Undergraduate Awards 2014

Congenital Heart Block: Epidemiology, Etiology and Pathogenesis

Category: Medical Sciences
Abstract

Congenital heart block (CHB) is the most severe manifestation of Neonatal lupus erythematosus syndrome, which is often described as a model of passively acquired autoimmunity. CHB constitutes a unique model where specific maternal autoantibodies target and mediate fetal organ-specific disease.

The essay will first review the studies that are at the basis of the definition of autoantibody-associated CHB and its differentiation from congenital heart block in fetuses with cardiac abnormalities. After analyzing the role of Neonatal lupus erythematosus in congenital heart block development, disease epidemiology will be exposed pointing out relevant problems resulting from the low incidence of CHB. Through the exposition of the last studies, a detailed overview of a possible process converting maternal antibody binding, to a profibrotic damaging event, will be provided. Fetal factors, including genetic predisposition and candidate polymorphisms evaluated to date, will be briefly discussed. This essay will also present some of the future perspectives discussed during “The 14th Congress of the European League Against Rheumatism” held in Madrid from 12th to 15th June 2013.

Cover picture: Artwork by New York artist Rachel Urkowitz (email: rachelurk@earthlink.net) representing autoantibodies that cross the placenta and cause congenital heart block in the fetus.
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>AV</td>
<td>Atrioventricular</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
<td></td>
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<td>CHB</td>
<td>Congenital heart block</td>
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<tr>
<td>CR</td>
<td>Complement receptor</td>
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</tr>
<tr>
<td>DMNQ</td>
<td>2,3-dimethoxy-1-naphthoquinone</td>
<td></td>
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<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
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<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5′-triphosphate</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen system</td>
<td></td>
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<tr>
<td>HT</td>
<td>Hydroxytryptamine</td>
<td></td>
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<tr>
<td>I&lt;sub&gt;CaL&lt;/sub&gt;</td>
<td>L-type calcium channel current</td>
<td></td>
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<tr>
<td>I&lt;sub&gt;CaT&lt;/sub&gt;</td>
<td>T-type calcium channel current</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
<td></td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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</tr>
<tr>
<td>IKs</td>
<td>Slow delayed rectifier potassium current</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>ISRE</td>
<td>Interferon-stimulated response element</td>
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<tr>
<td>I/V</td>
<td>Current/Voltage</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
<td></td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
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</tr>
<tr>
<td>NLE</td>
<td>Neonatal lupus erythematosus</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
<td></td>
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<tr>
<td>pA</td>
<td>Picoampère</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td>Sinoatrial</td>
<td></td>
</tr>
<tr>
<td>Smac</td>
<td>Smooth muscle actin</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>Sjögren’s syndrome</td>
<td></td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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Introduction

A major influence in the choice of the topic was exerted by a two-month research internship included in the Physiology and Experimental Medicine program of my University.

This experience gave me the opportunity not only to get to know CHB, but also to meet high profile researchers able to show me the most intriguing aspects of this disease.

Congenital heart block is regarded as a passively transferred autoimmune disease where maternal antibodies (Abs) cross the placenta and are, potentially, initiators of a damaging process in the fetal heart.

The main aspect that fascinated me was that the study of CHB is not only included in the so-called “translational research” in which the clinical observations are examined and tested in the laboratory. On the contrary, it should also be defined as “integrational research” in which the most intriguing and challenging purpose is to fit together different critical aspects, even those that seem to be at odds.

In CHB two aspects are difficult to reconcile: the clinical and the cellular one.

The most difficult clinical observation to understand is the fact that, in twin pregnancies, only one twin can be affected. For the cellular aspect, the specific anticorpal targets and the damaging process have yet to be properly defined.

The fact that questions regarding critical aspects of CHB have not yet found their answer could appear disorienting at first, but it can also be seen as an open field of possibilities for researchers to utilize an array of techniques to understand a complex disease.

Through the exposition of the last studies on CHB, this essay will try to analyze critical aspects of this disease providing an overview of where we are today in the iter leading to the conceptualization of its pathogenesis.
Neonatal lupus erythematosus and CHB

From the first time it was reported by Morquio in 1901\(^2\) and its first diagnosis ante partum by Plant and Steven in 1945\(^3\), congenital heart block, initially encased only in the interest of the disciples of cardiology, has become important as a model of passively acquired autoimmunity. Carter et al.\(^4\) reported the occurrence of CHB in association with tumors such as mesotheliomas, Machado et al.\(^5\) stated its occurrence in association with structural heart disease, such as atrioventricular septal defects, left atria isomerism, and abnormalities of the great arteries.

Reports of CHB in children whose mothers had autoimmune diseases\(^6\) and the finding that the maternal sera contained Abs against Ro ribonucleoproteins\(^7\) solidified Aylward’s clinical observation of 1928\(^8\). He showed the occurrence of CHB in two children whose mother suffered from “Mikulicz’s disease” (generally considered to be identical to Sjögren’s syndrome).

It is now well accepted that congenital heart block, when occurring in a structurally normal heart, is the most severe manifestation of Neonatal lupus erythematosus syndrome. Neonatal lupus erythematosus syndrome is a model of passively acquired autoimmunity developing in fetuses of mothers positive for the Abs reactive to 52 or 60 kD Ro and/or 48 kD La ribonucleoproteins.

The woman may have systemic lupus erythematosus or Sjögren’s syndrome or, as nearly half the cases, may be asymptomatic: fetal or neonatal disease appears to be totally independent of maternal health status. Maternal Abs begin crossing the placenta as early as 11 weeks of gestation and, when accumulating in the fetal circulation induce pathology in the developing child. NLE includes several clinical manifestations, congenital atrioventricular block, NLE rash, and various liver and blood cell abnormalities in the newborn. While the non cardiac manifestations are transient and resolve as maternal Abs are cleared from neonatal circulation, a complete atrioventricular block, in a structurally normal heart, is considered permanent.

More than 80% of cases are detected before 30 weeks of gestation, with a peak of incidence between 20 and 24 weeks. The degree of heart block varies, depending on the severity of the disease, from 1\(^{st}\) degree AV block, where the conduction is delayed, to 3\(^{rd}\) degree AV block, where the impulse from the SA node is completely interrupted.
It has been verified that incomplete heart block could progress after birth, even after maternal antibody clearance, toward a third degree CHB\textsuperscript{3}. However spontaneous reversibility of first degree block has also been reported. These findings suggest that CHB is not an all or nothing phenomenon but it is a progressive disease, ranging in different levels of cardiac involvement, and that there is a serological-genetic threshold below which the incomplete block does not result in a more severe block.

Maternal sera were assessed in order to screen for a specific antibody profile associated with a higher risk of developing CHB. Results demonstrate that, while anti-Ro60 and anti-La Abs have been found at higher levels in mothers whose child develops cutaneous lupus, anti-Ro52 Abs have a major role in predicting fetal CHB. Systematic analysis permitted identification of an antibody subpopulation against p200 (amino acid 200-239 of Ro52 protein) which was associated with a higher risk to develop the disease\textsuperscript{10}.

The most intriguing aspect of CHB that can raise many hypothesis is the fact that, considering the potential pathogenic role of anti-Ro/La Abs, CHB has a low incidence. This can obviously suggest that a specific maternal antibody profile is responsible for mediating injury in the fetal heart. The low rate of recurrence of CHB in subsequent pregnancies (15\%)\textsuperscript{11} demonstrates that the presence of these Abs does not, on its own, imply induction of the fetal disease. This latter consideration is also supported by the discordance of CHB in identical twins reported by Buyon et al.\textsuperscript{1}

It is arguable that Abs can evolve multiple epitope specificities over time but it is most likely that fetal factors and uterine environmental influences are implied in the onset of CHB and that Abs are necessary but not sufficient to trigger the process leading to fibrosis.
Epidemiology

Congenital heart block, not accompanied by any cardiac anatomic abnormalities, is a rare disease with an incidence of 1/1500-1/20000 in the general population\textsuperscript{12}. Its incidence rises to 5/100 in patients with lupus\textsuperscript{11}.

Because of the rarity of the disease, clinical data are limited: the previous largest cohort of CHB was reported by a rheumatology group based in Toronto and comprised 64 affected families\textsuperscript{13}. Anti-Ro or anti-La autoantibodies were detected in only 60\% of 53 tested mothers.

In September 1994 a National Research Registry for Neonatal lupus erythematosus was established by the National Institute for Arthritis, Musculoskeletal and Skin Diseases to provide a large database of clinical data. To be an eligible case for enrollment in the registry, a mother should have a child with any manifestations characteristic of NLE, such as cardiac abnormalities, skin rash, hemolytic anemia, leukopenia, thrombocytopenia or cholestatic liver disease. There were no restrictions on the maternal health status. That database comprises affected cases detected between 1979 and 1997.

Of the 119 mothers with CHB children, present in the Neonatal lupus erythematosus National Research Registry, Buyon et al. in 1998\textsuperscript{11} examined 105 mothers positive to anti-Ro/La Abs and their children, regardless the presence of NLE cutaneous manifestations. They found a mortality rate of 19\% (22 children of the cohort evaluated died); as expected, mortality rate reduction correlated to the birth at later gestational age. Excellent prognosis was assessed in CHB children surviving neonatal period: a 79\% 3-year survival rate was defined. 63\% of the surviving infants required pacemakers.

The recurrence rate of a CHB child after a previous affected pregnancy was 16\%. However, recent assessment evaluated a recurrence rate of 18\% of 100 pregnancies after a CHB child\textsuperscript{14}. Even though for NLE a gender-based difference in frequency and prognosis was previously defined, such a difference could not be assessed for CHB.

With the regard to ethnicity, in this study 76\% of the mothers with affected children were Caucasian, 10\% were African or American, 11\% were Hispanic, 2\% were Asian or pacific islander. CHB detection was comprised, most frequently, between 20 and 24 weeks of gestation and no cases were detected before 17 weeks (Figure 1).
This study also showed that incomplete heart block does not remain fixed, but it can progress to a more severe heart block or complete heart block even after birth. Furthermore, this finding was corroborated by results reported by Askanase and al.\textsuperscript{9} of injury to the conduction system. This latter finding stresses the importance of ECGs for all the anti-Ro/La Abs exposed infants in order to detect the effect on the fetal hearts.

Results presented should be helpful to define the most suitable monitoring period of pregnancies of mother whose sera contain anti-Ro/La Abs as well as a non-invasive diagnostic technique and a proper management.

Figure 1 - Time of detection of CHB. *In one child, third-degree block was diagnosed at age 2 years 7 months; in the other, first-degree block was diagnosed at age 10 years.\textsuperscript{11}
Etiology: Ro/La antigens and proposed hypothesis of CHB

At present, there is no direct evidence that maternal Abs cross the placenta and bind to Ro or La protein \textit{in vivo}. However, it is generally accepted that the pathogenetic mechanism of NLE-CHB involves the transplacental passage of maternal anti-Ro/La autoantibodies via Fcγ receptors into the fetal circulation\textsuperscript{15}; afterwards the antigen binding on the fetal heart.

Ro and La antigens have been extensively characterized at the molecular level. La antigen is a single 48 kD protein while Ro antigen consists of a 52 kD protein (Ro52) and a non-homologous 60 kD protein (Ro60).

La antigen does not share antigenic determinants with either Ro52 or Ro60\textsuperscript{16} and it is implied in facilitating the maturation of Rna polymerase III transcripts. Cloning of Ro60 identified a zinc finger and a Rna-binding protein consensus motif\textsuperscript{17}. It has been suggested that Ro60 may function as part of a novel quality control or discard pathway for 5S rRNA transcription.

In addition to the already mentioned La and Ro60, another protein, Ro52, is believed to be the target of maternal Abs in CHB. The full length protein, 52α belongs to the TRIM family. TRIM family proteins share consensus domains consisting of two zinc-binding domains, a RING motif, a B-box and a coiled-coil region (Figure 2). The zinc-binding region as well as the linker between the RING and the B-box have been shown to be crucial for the full folding of the protein\textsuperscript{18}.

Clancy et al.\textsuperscript{19} have identified a smaller transcript, 52β, resulting from the alternative splicing of exon 4 encoding aa 168-245 inclusive of the leucine zipper, whose expression is maximal at the time of cardiac ontogeny, when maternal Abs gain access to the fetal circulation. However, the protein 52β has not been detected or demonstrated to be expressed endogenously; only the transcript has therefore been studied.

While the function of Ro60 and La antigen has been known for some time, the function of Ro52 has remained elusive. Recently this
protein revealed its role as a E3 ligase in the ubiquitination process. Different substrates have been demonstrated to undergo Ro52-mediated ubiquitination including IgG and IRF-8. The latter substrate is a transcription factor; its binding to the IFN-stimulated response element (ISRE) regulates the transcription of type I IFNs stimulated genes. It has been reported to have a negative regulatory role in cells of the immune system, such as macrophages, dendritic cells and B cells.

Furthermore ex vivo and in vitro studies demonstrated overexpression of Ro52 in stably transfected B cells leading to a decrease of proliferation and increase in sensitivity to CD40-mediated cell death, suggesting a biological role of Ro52 in cell growth and/or apoptosis.

Higher risk has been suggested in women in whom the antibody response is directed to amino acids 200-239 (p200) of the Ro52 protein rather than to Ro60 protein.

Low incidence of anti-Ro60 antibody positivity in mothers of children with CHB can be referred to its conformation-dependent binding. In fact the use of native Ro60 in immunoarrays for detecting anti-Ro60 Abs in patient serum has shown that anti-Ro60 Abs are more commonly present in sera than previously appreciated.

Even though anti-Ro60 Abs may play an important role in CHB pathogenesis, anti-Ro52 or subspecifically anti-p200 Abs emerges as the strongest candidate for a CHB risk marker.

However, Abs are necessary but not sufficient to trigger the final pathway leading to injury. Considering the potential pathogenicity of Abs, the low frequency of CHB suggests that not all anti-Ro/La Abs are likely to mediate injury and that a specific unique epitope is recognized.

The low recurrence rates in subsequent pregnancies and the discordance of CHB in identical twins shifted investigators’ attention to fetal and/or environmental contribution.

Varied expression of the fetal epitope may explain the low disease rate (1-2%). Most likely, a combination of antibody specificity, as well as fetal genetics, and target expression is involved.

Two principal mechanisms, by which heart block is induced by maternal anti-Ro/La Abs have been proposed: the binding of abnormal cardiomyocyte surface expressed Ro/La antigens and the binding of cross-reactive self-antigens on the cardiomyocyte surface.

Because of the cellular location of Ro and La autoantigens, the accessibility of these intracellular proteins to their cognate extracellular Abs has to be elucidated. Up to now,
apoptosis in the target tissue, because of its selective involvement in embryogenesis and morphogenesis, seems the most reasonable consideration to account for this problem. Cross-reactivity between one or any of the anti-Ro/La Abs and a cardiac surface expressed molecule may provide a molecular explanation for pathogenicity. Although no definite target for heart block-inducing Abs has been identified; several molecules present in the cardiac tissue have been suggested to be bound by maternal Abs. These molecules, which have been given the most attention as potential targets, include the atrial 5-HT$_4$ receptor and voltage-gated calcium channels.

**Bringing intracellular antigen to maternal antibody: the apoptosis hypothesis**

Apoptosis is a controlled and programmed cell death. It occurs either during embryogenesis, where it is implied in morphogenesis and physiological organ shaping, or in tissue turnover. It can be triggered by the binding of nuclear receptors by glucocorticoids, heat, radiation, viral infection, nutrient deprivation, hypoxia and increased intracellular calcium concentration. Even though the physiologic triggers of apoptosis in fetal CHB tissues are unknown, every apoptosis-inducing agent triggers a common cellular final pathway leading to identical morphological and biochemical changes.

Apoptosis was thought to be immunologically silent since tissue remodeling, during embryogenesis, does not trigger inflammation. However if there are Abs circulating, apoptotic cells can be opsonized and they could be recognized by macrophages because they expose phagocytotic molecules. This triggers the abnormal release of cytokines resulting in an inflammatory response. Applicability of apoptosis to the pathogenesis of CHB as a linker between antibody and subsequent fetal cardiac tissue fibrosis is bolstered by several observations.

During apoptosis the cell undergoes redistribution of molecules, such as proteins, in its compartments. Translocation of calreticulin$^{24}$, the 60-kD heat-shock chaperonin protein$^{25}$ and neutrophil myeloperoxidase$^{26}$ from their location to the apoptotic cell membrane has been reported.

Casciola-Rosen et al.$^{27}$ first demonstrated that Ro and La are present in surface blebs of apoptotic keratinocytes. This observation was also extended to the developing fetal heart. Tran and al.$^{28}$ conducted in 2002 the first *in vivo* study demonstrating the subcellular translocation of La autoantigen during apoptosis in the fetal heart. Although apoptosis was previously thought to occur in the fetal heart only postnatally, they could detect apoptotic
cells in the AV node, SA node, AV bundle, insertions of the heart valves and working myocardium of normal mouse fetuses on days 15, 17 and 19 of gestation. No apoptosis was detected in the adult heart conduction system and working myocardium. This can give explanation to the developmental phase-dependence of CHB (since it has never been reported in the maternal heart despite the presence of the same fetal Abs).

A mechanism underlying La protein redistribution has been proposed. La protein is a multifunctional molecule implied in biogenesis of Rna polymerase III, and it is known to shuttle between cell nucleus and cytoplasm. This protein is able to reenter the nucleus, after performing its cytoplasmatic role, because of 383-408 amino acids sequence. This sequence, mapped at the COOH-terminus, is a nuclear localization signal (NLS). In early apoptosis La protein is thought to undergo proteolytic-caspase-mediate cleavage at the COOH-terminus and to lose its NLS, resulting in its redistribution near the cell membrane.

Neufing and al.\textsuperscript{29} demonstrated, \textit{in vivo} in a murine xenograph model and \textit{in vitro} in cultured human fetal cardiocytes, that human Abs targeting the COOH-terminal La epitope did not bind apoptotic cells. However the inability to bind that epitope is not yet attributable to the loss of C-terminal region of the molecule by caspase-dependent cleavage. Hence, COOH-terminal epitope inaccessibility suggests that this region of the molecule may be involved in membrane attachment and externalization of NH\textsubscript{2}-terminal.

Figure 3 - Cellular topology of Ro and La in nonpermeabilized nonapoptotic and apoptotic cultured human fetal cardiocytes. PI is seen as red fluorescence, Ro/La as green fluorescence, and overlapping pixels are orange-yellow. The strong green signal combined with PI staining of the nucleus is seen as yellow (A). In B, La is markedly decreased in the nucleus and increased in the cytoplasm of an apoptotic cardiocyte, while concentrating in blebs. In D, Ro is seen in the nucleus and cytoplasm of nonapoptotic cells. In apoptotic cells, there is translocation of Ro to the cell periphery and strong staining of blebs (E). Low power views of permeabilized apoptotic cardiocytes stained with anti-La (C), anti-Ro (F) are shown for reference. Images were taken 6 h after induction of apoptosis with 0.5 mM staurosporine.\textsuperscript{31}
This part of the protein is in fact predicted to encode a GAG attachment side, which is known to target proteins present in the extracellular matrix and cell surface and to mediate molecules interaction. Neufing and al. proposed a mechanism whereby during apoptosis La protein undergoes the cleavage of NLS and the dephosphorilation of Ser\textsuperscript{366}, leading to La accumulation in cell cytoplasm and exposure of GAG side chain. GAG side chain can interact with accessory protein. Annexins, a family of Ca\textsuperscript{2+}-dependent GAG-binding proteins, are potential candidates as vehicle for the translocation of La to the extracellular cell surface during apoptosis. Annexin I in fact has been demonstrated to be recruited from the cytosol to the cell surface during apoptosis\textsuperscript{30}.

Recently Miranda-Carus and al.\textsuperscript{31} assessed the accessibility of Ro and La antigens to maternal autoantibodies in apoptotic human fetal cardiocytes. They showed remarkable changes in cardiocyte topology of La and Ro during apoptosis (Figure 3), independently of the apoptosis inducer used (staurosoprine or DMNQ). Not surprisingly, apoptotic cells stained with human anti-La and anti-Ro Abs revealed La and Ro presence in the periphery and surface blebs of apoptotic cardiocytes.

Apoptosis could explain intracellular antigens accessibility to maternal autoantibodies and it is consistent with spectrum and unique fetal expression of CHB. However, apoptosis hypothesis is not able to explain the reasons for the selective vulnerability of specific fetal organs (heart, skin, liver) and the low rate (1-2%) of CHB in offspring of mothers with anti-Ro/La Abs.

Apoptosis does not readily account for all the clues observed at the bedside; it may be one fetal factor among several, able to induce inflammation and calcification reported in CHB fetal heart tissue.

**Serotonergic hypothesis**

The serotoninergic receptor belongs to the G protein coupled transmembrane receptor family. After ligand binding, these receptors could activate different intracellular pathways resulting in the production of second messengers or acting on ion channels permeability. The serotonin receptor is expressed on the membrane of human\textsuperscript{32} fetal cardiomyocytes; when serotonin binds that receptor, the conformational change induces a specular changing in its coupled G protein. The G protein is composed by three subunits: \(\alpha\), \(\beta\) and \(\gamma\). The \(\alpha\) subunit is a guanosine nucleotide binding protein: when binding to GDP the protein is in its inactive form and when to GTP it is in its active form. The \(\alpha\) subunit and the \(\beta\)-\(\gamma\) complex trigger, when activated, different intracellular pathways. So, the
conformational change in the serotoninergic receptor, due to its ligand binding, causes the activation of the G protein. The α subunit is implied in the increasing production of cAMP acting on adenililiciclase, a transmembrane enzyme. The increased cAMP levels have been stated to activate protein kinase A (PKA). PKA might phosphorilate L-type calcium channel, thus, enhancing inward calcium current.

Because of its presumed role in calcium homeostasis, the serotoninergic receptor might be implied in the heart conduction system dysfunction seen in CHB patients. This consideration led Eftekhari et al.33 to assess a study to define whether the serotoninergic receptor is implied in CHB pathogenesis.

Screening sera from patients with NLE, they could find a high anticorpal prevalence against the serotoninergic receptor correlating with the presence of anti-Ro52 Abs. To verify the presence of overlapping regions, they used a homology scanning program. A homologous region was found between Ro52 (amino acids sequence 365-382) and aa165-185 of the serotonin receptor, corresponding to the second extracellular loop (the G protein coupled receptor immunodominant region). To evaluate the cross reactivity, they used a peptide derived from the serotonin receptor which overlaps the homologous region. Raising antibody against that peptide in a rabbit model, they found that the affinity purified antibody recognize Ro52 and that the recognition was blocked when the Abs were preincubated with the serotoninergic receptor.

These findings may confirm the presence of cross reactivity between Ro52 and the serotoninergic receptor expressed on the surface of fetal cardiomyocytes.

When serotonin binds its pharmacologically defined 5HT_4 receptor, expressed on human atrial cardiocytes membrane, exerts positive chronotropic, ionotropic and lusitropic effect on the heart. Its action might be due to the enhancement of the calcium inward current, through L-type calcium channel. The engagement of serotonin receptor might lead, through a cAMP-mediated intracellular pathway, to the phosphorilation of key proteins involved in excitation-contraction coupling; one of these proteins is L-type calcium channel.

A mechanism, by which the serotoninergic receptor might play a role in CHB pathogenesis, should imply the binding of maternal Abs to serotonin receptors expressed on the fetal cardiomyocyte surface. This binding should cause the inhibition of 5HT_4 receptor and the loss of activation of the intracellular pathway leading to the enhancement of inward calcium current. The resulting current inhibition may play a role in cardiac electrical dysfunction.
The novel hypothesis that the response of maternal Abs against the serotoninergic receptor is directly relevant to the pathogenesis, was not supported by Buyon\'s study in 2002\textsuperscript{32}. Buyon et al. could identify the functional activity of the serotoninergic receptor in fetal cardiomyocytes and its role in calcium homeostasis; however, they could not demonstrate specific reactivity with the serotonin receptor peptide tested previously by Eftekhar et al. In order to define the role of the serotonin receptor in CHB pathogenesis and the reason of these conflicting results, a further study was established\textsuperscript{34}.

The discrepancies between Eftekhar\'s and Buyon\'s results might be due to differences in the assay set up and to the conformational structure of the serotonin receptor peptide tested. Only 16\% of mothers of child with CHB screened presented anti-5HT\textsubscript{4} Abs; on the contrary 98.6\% of them presented anti-Ro52 Abs. Furthermore anti-serotonin receptor peptide antibody had only 16\% sensibility and 75\% specificity for Neonatal lupus erythematosus. This findings suggest that anti-serotonin receptor peptide Abs may contribute to the electrophysiological disturbances compatible with CHB, but they do not have a predictive role for CHB comparable to that of anti-Ro52 Abs. Moreover, if the immunological response is seen as a dynamic phenomenon, then it is arguable that B cells produce cross reactive Abs against both Ro52 and 5HT\textsubscript{4}. During the immune response Abs develop specificity towards Ro52, in most of the cases and rarely towards 5HT\textsubscript{4}.

To assess the role of those Abs in physiologic and pathologic conditions, it would be useful to define, through histological comparison, the differences detectable in fetal hearts from mothers who have anti-Ro52 Abs and mothers with anti-5HT\textsubscript{4} receptor Abs.

\textit{Voltage-gated calcium channel hypothesis}

The role of calcium as a second messenger in cellular activities, particularly its importance in apoptosis\textsuperscript{35} and cardiomyocytes excitation-contraction coupling\textsuperscript{36}, give enough clues to guess a possible role of calcium homeostasis disturbances in CHB pathogenesis.

Furthermore, the clinical findings in CHB children showing various degree of AV block and the importance of calcium channels in pacemaker activity indicate a role of these channels in CHB development. Since the hallmark of autoantibody-associated CHB is complete AV block, researchers initially focused on the AV node; novel experimental models and clinical findings suggest that also the SA node might be affected in CHB.
Sinus bradycardia was found in Ro/La immunized mice\textsuperscript{37}. Brucato et al.\textsuperscript{38} and Menon et al.\textsuperscript{39} were able to detect sinus bradycardia in infants from anti-Ro/La Abs positive mothers. These experimental and clinical data highlight the implication of SA node in altered conductive activity seen in CHB disease. Indeed, human fetal autopsies\textsuperscript{40} showed calcification of SA node, pointing out its involvement in disease functional development.

SA node pacemaker activity might be due to the interplay of different currents that define different stages of nodal cardiocytes action potential. A general model to explain SA node pacemaker activity will be presented. It should be remembered that currents involved in heart impulse generation, and their interplay are still a matter of debate.

Once the nodal cell is electrically stimulated, it undergoes the so-called rapid depolarization phase, characterized by L-type calcium channels and, to a lesser extent, sodium channels opening, resulting in inward cation flux. This cellular cation influx causes the depolarization of cardiocytes from a resting membrane potential of -60 mV to a membrane potential of +10/+20 mV.

After reaching the spike of +10/+20 mV, the repolarization takes place: L-type calcium channels and sodium channels close and the slow delayed rectifier ($I_{Ks}$) potassium channels are still open, bringing the membrane potential to a more negative voltage.

One of the aspects that differentiate nodal cell from atrial and ventricular cardiomyocytes is the diastolic depolarization. After repolarization, the nodal cell membrane potential slowly becomes more positive. This change in voltage is due to the activation of the hyperpolarization-activated inward current (operative in early phase), L-type and T-type calcium currents (operative in late phase) and the persisting delayed rectifier potassium current.

If calcium channels are implied in CHB etiopathogenesis, two conditions have to be satisfied: they have to be expressed in fetal heart and recognized by maternal Abs.

Focusing on the major time-dependent currents, implied in SA node pacemaker activity, Hu et al.\textsuperscript{41} tested the currents affected by IgG positive maternal sera and their implication in fetal heart negative chronotropic influence. Application of IgG positive maternal sera to isolated rabbit SA node cells did not affect both delayed rectifier potassium current ($I_{K}$) and hyperpolarization-activated inward current ($I_{f}$). However it reduced the peak of L-type calcium channel current ($I_{CaL}$) from 191 to 112 pA and affected T-type calcium channel current ($I_{CaT}$) to a lesser extent.
L-type calcium channels are heterotrimeric transmembrane proteins composed by α1, β and α2/δ subunits. α1 subunit contains the voltage sensor domain, the selectivity filter, the ion conducting pore and the binding site for calcium channel blockers. The other subunits may be implied in controlling channel gating. Hu and al. demonstrated that the current inhibition did not affect I/V (current/voltage) relation or activation curve (voltage dependence remained unchanged). This finding might demonstrate that the alteration, induced by maternal IgG, does not affect channel gating. This consideration may suggest that α1C and α1D subunit (Figure 4) might be implied in CHB patients ECG abnormalities.

Up to now, it is not possible to pharmacologically separate α1C and α1D current in native cells. However, to define the effect of IgG on α1D subunit, Qu et al. developed an expression system that allowed them to identify IgG α1D current inhibition. Furthermore, they could detect from young rabbit heart tissue α1D L-type calcium channel mRNA in SA node, atria and AV node. On the contrary, α1C L-type calcium channel mRNA was universally present in the heart. From these results, α1D subunit is presumed to play a more incisive role than α1C one in sinus bradycardia detected in CHB-affected patients. In addition Mangoni et al. showed that SA node I_{CaL} density was decreased by 75% in α1D calcium channel subunit knockout mice compared to wild type. Moreover, a functional reason is at the basis of α1C and α1D subunits different roles in SA node action potential and cardiac electrical impulse generation. SA nodal cells diastolic depolarization occurs between -60 and -40 mV while α1C calcium channel subunit activates at a more positive voltage (-40 and -30 mV).

These biophysical property and the fact that α1D calcium channel knockout mice exhibit sinus bradycardia could highlight the role of this subunit in CHB development. These results may provide functional basis for CHB-associated sinus bradycardia and highlight

![Figure 4 - Schematic structure of L-type Ca channel α1D subunit. α1D subunit is organized in four domains, I–IV, each consisting of transmembrane segments S1–S6 connected with a small stretch of amino acids. The extracellular loop between transmembrane segments S5–S6, E-Loop, is the ion conductance pore and selectivity filter.](image)
the importance of calcium currents dysregulation in CHB disease. In addition, they point out the importance of finding a calcium channel agonist to treat, or at least to ameliorate the severity of CHB patients conductive abnormalities.

In this regard, Qu et al.\textsuperscript{42} showed in 2005 the restoration, above the basal level, of the inhibitory effect of maternal antibody on calcium current, in mice with CHB treated with a L-type calcium channel agonist (Bay K8644).

Efforts should be directed towards restoration or enhancement of inward calcium current, suggested to be affected by maternal Abs and to play a role in CHB development.

A presumable sequela that \textit{in vivo} leads to CHB pathogenesis might be proposed: circulating maternal Abs may recognize a certain epitope present in L-type calcium channels, expressed on fetal cardiomyocyte membrane. This binding inhibits calcium inward current, fundamental for nodal cells action potential, electrical impulse generation and working myocardium excitation-contraction coupling. Chronic calcium channel exposition to maternal Abs might lead to channel internalization, decreased cell membrane channel density and altered intracellular calcium concentration. Abnormal calcium levels could account for apoptosis. The latter may trigger inflammation process leading to tissue calcification or might be implied in intracellular antigens translocation to the cell membrane. This new location may enable maternal Abs to recognize cognate antigens leading to tissue inflammation and calcification.

Determining the specific epitopes bound by maternal autoantibodies is a critical first step in understanding the pathogenesis of this autoantibody-mediated disease and may lead to a novel screening and to new diagnostic and therapeutic strategies as well. Preventing CHB development could be achieved by defining a specific decoy to maternal Abs. This peptide would bind maternal Abs and inabililtate their binding of fetal cardiac epitopes.
Pathogenesis

*Opsonization of cardiocytes: the role of macrophages*

The traditional paradigm implied in Abs-mediated tissue damage is that anticorpal binding leads to the formation of immune complexes in the vicinity of the fetal cardiac conduction system. Immune complexes can account for complement system activation and thereby can be related to proinflammatory/profibrotic factors clearance by infiltrating macrophages and cytokine secretion.

Miranda-Carus et al.\(^{44}\) evaluated whether IgG-apoptotic cell complexes interfere in the physiologic tissue macrophages scavenging function, converting it into a proinflammatory/profibrotic event.

Apoptotic cells internalization by macrophages has always been thought to be immunosuppressive because of its inhibition of proinflammatory cytokine release.

Caron et al.\(^{45}\) identified two distinct phagocytosis pathways, defined by different receptors and controlled by two different intracellular cascades, both involving RhoGTPase.

![Figure 5 - Proposed CHB pathologic cascade leading from inflammation to fibrosis.](image-url)
Macrophages scavenging function, via Fcγ mediated uptake, is thought to be proinflammatory. On the contrary, type II phagocytosis, mediated by the complement receptor 3 (CR3), is not accompanied by inflammation.

Miranda-Carus et al.\textsuperscript{4} demonstrated that macrophages release of Th1 cytokine, TNFα, after opsonized apoptotic cardiocytes ingestion, increased by 3-5 fold over basal levels. This increased release was consistent with type I phagocytosis. These findings suggest that the presence of opsonized apoptotic cardiocytes induces macrophages phenotypic changes. This leads to macrophages activation resulting in cytokines release and therefore in inflammatory sequela development.

**Final stages of injury: from the transdifferentiation of fibroblasts to unresolved scarring of the AV node**

Physiologically, the healing of a wound is a well orchestrated process, in which cross-talk between “repair” cells is fundamental to achieve a correct recovery. During wound healing two phases take place; proliferation and remodeling. The proliferative phase is important for the formation of the granulation tissue whereas the latter is necessary to permit connective tissue matrix maturation. Fibroblast transdifferentiation to myofibroblast\textsuperscript{46} is a fundamental step in the granulation tissue correct formation. However, persistency of myofibroblasts can account for disruption of the healing equilibrium and for scarring tissue appearance.

Histopathologic studies showed a typical spectrum of CHB in affected heart fetuses including AV nodal replacement by fibrosis or fatty tissue\textsuperscript{47}, fibrous structures in the conduction tissue and working myocardium altered contractility secondary to endocardial fibroelastosis\textsuperscript{48}.

Smac-positive and CD68-positive infiltrated, indicative of myofibroblasts and macrophages respectively, was shown in the ventricular tissue of a term male infant, diagnosed with AV block at 19 weeks and dying at birth\textsuperscript{49}. Furthermore, the absence of myofibroblasts or macrophages was demonstrated in ventricular tissue from a 24-week healthy abortus and a term neonate died at birth of noncardiac causes (Figure 6). As a consequence, further studies were conducted. Clancy et al.\textsuperscript{50} incubated fibroblast primary cultures with a supernatant composed of opsonized apoptotic cardiocytes and macrophages. This way, they could test whether fibroblast differentiation to a scarring phenotype leading to fibrosis might be a consequence of opsonized apoptotic cells phagocytosis.
When cocultured with supernatants from opsonized apoptotic cells and macrophages, fibroblasts showed a markedly higher expression of Smac and enhanced proliferation. As already mentioned, myofibroblasts appearance is a necessary step in wound healing, particularly during granulation tissue formation. However, their persistence in wound site leads to fibrosis. The transdifferentiation from fibroblast to myofibroblast includes expression of Smac, of the embryonic form of smooth muscle myosin M chain, of αvβ3 integrin and types I and II collagen release. Myofibroblast role in unresolved scarring is due to αvβ3 binding to collagen. This interaction, in fact, interferes with lattice contraction and prevents matrix organization leading to fibrous tissue formation.

Furthermore, fibroblast exposition to TGFβ, obtained from macrophages cocultured with opsonized apoptotic cardiomyocyte, led to overexpression of Smac (Figure 7) and markedly attenuate proliferation. Conversely, TNFα had no effect neither in proliferation nor in Smac expression. These results are supported by a previous study in which TGFβ injection in rats induced Smac-positive cells infiltration and granulation tissue formation.

The importance of cytokine ratio in cross-talk between macrophages and fibroblast and their relevance in fibroblast transdifferentiation have yet to be defined.

Figure 6 - Detection of macrophages and myofibroblasts in CHB. Longitudinal sections through the left ventricle from the affected neonate were stained using H&E (A and B), anti-SMAC (C and D), and anti-CD68 (G and H). Densely packed myofibroblasts (C and D, SMAC-positive cells) are present in thickened fibrous subendocardial areas adjacent to small clusters of macrophages (G and H, CD68-positive cells). Sections from the left ventricle of a normal 24-wk abortus were also stained with anti-SMAC (E) and anti-CD68 (I), as were sections from the right ventricular endomycocardium (one-third of the way from AV valve to apex) of a term neonate dying at birth of noncardiac causes (F, anti-SMAC; J, anti-CD68).
In conclusion, these *in vivo* and *in vitro* studies, support the speculation that *in vivo* IgG-apoptotic cell complexes form near the heart conduction system. These complexes lead to proinflammatory/profibrotic factors clearance by infiltrating macrophages and cytokines secretion that modulate fibroblasts into scar promoting myofibroblasts. This suggests that CHB results from unresolved wound healing.

In areas of high remodeling such as heart electrical conduction system, apoptosis can be consistently appreciated during development. Perturbation of the clearance of those apoptotic cells, due to Abs binding, can account for the skewing of IgG-apoptotic cells complexes from a non inflammatory phagocytosis towards a proinflammatory clearance by infiltrating macrophages. Diverting macrophages physiological clearance towards inflammatory pathway implies the release of cytokines, such as TGFβ, contributing to the exuberant scarring, due to transdifferentiation of fibroblasts into myofibroblasts.

It is logical that the most vulnerable organs are those with minimal regenerative capacities, such as the heart and, particularly, its terminally differentiated conduction system. Furthermore, scar tissue formation is implied in pacemaker and electrical dysfunction leading to permanent heart block.

![Figure 7 - Effect of supernatants from cocultures of macrophages on transdifferentiation (SMAc staining) of cardiac fibroblasts. Cultured fibroblasts were incubated in the absence (A and E) or presence (B–D and F–H) of supernatants. The supernatants were derived from cultured macrophages alone (B and F), macrophages incubated with nonopsonized apoptotic cardiocytes (C and G), or macrophages incubated with opsonized apoptotic cardiocytes (D and H).](image-url)

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Contributory genetic factors

The low incidence of CHB suggests that, in its pathogenesis, maternal Abs are a required but not sufficient factor, pointing out the necessity to investigate the role of other influences.

Previous studies focused on the role of maternal disease severity and infections during pregnancy as risk factors. However, both of them were not found to contribute to the disease. Maternal and fetal genetic constitutions might play a determining role. Both maternal and fetal genetics might be involved in CHB pathogenesis: at the maternal level determining antibody specificity; at the fetal level determining fetal CHB susceptibility.

Investigating maternal HLA genes involved in antibody specificity Gottenberg and al. found that HLA DRB1 02 and HLA DRB1 03 are strongly associated with the presence of maternal La Abs, regardless of the maternal health status. Moreover, two studies in 1999 identified that HLA alleles enriched in mothers with CHB children are DRB1 03, DQB1 02, DQA1 05 and HLA Cw7.

Fetal alleles that might be involved in CHB susceptibility are potentially those of all genes coding for proteins implied in antigen presentation, inflammation control and fibrosis pathways. Previous studies have demonstrated an increased frequency of Class I HLA-Cw3 in CHB children compared to their healthy siblings and the role of fetal profibrogenic TGFβ allele and TNFα promoter region A polymorphism in developing the disease.

Recently Strandberg et al. assessed four different Ro52 immunized rat models. Three of them shared the same MHC allele RTAV1(DA.AV1, PVG.AV1, LEW.AV1) while the fourth had different MHC allele RT1 but shared with the others the same genetic background. These strains have already been demonstrated to have different susceptibility to other autoimmune disease, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis.

They could assess that maternal MHC alleles are fundamental for determining the anticorpal specificity, through antigen presenting pathway. Once maternal Abs are transferred, fetal MHC alleles are crucial for the different susceptibility to the cardiac injury.

Moreover, as already shown for other autoimmune disease, they could demonstrate that maternal or paternal inheritance origin plays a role in fetal susceptibility. In fact pups with maternal origin alleles, had a significantly longer PR interval when compared to pups with
paternal origin alleles. This latter finding points out the presence of a further regulatory level in CHB development out of a pure genetic constitution, plausibly lying in the epigenetical background.

Further studies should be carried out to better define the role of different maternal and fetal MHC molecules, and to test the presence of other contributory alleles out of MHC locus. In fact, genes involved in the inflammatory and fibrotic pathway could be risk factors.

When considering CHB disease, the role of placenta genes in determining fetal environment should be taken into account. Uterine environment might play an important role in defining whether a fetus may or may not develop AV block.
Future directions

From 12th to 15th June 2013, the “The 14th Congress of The European League against Rheumatism” held in Madrid, brought together leading scientists in the field. During this meeting CHB experts addressed some of the questions which still remain open for this fatal disease.

Since CHB has never been detected in mothers with CHB children, the target of maternal Abs, as already pointed out, may likely be an AV node antigen, whose expression is developmentally regulated. Hoxha et al.61 directed their efforts towards the definition of human developmentally regulated molecules in the AV node. Those molecules were found to be important in determining the phenotype of AV nodal cells, and present at the time of maternal Abs supposed passage through placenta.

One of the molecules found to be developmentally regulated in the AV node is the T-type calcium channel α1G subunit. Strandberg et al.62 are assessing cross-reactivity of anti-Ro/La Abs positive maternal sera with a peptide from T-type calcium channel α1G subunit. If they will detect cross-reactivity, this finding might suggest that α1G subunit might have a possible implication in CHB.

It should be highlighted that, even if the hallmark of autoantibody-associated CHB is complete AV block, recent clinical38,39, experimental models37 and histological findings40 suggest a novel implication of SA node in CHB pathogenesis. Because of its membrane potential window of activation (-70/-60 mV63), T-type calcium channel current might play a role in the late phase of SA node diastolic depolarization. Further studies are required to assess whether antibody binding to T-type calcium channel might be implied in sinus bradycardia, recently detected in CHB patients.

Some molecules expressed in the heart, such as βmyosin heavy chain, have been demonstrated to undergo re-expression under pathological conditions; the same mechanism could involve maternal antibody epitopes implied in CHB. With this regard, Tsang et al.64 could demonstrate the role of cytomegalovirus in inducing cell surface expression of Ro52 antigen. Cytomegalovirus infection might be a possible inductor to evaluate for CHB development.

Yan et al.65 have started to define potential targets of CHB pathophysiology through a review of a database subset of small molecules implied in cardiac conductive activity.
Their purpose is to identify proteins involved in a biological network system related to cardiac conductive activity, and to predict possible CHB drug targets.

The international workshop turned out to be an important forum to debate about novel developments in CHB field. The importance of this meeting resides in the possibility for scientists, with different backgrounds, to compare their points of view. This comparison will help to better understand the basis of this disease and to develop optimal management and treatment to improve patient outcome.
Conclusions

Despite a century of advances since Morquio’s first CHB report, the challenge continues with the quest for understanding this complex disease in all its facets.

The essay has characterized the features of autoantibodies-associated CHB and its differentiation from CHB in fetuses with cardiac abnormalities. Presumable targeted antigens and a possible process responsible for fetal cardiac injury have been presented. Despite the fact that promising new leads in target cardiac antigen definition have been proposed, up to now, Ro52 remains the strongest candidate. However, focus remains on defining the way to account for the accessibility of this intracellular antigen.

Moreover, it should be outlined that most CHB studies considered in this essay were based on in vitro models. It is therefore complicated to define whether the results they expose reflect characteristics of heart injury seen in CHB children.

CHB is a disease not yet completely understood as target antigens and additional contributory factors still remain open questions.

Major efforts should be directed towards the screening of genetic and epigenetic polymorphisms linked to CHB development. A regulatory mechanism both at a genetic and at an epigenetic level might explain some of the critical clinical aspects pointed out in this essay, such as low incidence and twin discrepancy in disease developing.

During my summer internship, I began to understand the difficulties in investigating the complexity of this disease. Due to the rarity of CHB and the ethical issues involved in acquiring aborted fetuses, there is a shortage of diseased heart tissue samples, as well as maternal sera with pathogenic Abs to conduct research. This also affects the ability to obtain sufficient statistical power. Therefore, it would be useful to create a network among researchers studying this disease. Through collaboration, they would be able to share samples as well as clinical data.

Once a network has been established, the possibility for dissecting the individual components in CHB inflammatory cascade would increase. Moreover, this collaboration and knowledge translation would benefit families, help to define research strategies, and, maybe, establish management practices of pregnancies at risk of developing CHB as well as treatment for those in which CHB was detected.
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