups in the group injected with 4 mg patient IgG had a significantly higher MPI (Figure 19F) than the group injected with 4 mg control IgG. It was not possible to compare the data with the PBS group due to the absence of available recordings in the experiment. We did not observe bradycardia and/or prolongation of AV time (Figure 20A and B) in the group injected with 2 mg patient IgG. However, also in this group, with lower injected amount of IgG, a significant prolongation of ICT, IRT and ET time (Figure 20C, D, E) compared to the group injected with 2 mg control IgG was observed. Also, there was a significant increase of MPI (Figure 20F) with respect to the group injected with 2 mg control IgG. It was not possible to compare the data with the PBS group due to the absence of available recordings in the experiment.

We also observed that in the group injected with 4 mg patient IgG one of the rats had nine dead fetuses, while there were no dead fetus in the group injected with 2 mg patient IgG. Among the rats injected with control IgG, there was only one dead fetus in the group injected with 2 mg.

Significant bradycardia (p=0.008) and AV time prolongation (p=0.01) were present in the group injected with 4 mg CHB-inducing mouse monoclonal Ro52-antibody Ab31 compared to pups injected with PBS. Moreover, was observed a significant prolongation of ET time (p=0.004) too; it was not possible to compare the data regarding ICT, IRT and MPI with the PBS group due to the absence of available recordings in the experiment. There was no
significant difference in AV, ICT, IRT, ET time and MPI in the group injected with 4 mg patient IgG compared with the group injected with 4 mg CHB-inducing mouse monoclonal Ro52-antibody Ab31, except a significant bradycardia (p=0.04).

2.2 ECG recordings in the pups of rats
As shown in Figure 21A the pups from the group injected with 4 mg patient IgG present a significant reduction of mean heart rate than the group injected with 4 mg control IgG. Moreover, the pups from the 4 mg patient IgG group present a significant prolongation of the PR and RR intervals compared with the group injected with 4 mg IgG control sera (Figure 21B and C). The pups from the group injected with 4 mg CHB-inducing mouse monoclonal Ro52-antibody Ab31 present a significant prolongation of PR interval (p=0.0005) compared to the group injected with PBS. Moreover, the pups from the group injected with 4 mg CHB-inducing mouse monoclonal Ro52-antibody Ab31 showed a significant higher heart rate (p=0.02) then the group injected with 4 mg patient IgG. While there was no significant difference on PR and RR intervals between the group injected with 4 mg CHB-inducing mouse monoclonal Ro52-antibody Ab31 and the group injected with 4 mg patient IgG.
Figure 19. Echocardiographic recordings from the pups from 4 mg patient IgG group (n=8), 4 mg control IgG group (n=25), 4 mg monoclonal antibody Ab31 group (n=7) and PBS group (n=18); AV=atrio-ventricular; ICT=isovolumetric contraction time; IRT=isovolumetric relaxation time; ET=ejection time; MPI=myocardial performance index.
Figure 20. Echocardiographic recordings from the pups from 2 mg patient IgG group (n=14), 2 mg control IgG group (n=12), 4 mg monoclonal antibody Ab31 group (n=7) and PBS group (n=18); AV=atrio-ventricular; ICT=isovolumetric contraction time; IRT=isovolumetric relaxation time; ET=ejection time; MPI=myocardial performance index
2.3 The anti-Ro52 reactivity in pups

Pups in both groups injected with patient IgG had significant higher reactivity to Ro52 than groups injected with healthy donor IgG (p<0.0001). Surprisingly, the group injected with 2 mg patient IgG had significant higher levels of Ro52 antibodies than the group injected with 4 mg IgG patient sera (p=0.001). This may depending on the timing of blood collection from pups before/after suckling.
3. Discussion

In this study we demonstrate that injecting pregnant rats with human polyclonal IgG from a patient with a child affected with CHB results in bradycardia and in prolongation of AV-time in a dose dependent manner.

In fact the group injected with 4 mg patient IgG presented a significant bradycardia and prolongation of AV time together with a decreased cardiac performance due to the prolongation of ICT and ET and an the increase of MPI in the US recordings. Moreover, a significant bradycardia and a prolongation of PR/SQRT RR and RR intervals in the ECG recordings were observed. These results confirm observations in studies of children affected with CHB that reported an association between high levels of anti-SSA/Ro
antibodies and risk of developing CHB (49, 50, 54). Moreover, a prolongation of ICT time, sign of decreased cardiac performance due to the reduction of myocardial contractility, was observed by Bergman et al (144) in a large proportion of fetuses of mothers with positivity for anti-Ro52. Also, the authors reported a prolonged AV time in this fetuses then in those of negative mothers. So, the similarities of different cardiac measurements between our animal model and of those of human studies confirm that it is a really valid model capturing many of the features of the human disease, and could be useful for this to better understanding the pathogenicity and exploring novel therapeutical approaches of CHB. The group injected with 2 mg patient IgG presented only a significant prolongation of ICT, IRT and ET together with an increase of MPI in the US recordings; as a consequence 2 mg patient IgG injection is associated only with a decreased cardiac performance. This is probably due to a mild myocardial injury without involvement of the cardiac conduction system. These results confirm the findings by Bergman et al (144) showing signs of decreased cardiac performance in human fetuses exposed to anti-Ro52/SSA antibody.

Finally, we did not observe third degree AVB, maybe this has occurred as pups die when they develop it and/or maybe we had not the right genotype. We can try different strains in the future, perhaps based on what human genetics will identify as susceptibility genes. In fact, there has been reported that beside the maternal MHC controlling antibody specificity, fetal MHC-encoded genes influence fetal susceptibility to CHB (71, 97).

4. Conclusion

Our data suggest that an animal model for CHB can be established by a simple technique of passive transfer of human IgG from a patient with a child with CHB. This model will be useful for exploring and understanding the pathogenesis of CHB induced by human Ro/La antibodies and to evaluate novel therapeutic approaches and drugs. Further, data obtained in this animal model of CHB suggest that high levels (4 mg) of anti-SSA/Ro and
anti-SSB/La antibodies induce bradycardia, AV time prolongation and a decreased cardiac performance. Even at low levels (2 mg) these antibodies cause a decrease in cardiac performance.
Part III
1. Patients and methods

1.1 Study population

Six consecutive cases of anti-SSA/Ro and anti-SSB/La-related CHB; 3 cases with 2nd degree CHB and 3 cases with 3rd degree CHB attending the Rheumatology Unit of University-Hospital of Padua were treated with betamethasone, plasmapheresis, and IVIG. Moreover, the newborns were also treated with IVIG soon after birth and continued until maternal antibody levels became undetectable. The human ethics Committee of the University of Padua Medical Centre approved the study protocol. The patients were informed about the disease risks and had signed a written consent form.

1.2 Transplacental treatment strategy

A. Maternal Steroid Therapy

Oral Betamethasone (4 mg/day) was prescribed to pregnant women diagnosed with CHB. After delivery Betamethasone was switched to Prednisone (25 mg/day), a therapy which was gradually tapered during the following 7 weeks unless the mother’s clinical condition required continuous steroid treatment.

B. Plasmapheresis Treatment Protocol

Plasmapheresis sessions were performed in the Apheresis Unit of Blood Bank of the University-Hospital of Padua. The procedures were carried out using a Cobe Spectra (Gambro BCT, Lakewood, Co, USA), a continuous blood cell flow separator, according to the following timetable: daily sessions at onset for the first 2 days, and thereafter it was performed weekly until the delivery date with the last session coinciding with the day before the planned delivery. During each session 70-100% plasma volume was
exchanged with 4% albumin saline solution. Anticoagulation was ensured with ACD-A in 1:12 - 1:15 ratios. Only subcutaneous veins of the arms were used as blood access points.

C. IVIG Treatment Protocol during Pregnancy

IVIG infusions (1 g/Kg/day) were scheduled at 15 day intervals rather than on two consecutive days every month, the usual timing to treat autoimmune disorders. The infusions were administered using that timetable in the attempt to reduce the amount of IVIG being removed weekly by the plasmapheresis sessions. The following were considered contraindications to treatment: immunoglobulin A deficiency, renal failure and previous intolerance/allergy to IVIG. Low dose aspirin (100 mg/day), which was suspended a week before the planned delivery, was empirically administered to minimize the IVIG’s thrombophilia side effects.

1.3 IVIG Treatment Protocol for Newborns

Treatment was begun as soon as possible after birth in the event of normal IgA levels and positivity to passive maternal antibodies. IVIG infusions (1 g/Kg/day) were scheduled at 15 day intervals rather than on two consecutive days every month in the attempt to prevent blood viscosity and excessive plasma volume build-up in the neonates. Slow infusions lasting at least 8-10 hours and opportune hydration throughout the infusion were monitored. IVIG sessions were continued on this timetable until passive maternal antibodies became undetectable on ELISA assay. However, when the IgG serum level was higher than that registered at birth, the IVIG infusion took place once a month.

1.4 Monitoring during Pregnancy

All the patients underwent a physical examination, foetal ultrasound studies, and routine biochemistry testing every 2 weeks from the time therapy was begun until delivery. Foetal
echocardiographies were performed weekly starting from the time CHB was detected to the end of the pregnancy.

1.5 Detection of antibodies
Maternal sera were collected at the time CHB was detected and at delivery, and newborn sera were collected at delivery and before every IVIG infusion. All samples were tested for 52 and 60 kd anti-Ro/SSA and anti-La/SSB using commercial ELISA kits (Technogenetics, Italy). The results were verified using an in-house counterimmunoelectrophoresis method and a commercial line-blot assay (Inno-Lia, Innogenetics, Belgium).

1.6 Statistical Analysis
Statistical analysis was performed using Spearman's Rho test. A p-value <0.05 was considered significant.

2. Results
2.1. 2nd degree CHB
Case no.1
A pregnant 32-year-old female was referred to our Rheumatologic Centre in January 2009 during the 20th week of her first pregnancy because of fetal bradycardia detected by fetal ultrasound. The patient had been diagnosed with Sjögren's syndrome two years earlier. A fetal echocardiography carried out at the time of referral confirmed the finding of a second-degree AV block with a mean ventricular rate of 74 beats per minute (bpm) and a mean atrial rate of 142 bpm (Figure 23 A). Fetal cardiac anatomy and function were normal, and no pericardial or pleural effusions or ascites were detected. Treatment with betamethasone (4 mg/day) and weekly plasmapheresis sessions was started. Two weeks later, at the 22nd week, the mean heart rate increased from 74 bpm to 124 bpm (Figure
and a fetal echocardiography showed episodes of sinus rhythm (mean rate 160 bpm) alternating with a 2:1 second-degree AVB. At the 25th week IVIGs (1 g/kg) were added to the therapy regimen. IVIG infusions (Kedrion, Italy) were scheduled at 15 day intervals rather than on two consecutive days every month, which is the usual scheduling timetable. Just as in a previous protocol (145) the infusions were administered with this timing in an attempt to reduce the amount of IVIG that was removed weekly by the plasmapheresis sessions. Low dose aspirin (100 mg), which was suspended a week before the planned caesarean section, was empirically administered to counteract IVIG-related thrombophilia.

A normal sinus rhythm (Figure 23 B) was recorded by fetal echocardiography at the 26th week. As indicated in Figure 24, serial echocardiographies, performed regularly until the time of delivery, confirmed a stable mean heart rate ranging between 148 and 175 bpm and a PR interval ranging from 0.110 to 0.125 seconds.

A caesarean section was planned at the 35th week of gestation because of a mild growth restriction and a normal male infant weighting 1905 g was delivered. The infant, who’s Apgar score was 8 at five minutes, appeared healthy at birth, and no signs of cutaneous lesions were noted after ultraviolet light exposure during the follow-up. His anti-SSA/Ro antibody levels (52 and 60 kd) were 95 and 280 units/ml, respectively, while anti-SSB/La testing was negative. All standard blood tests, including immunoglobulin (Ig) G, IgA, and IgM levels, were normal for his age. ECG monitoring after birth showed a sinus rhythm with a first-degree AV block and a mean heart rate of 158 bpm during the first 19 hours after birth. At that point a variable Mobitz I AV block appeared on ECG monitoring, which was confirmed by an ECG (Figure 25 A). IVIG therapy (1 g/kg per day on two consecutive days) was immediately administered to the newborn to counteract the effects of passive maternal antibodies and six days later a sinus rhythm with a first-degree AV block was recorded by an ECG (Figure 25 B).
**Figure 23.** M-mode fetal echocardiography recordings. A) At the 20th week, the finding of an second-degree atroventricular (AV) block. B) At the 26th week there was a normal sinus rhythm after five weeks of betamethasone, five plasmaphereses, and one intravenous immunoglobulin (IVIG) infusion. a: atrial contraction, v: ventricular contraction.

**Figure 24.** Mean fetal heart rate during the combination therapy. There is a significant correlation between mean heart rate increase and weeks of treatment. B: betamethasone, P: plasmapheresis, IVIG: intravenous immunoglobulins, CHB: congenital heart block, NSR: normal sinus rhythm with a first-degree AV block.

This finding was confirmed by a 24-hour Holter ECG. Single IVIG infusions (1 g/kg per day) were scheduled at 15 day intervals rather than on two consecutive days every month.
This was done in the attempt to prevent blood viscosity and excessive plasma volume build-up. IVIGs were continued on this time schedule until the infant was four months old, at which time anti-52 kd and 60 kd SSA/Ro antibody levels became undetectable. A Holter ECG recently carried out, now a healthy 3 year and a half-old male, identified a sinus rhythm with a normal PR interval.

![Figure 25](image)

Figure 25. (A) An electrocardiogram performed 19 hours after the infant’s birth shows a variable Mobitz I AV block. (B) An electrocardiogram performed after two IVIG infusions shows a sinus rhythm with a first-degree AV block.

**Case no. 2**

A 33-year-old female was diagnosed with undifferentiated connective tissue disease because of dry eyes and the presence of anti-SSA/Ro (52 and 60 kd) and anti-SSB/La antibodies. She was not receiving any kind of therapy. A normal heart rate (162 bpm) and anatomy were recorded during routine fetal ultrasound performed at the 18th week of gestation (Figure 26) of her first pregnancy. At the 20th week a ultrasound revealed bradycardia, confirmed by a fetal echocardiography which detected a second-degree AV block with a mean ventricular rate of 80 bpm and mean atrial rate of 160 bpm. At that time betamethasone was administered at a dose of 8 mg/day for three days and then continued at 4 mg/day, but it did not achieve any modification in the heart rate, as demonstrated by
the six echocardiographies carried out over the next two weeks. The patient was referred to our Rheumatologic Centre in October 2010 during the 22nd week of her pregnancy and a fetal echocardiography confirmed a 2:1 AV block and registered a heart rate of 75–80 bpm (Figure 26). A combination therapy was begun at the 23rd week of that pregnancy (betamethasone 4 mg/day, weekly plasmapheresis, and IVIG 1 g/kg every 15 days). The regimen was continued throughout the rest of the pregnancy until delivery. At the 24th week the mean heart rate began increasing (Figure 26) and at the 27th week an echocardiography detected a normal sinus rhythm with a mean heart rate of 171 bpm.

Figure 26. Mean fetal heart rate during the combination therapy. There is a significant correlation between mean heart rate increase and weeks of treatment. B: betamethasone, P: plasmapheresis, IVIG: intravenous immunoglobulins, CHB: congenital heart block, NSR: normal sinus rhythm with a first-degree AV block.

Serial echocardiographies confirmed the improvement in the fetal heart rate, which continued until the 34th week (Figure 26), when a premature rupture of the membranes occurred and a 2450 g female was delivered by uncomplicated caesarean section. The infant’s Apgar score at five minutes was 9. The infant appeared healthy at birth and the results of all standard blood tests, including IgG, IgA, and IgM levels, were normal for her age. Anti-52 kd and 60 kd SSA/Ro antibody levels were 559 and 420 units/ml,
respectively, while the anti-SSB/La titres were borderline (21.1 units/ml). ECG monitoring showed a sinus rhythm with a first-degree AV block. Eighteen hours after birth the infant underwent two consecutive days of IVIG infusions (1 g/kg per day) and from that time until she was four months old, when maternal anti-52 kd and 60 kd SSA/Ro antibodies became undetectable by ELISA, she was administered IVIG at 15-day intervals. A Holter ECG recently carried out, now a healthy 23 month-old female, revealed a 1st degree block alternating with a 2nd degree Wenckebach type block with a mean heart rate of 94 beats per minute (bpm).

Case no. 3
In November 2011 a 32-year-old woman in her 30th week of gestation was referred to our Centre. The patient had been diagnosed with undifferentiated connective tissue disease characterized by Raynaud’s phenomenon and positivity for anti-Ro/SSA and anti-La/SSB in 1992. A 3rd degree CHB was detected (with an atrial rate of 154 bpm and a ventricular rate of 60 bpm) at the 20th week of gestation, which reverted to a normal sinus rhythm the following day in the absence of any treatment. Nevertheless, oral Betamethasone (4 mg/day) was initiated and continued throughout the remainder of her pregnancy. Twice weekly echocardiographic evaluations were scheduled. Short periods (one minute duration) of a 2nd degree block were detected during fetal echocardiographies at the 23rd and 29th weeks of gestation. During the 30th week of gestation a 2nd degree CHB, which was stable for a 30 hour period (with an atrial rate of 155-160 bpm and a ventricular rate of 67-80 bpm), was registered. Plasmapheresis and IVIG infusions were added to Betamethasone therapy. There was a partial recovery characterized by a normal conduction alternating with a 2nd degree block over the following three week period. At the 33rd week of gestation the treatment protocol was intensified in the attempt to achieve a stable reversion of the block. Plasmapheresis was performed every 5 days and IVIG infusions every 11 days. An intensive treatment was possible as maternal serum IgG
levels were always within the normal range. A normal atrioventricular conduction with a mean heart rate of 150-165 bpm, which remained stable until delivery, was detected at the 34th week of gestation. A caesarean section was performed a week later in view of intrauterine growth restriction (IUGR) and oligohydramnios. A healthy female infant weighing 1730 g (10% le) and with an Apgar score of 9 at 5 minutes was delivered. The first postnatal Holter ECG registered a stable sinus rhythm with a normal PR interval and a mean heart rate of 140 bpm. Levels of passive 52-60 kd anti-Ro/SSA and anti-La/SSB antibodies were 1100, 510, and 1327 U/ml, respectively. IVIG infusions were begun 41 hours after birth and continued fortnightly. The infant is now 12 months-old, has caught up with her full term peers, and a recent ECG showed a normal sinus rhythm a mean heart rate of 131 bpm.

2.2. 3rd degree CHB

**Case no. 4**

A 36-year-old woman was referred to our Centre in October 2010 during the 22nd week of her sixth pregnancy. Diagnosed with Basedow’s disease in 2003 and with Sjögren’s syndrome in 2008, the patient’s obstetric history revealed one normal pregnancy, two early spontaneous abortions, and two intrauterine deaths at the 18th and 36th weeks, respectively. A normal heart rate and anatomy were recorded at the 18th week of gestation during the first fetal echocardiography. Fetal ultrasound at the 20th week of gestation showed a normal heart rate. An echocardiography at the 22nd week revealed a 3rd degree CHB with an atrial rate of 150 bpm and a ventricular rate of 52 bpm. Cardiomegaly and a mild pulmonary valve insufficiency were noted. Combination therapy was begun. Throughout the remainder of the pregnancy heart rate and cardiomegaly remained stable and there were no signs of heart failure or endocardial fibroelastosis (Figure 27). At the 33rd week of gestation an elective caesarean section was carried out in view of mild
oligohydramnios and growth restriction. A male infant with a birth weight of 1810 g (10% le) and an Apgar score of 8 at 5 minutes was delivered. A postnatal echocardiography confirmed a 3rd degree CHB with normal systolic function. Passive 52-60 kd anti-Ro/SSA and anti-La/SSB antibodies were 726, 901 and 195 U/ml, respectively. IVIG infusions were begun 14 hours after birth and continued until, five months later, antibodies were no longer detectable at ELISA assays. During that time period the neonate’s heart rate and clinical conditions remained stable. A pacemaker was implanted when he was 10 month- old and now, at 24 months, he is a healthy infant with a body weigh 50th le and a left ventricle ejection fraction (EF) 68%.

Figure 27. Mean fetal heart rate during the combination therapy. B: betamethasone, P: plasmapheresis, IVIG: intravenous immunoglobulins, CHB: congenital heart block, NSR: normal sinus rhythm with a first-degree AV block.

Case no. 5

A 25 year-old woman at the 27th week of her third pregnancy (the previous two were normal) was referred to us in April 2011 in view of fetal bradycardia detected during a fetal ultrasonography. A fetal echocardiography carried out at that time revealed a 3rd degree CHB (with a mean atrial rate of 160 bpm and a mean ventricular rate of 63 bpm) associated to cardiomegaly. A fetal block with arthralgias, acrocyanosis and high levels of
antibodies to 52-60 kd Ro/SSA and La/SSB (12134, 5494 and 446 U/ml, respectively) led to a diagnosis of undifferentiated connective tissue disease. Combination therapy was begun immediately and a week later the mean ventricular rate increased to 75 bpm. Subsequent weekly echocardiographies confirmed a 3\textsuperscript{rd} degree CHB and showed a further increase in the ventricular rate reaching 85 bpm with a regression of cardiomegaly. At the 35\textsuperscript{th} week a sudden placental abruption took place and a caesarean section was carried out. A healthy female weighing 1790 (10\% le) with an Apgar score of 8 at 5 minutes was delivered. A postnatal ECG confirmed a 3\textsuperscript{rd} degree CHB with a mean heart rate of 85 bpm and an echocardiography registered a normal systolic function with no signs of further cardiac complications. As expected, the neonate was positive for passive 52-60 kd anti-Ro/SSA and anti-La/SSB antibodies (829,1347,105 U/ml, respectively), and IVIG infusion was begun and continued during the next 6 months until maternal antibodies were no longer detectable. Now, at 18\textsuperscript{th} months, the infant enjoys good health. A recent echocardiography registered a left ventricle EF 78\%. and a Holter ECG showed a mean heart rate of 67 not requiring pacing.

Case no. 6

A 34 year-old woman was referred to our Rheumatologic Centre in April 2011 at her 24\textsuperscript{th} week of gestation in view of bradycardia (119 bpm) detected during a fetal sonography 19 days earlier. The patient reported two previous normal pregnancies and one early abortion. An echocardiography carried out a few days later revealed a 3\textsuperscript{rd} degree CHB with a ventricular rate of 60 bpm, cardiomegaly, mild insufficiency of the mitral and tricuspidal valves, and mild pericardial effusion. The patient was healthy and 52-60 kd anti-Ro/SSA antibodies at the time CHB was detected were 94 and 658 U/ml, respectively. Combination therapy was begun as soon as the ventricular rate registered between 56 and 60 bpm with an atrial rate of 130-150 bpm and the valves and pericardial effusion were stable. At the 36\textsuperscript{th} week a mild intrauterine growth restriction (IUGR) was registered and a planned...
caesarean section was carried out. A male infant weighing 2315 g was delivered in good clinical condition. His Apgar score was 9 at 5 minutes and an echocardiography, carried out soon after birth, confirmed a 3\textsuperscript{rd} degree CHB, without cardiomegaly and with normal ventricular function. Passive 52-60 kd anti-Ro/SSA antibodies were detected in his blood (43 and 65 U/ml, respectively) and IVIG infusions were administered for 5 months. During that period the mean ventricular rate was stable ranging between 57 and 67 bpm and control echocardiographies were stable. The infant is now 17\textsuperscript{th} months old and his heart rate (stable at 60 bpm) and haemodynamic condition (left ventricle EF 71\%) do not require pacing.

**3. Discussion**

Currently no therapy is found to be effective in the treatment of 2\textsuperscript{nd} and 3\textsuperscript{rd} degree CAVB induced by maternal antibodies (146); also because the rarity of the disease does not allow for controlled clinical trials.

The aim of this study was to evaluate the outcome of a combined therapy protocol utilizing daily Betamethasone, weekly plasmapheresis, and fortnightly IVIG in six cases of CHB during pregnancy and IVIG fortnightly in neonates after birth. The foetuses affected with 2\textsuperscript{nd} degree block reverted to a normal atrio-ventricular conduction during pregnancy while those with a 3\textsuperscript{rd} degree block remained stable (case 4), showed a higher ventricular rate (case 5), or an improvement in cardiac function (case 6), which improved their chances for survival and delayed the need for a pacemaker. No severe cardiac complications such as impaired ventricular function, hydrops or cardiomyopathy were observed in the foetuses with 3\textsuperscript{rd} CHB. The follow-up of the children affected with 2\textsuperscript{nd} degree CAVB revealed a complete regression of the block in case 1 and 3 and no signs of relevant worsening in case 2. It might be that the 2\textsuperscript{nd} degree block of case 2 did not revert to a stable normal sinus rhythm because of the late beginning of the combined therapy (136). In fact, for two
weeks after CAVB detection only betamethasone was administered to the mother and it did not achieve any modification in the heart rate. The infants affected with 3\textsuperscript{rd} degree block (cases 4, 5, and 6) remained stable and until now only one has required a pacemaker at the age of 10 months. It is also interesting to observe that no side-effect of combined therapy was registered in any case.

The novelty characterizing this therapeutic strategy was the combination of three treatments whose effects were exploited to neutralize anti-Ro/SSA and anti-La/SSB antibodies and their pathologic effects: a) Betamethasone was used to reduce inflammation of the foetal conduction system and the myocardium; b) Plasmapheresis was employed to remove a large part of the offending autoantibodies from the maternal-foetal circulation; and c) IVIG was used to counteract the remaining autoantibodies. It is also possible that IVIG therapy treats foetal cardiac injury thanks to its transplacental passage.

The idea of continuing IVIG treatment after birth was based on several reports describing postnatal progression of incomplete blocks and the occurrence of other cardiac complications (135, 140, 141, 147). Our group was first faced with that decision while we were monitoring case 1 in this report. After a short period of 1\textsuperscript{st} degree block at birth, after nineteen hours he progressed to a 2\textsuperscript{nd} degree Wenckebach type block and therefore the decision was taken to prescribe IVIG with a prompt regression to the initial 1\textsuperscript{st} degree block. Also employed in the treatment of other neonatal diseases such as alloimmune thrombocytopenia, IVIG is, in fact, considered safe therapy for foetuses and neonates. Plasmapheresis, instead, cannot be recommended for routine use in neonates because of problems connected to vascular access and prolonged steroid therapy could induce serious side effects in newborns (148).

The efficacy of the combination treatment strategy described here is probably connected to the precocious timing that was used in these cases. It should, in fact, be begun as soon as a 2\textsuperscript{nd} degree CHB is detected and before irreversible and deleterious effects on the
atrio-ventricular node tissue have taken place (107). Thus, according to most authors then, foetuses exposed to maternal anti-Ro/SSA and/or anti-La/SSB antibodies should be carefully monitored by weekly echocardiographies between the 18th and 24th weeks of gestation. If this combined therapy is also effective in treating 3rd degree CHB remain to be proven. It might well be that blocking the progression of heart injury results in an increase of basal heart rate thus delaying the necessity of pace-maker implantation. In any case strict adherence to timing and dosage is a primary determinant of treatment success.

4. Conclusion
Overall, lessons learned from these case series are: a) the efficacy in treating 2nd degree CAVB, and b) the safety of plasmapheresis and IVIG therapies. The low number of treated cases, the relatively short follow-up (mean 22.7 months ± 10.4, range 12-42 months) and the high cost of the procedure can all be considered limits. If these results are confirmed by large-scale studies, this protocol could lead to improved outcomes in the treatment of this devastating disease.
Part IV
THE EFFECT OF THE COMBINED TREATMENT протокол на antibody levels

1. Patients and methods

1.1 Study population

Sera from 6 mothers of foetuses with CHB, attending the Rheumatology Unit of University-Hospital of Padua treated with the combined therapy protocol were collected prospectively from 2009-2012 and stored at -80°C. There were 3 samples collected before, during and at the end of the treatment from all the patients, and serial sera before and after each plasmapheresis from 4 mothers. The anti-Ro52, anti-p200 and anti-La antibody levels were investigated.

1.2 Detection of antibodies

Anti-Ro52 and anti-p200 antibodies were detected with an in house ELISA as previously described (Part I). Anti-La antibodies were detected with a commercial ELISA kit (Orgentec, Germany). Anti-tetanus antibodies were detected with an in house ELISA as follows: high-binding 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µl/well of tetanus 1:250 diluted in carbonate buffer, pH 9.6. Plates were washed three times with PBS/0.05% Tween-20 (TPBS) and blocked with 200 µl of TPBS/5% milk powder per well for 2 h. After three washings with TPBS, the plates were incubated with patient sera diluted 1:500 in PBS/0.05% Tween-20/1% milk powder for 2 h. Plates were washed five times in PBS/0.05% Tween-20 and then incubated with AP-conjugated rabbit-antihuman IgG antibody (Dako) for 2 h. Plates were washed again three times, and phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer pH 9.8 was added. Absorbance was measured at 405 nm after 1,5 h. All steps were performed in room temperature except coating which was performed at +4°C.
1.3 Statistical Analysis

Statistical analysis was performed using Wilcoxon and two way ANOVA tests. Linear regression was performed to evaluate the decrease of antibody levels compared to the basal value. A p-value <0.05 was considered significant.

2. Results

2.1 Anti-Ro52 and anti-p200 antibody levels in women treated with a combined therapy protocol

The effect of the combined therapy protocol on autoantibody levels was analyzed as follows. First, we investigated the anti-Ro52 and anti-p200 antibody levels at 3 time points during pregnancy (before, somewhere during and at the end of the treatment). Surprisingly, we observed that Ro52 autoantibody levels were not affected by the plasmapheresis procedure. Anti-p200 levels displayed a moderate decrease, which however was significant (Figure 28).

![Figure 28](image-url)

**Figure 28.** (A) The anti-Ro52 and (B) anti-p200 antibody levels in women treated with the combine therapy protocol.

2.2 Antibody levels before and after plasmapheresis

As plasmapheresis is a well established method to effectively deplete circulating IgG, we proceeded to investigate how plasmapheresis affects autoantibody levels. In the next step,
we analyzed the anti-Ro52, anti-p200 and anti-La antibody levels before and after each plasmapheresis in four patients treated with the combined protocol. The analysis demonstrated that the plasmapheresis procedure indeed did decrease autoantibody levels in several patients when comparing samples taken directly before and after a plasmapheresis procedure, but also that the effect was very modest in patients with high titer autoantibody levels. For example, the levels of anti-Ro52 antibodies significantly decreased in patient N.1 (Figure 29A) and patient N.3 respectively, p<0.0001 and p=0.004, while they did not decreased significantly in patient N.2 (Figure 29B) and N.4. The linear regression shows that the patients with high levels of anti-Ro52 antibodies (patient N.2 and N.4) have a less decrease of their levels after plasmapheresis (Figure 29C).

The levels of anti-p200 antibodies significantly decreased in all the patients, p=0.0001 (Figure 29D), p=0.02 (Figure 29E), p=0.008 and p=0.03, respectively. However, the linear regression (Figure 29F) shows that the patient with high levels has less decrease of anti-p200 antibody levels after the plasmapheresis.

The anti-La antibody levels followed a similar pattern and significantly decreased in patient N.1, N.2 and N.3 p=0.0001 (Figure 30A), p=0.04 and p=0.001, respectively; while the levels of anti-La antibody did not decreased significantly in patient N.4 (Figure 30B). The linear regression (Figure 30C) shows that the patient with high levels has less decrease of anti-La antibody levels (patient N.4) after the plasmapheresis.

2.3 The role of IVIG on antibody levels

All the patient treated with the combine therapy was given IVIG fortnightly and plasmapheresis every week as reported in Figure 31.
Figure 29. The anti-Ro52 antibody levels in patient N.1 (A), patient N.2 (B) and linear regression showing the relative decrease of anti-Ro52 antibody levels in the first plasmapheresis (C). The anti-p200 antibody levels in patient N.1 (D), patient N.2 (E) and linear regression (F) showing the relative decrease of anti-p200 antibody levels in the first plasmapheresis.

Figure 30. The anti-La antibody levels in patient N.1 (A), patient N.4 (B) and linear regression (C) showing the relative decrease of anti-La antibody levels in the first plasmapheresis.

Figure 31. The treatment strategy; P=plasmapheresis; IVIG=intravenous immunoglobulin; WG=week of gestation

One possibility for explaining the surprisingly small effect of plasmapheresis on the autoantibody levels was that Ro/La antibodies were present in the IVIG infusions. To
exclude this possibility and to investigate the role of the IVIG on the antibody levels we evaluate the levels of total IgG before and after each cycle of plasmapheresis. Figure 32 illustrate the results from patient 1 who receive the first infusion of IVIG after the ninth cycle of plasmapheresis. As expected the levels of total IgG increases after the infusion of IVIG and significantly decrease after each plasmapheresis (p=0.0002).

![Graph showing IgG levels over plasmapheresis cycles](image)

**Figure 32.** The levels of total IgG tested in patient 1; *=IVIG infusion

As the IVIG is produced by pooling sera from blood donors we analyzed the IVIG (Kedrion, Italy) which we infused to our patients for anti-Ro52, anti-p200 and anti-La antibodies. No reactivity for either Ro52, p200 or La was observed in the IVIG. Moreover, as anti-tetanus is one of the more used vaccine in general population, we evaluate patient’s sera reactivity to anti-tetanus antibodies and found that their levels increased after infusion of IVIG as reported in Figure 33. Obviously, the IVIG from blood donors was positive for anti-tetanus antibodies. The anti-tetanus antibodies were negative in one patient who lacked anti-tetanus immunity in samples taken initially obtained anti-tetanus antibodies after IVIG treatment.
3. Discussion

In this study we evaluated the effect of the combined treatment on antibody levels. First, we investigated the anti-Ro52 and anti-p200 antibody levels in 6 treated patients at 3 time points of the pregnancy (before, during and at the end of the treatment) and surprisingly we found that only the anti-p200 levels were significantly decreased. In 4 out of these six patients serial sera obtained before and after each treatment were available, and we used these to evaluate the effect of plasmapheresis on Ro52, p200 and La autoantibody levels in detail. As anti-Ro60 antibodies are considered less central in the development of CHB (50) we did not detect those antibodies in the study. We found that plasmapheresis significantly decrease the anti-Ro52, anti-p200 and anti-La antibody levels in all cases except in patient N.2 and N.4 who have the highest levels of anti-Ro52 antibodies and in these patients there was not a significant decrease. In fact, a linear regression analysis demonstrated that patients with highest autoantibody levels have a less decrease in anti-Ro52 antibody levels. These results might be explained by the limit of ELISA assay which does not have a good resolution for highest antibody levels. Furthermore, as all patients
were treated with IVIG infusions we investigated the levels of total IgG before and after each plasmapheresis and observed that the levels of total IgG increased after the IVIG infusion. As IVIG is produced by a pooling sera from blood donors, we analyzed the anti-Ro52, anti-p200, anti-La and anti-tetanus antibody reactivity in a sample of the infused IVIG to exclude the possibility that the autoantibodies were being infused to the patients. We found only anti-tetanus reactivity and no Ro52, p200 or La antibodies within the IVIG. These latter results confirm that the changes in antibody levels depend on a) removal of pathogenetic antibodies by plasmapheresis treatment and b) novel production by the patient.

4. Conclusion

Our data indicate that weekly plasmapheresis reduces the levels of anti-Ro52, anti-p200 and anti-La antibodies in patients with lower autoantibody titers, but that it has a modest or no effect on autoantibody levels in patients with high titers of autoantibodies. It is not known how low autoantibody levels need to be for decreasing the risk of autoantibody-related pathology in CHB. In fact, there are no scientific evidence that depletion of Ro/La antibodies will have a beneficial effect although it is reasonable to hypothesize that it would.
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