Anti-Ro/SSA-p200 antibodies in the prediction of congenital heart block. An Italian multicentre cross-sectional study on behalf of the "Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni (FIRMA) Group"

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Abstract

Objective

To verify the association between the presence of specific anti-52 Ro/SSA-p200 antibodies and congenital heart block (CHB).

Methods

207 pregnant Italian women carrying anti-Ro/SSA Ab were retrospectively evaluated. Anti-p200 Ab were investigated in the mothers' sera by ELISA (Euro-Diagnostica,Wieslab SS-A p200).

Results

CHB occurred in 42 children (34 complete CHB), whereas 165 were not affected. All CHB cases were previously identified with an ELISA screening for anti-Ro/SSA 60 kD Ab. Anti-p200 Ab were more frequently positive (81.0% vs. 59.1%, p=0.013) and at a higher titer in CHB mothers (Absorbance ratio: 2.030 (0.208–4.052) vs. 0.925 (0.200–3.816); p=0.017). This association was maintained even when the 42 mothers of children with CHB were compared with a control group matched for age and diagnosis (80.9% vs. 50.0%; p=0.006). The presence of anti-p200 Ab provided an odds ratio (OR) for CHB of 2.98 (CI: 1.30–6.83), which was higher than that of other variables, such as maternal disease and other antibody specificities. CHB risk significantly decreased in the absence of this fine specificity (OR:0.34, CI: 0.15–0.77). However, while the predictive negative value related to anti-Ro/SSA 60 kD Ab ELISA was 100%, almost 20% of mothers negative for anti-p200 Ab delivered babies with CHB.

Conclusion

Anti-p200 antibodies seem to be associated with CHB with a higher probability than anti-Ro/SSA Ab, and therefore may be an additional test to identify mothers at higher risk to deliver affected children. An ELISA screening for anti-Ro/SSA 60 kD Ab is nevertheless mandatory given the probability of developing CHB also in the absence of anti-p200 Ab.

Key words

connective tissue disease, anti-Ro/SSA antibodies, anti-p200 antibodies, congenital heart block, neonatal lupus

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Introduction

Neonatal lupus (NL) is a rare syndrome associated with the transplacental passage of maternal anti-Ro antibodies (Ab). Congenital heart block (CHB) is the most severe complication of NL, causing a high rate of perinatal mortality (15–30%) and morbidity (1).

The pathogenesis of CHB remains not fully understood. According to the current hypothesis, placental passage of maternal anti-Ro Ab is responsible for foetal cardiocyte damage (2), but this condition is not sufficient for CHB development. In fact, many patients with anti-Ro Ab deliver healthy children, suggesting the involvement of various other genetic and perinatal factors (3, 4). The tendency to an impaired process of both foetal cardiocyte apoptosis, and apoptotic bodies opsonisation, could justify the inflammation persistence and consequent scarring (5, 6).

Recently, novel interest was raised by a small central portion of 52 kD Ro, from 200 to 239 amino-acids, simply named p200. According to some authors, p200 could represent the fine specificity of anti-Ro Ab associated to CHB. In 2002, Salomonsson described the presence of anti-p200 in 9 out of 9 mothers who delivered babies with CHB (7). In 2005, the same Authors demonstrated that anti-p200 Ab inoculation in rat pups was associated to cardiocytes Ca^{2+}-homeostasis dysregulation, leading to an increase in heart cell death, atrium-ventricular conduction prolongation and finally to CHB development (8). In the same year, also Clancy (9) evaluated the presence of anti-p200 Ab in a wide cohort of American anti-Ro positive mothers, but the previously reported observation was not confirmed. Finally, in 2008 Strandberg showed an association between high titers of maternal anti-p200 Ab and CHB in a wide multicentre and multi-ethnical study (10). More recently, an animal model demonstrated that the inoculation of anti-p200 Ab, but not of anti-52 kD Ro Ab without p200 peptide specificity, was able to cause heart block in rat pups (11).

The aim of our study is to verify the capability of anti-p200 Ab to predict CHB. A large Italian cohort of anti-Ro Ab positive mothers was studied in order to:

1. analyse the sensitivity, the specificity, the positive predictive value (PPV) and the negative predictive value (NPV) for CHB of anti-p200 ELISA compared to those of other immunoassays targeting unselected anti-Ro Ab (counter-immune-electrophoresis (CIE) and anti-Ro ELISA);
2. verify the association between maternal anti-p200 Ab and the occurrence of CHB;
3. verify the rate of anti-p200 Ab placental passage;
4. estimate the risk for CHB in children born to anti-p200 Ab positive mothers.

Material and methods

Patients

The study was performed in the frame of the "Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni" (FIRMA) Group. An Italian cohort of 207 anti-Ro/SSA Ab positive mothers from 6 different centres (Brescia, Milano, Pisa, Padova, Pavia and Bergamo) was retrospectively evaluated; the median age of the cohort was 32 years (range: 27–38); pregnancy outcome was a live-birth in 203 cases, whereas in 4 cases mothers decided to voluntarily interrupt the pregnancy, because of the occurrence of complete CHB (CCHB). A connective tissue disease (CTD) was diagnosed in 189 mothers – 70 Sjögren's syndrome (SS), 40 systemic lupus erythematosus (SLE) and 79 Undifferentiated connective tissue disease (UCTD) – while 18 women were asymptomatic. During pregnancy, prednisone treatment was administered to 47% of women with CTD, hydroxychloroquine to 32%, other immunosuppressive agents (cyclosporine-A and azathioprine) to 5% and low-dose aspirin to 60%.

CHB occurred in 42 babies (34 CCHB and 8 incomplete atrium-ventricular block, IAVB). Neonatal rash (NR) was observed in six babies, whereas no sign of NL was recorded in 159 children (HC: healthy children). The high occurrence of CHB in our cohort did not reflect the real incidence of the disease in the population of anti-Ro positive mothers, because full records of many anti-Ro positive pregnancies were not

available from different centres. For this reason, patients were not consecutively enrolled and the rate of CHB consequently resulted as overestimated.

Methods

Sera from all the mothers were preferentially collected during or nearby pregnancy, in a period ranging from 1989 and 2009, and retrospectively evaluated. All the 207 sera were tested for anti-Ro in a routine setting by at least one of the following assays: counter-immune-electrophoresis (CIE; home made assay (12); n=206), ELISA (DIASTAT™ anti-Ro (SS-A); Euro-Diagnostica; n=196) and immune-blotting for 52 and 60 kD Ro (IB; INNO-LIA ANA update, INNOGENETICS; n°33). Ro specificity was verified in CIE using a porcine spleen extract, whereas in ELISA an affinity-purified antigen (Ro 60 kD) from bovine thymus was coated in the plate. Anti-La Ab were determined by CIE assay in 125 samples; in 106 cases anti-La Ab were also tested in ELISA (DIASTAT™ anti-La (SS-B); Euro-Diagnostica).

Sera were retrospectively tested for anti-p200 Ab in ELISA (Euro-Diagnostica,Wieslab SS-A p200). A specific recombinant human p200 peptide was synthesised and furtherly coated to the plate, with a biotin-streptavidin system. Sera were firstly diluted and incubated to the plate; three washing steps followed. A conjugate of alkaline phosphatase-labelled Ab to human IgG was then added, and the detection of the antibodies was obtained with a substrate incubation (pNPP solution). Finally, a stop solution (containing EDTA and Tris) was added and the adsorbance of the plate was read by a spectrophotomer at 405 nm. All the determination of anti-p200 ELISA were performed in the same laboratory (Milano). Similarly, sera from 44 babies were collected at birth or within the first trimester of life and they were tested for anti-p200 Ab when the mother resulted positive for such antibodies. Local ethics committee authorisation was obtained before the evaluations on children sera. As negative controls, 55 sera from CTD women who were negative on CIE assay for anti-Ro Ab and positive for other anti-extractable nuclear antigens (ENA, 28 anti-Scl70 Ab; 9 anti-Sm Ab; 18 anti-Jo1 Ab) were tested by anti-p200 ELISA.

Statistical methods

Data were expressed as median (10^{th}-90^{th} percentile). Comparison between continuous variables was performed by Mann-Whitney test or by Wilcoxon Signed Rank test for paired data; Chi-Square test with Yates'correction was used to compare nominal variables. Whenever an association between CHB and multiple serological or clinical factors was observed, a multivariate analysis was performed. Anti-p200 Ab titer was expressed as the Absorbance Ratio (AR) calculated on a positive reference serum provided in the kit; according to this method the cut-off for anti-p200 Ab was ≥0.400 of AR (99^{th} percentile of negative controls). Sensitivity, Specificity, NPV and PPV were evaluated to compare different assays. Assuming a prevalence for CHB of 2% as reported in the literature (13), NPV, PPV were calculated with Bayes' theorem as follows (14): NPV: (prevalence of CHB * sensitivity for CHB)/[(prevalence of CHB*sensitivity for CHB)+[(1-prevalence of CHB)*(1-specificity for CHB)]]; PPV: (1-prevalence of CHB)* specificity for CHB/[(1-prevalence of CHB)*[(specificity for CHB)+(prevalence for CHB)*(1-sensitivity for CHB)]]. Simple regression for non parametric data was used to analyse the association between neonatal PR electrocardiographic interval at birth and anti-p200 Ab titer in mothers and in newborns.

The odds ratio (OR) with a 95% confidence interval (CI) was applied to estimate the risk for CHB in different antibodies specificity and in different maternal diseases. A *p*-value less than 0.05 was considered significant.

Results

Analysis of different anti-Ro Ab specificities

All the 207 mothers' sera resulted positive for at least one assay among CIE, ELISA or IB for anti-Ro Ab (196 positive to CIE, 189 to ELISA, 23 to 52 kD Ro IB and 30 to 60 kD Ro IB). A double positive test was observed in 195 sera (182 CIE and ELISA; 4 ELISA and IB; 9 CIE and IB), while three positive tests were seen in 18 mothers. A positivity for both anti-Ro and anti-La Ab was also observed in 44 out of 125 (35.2%) on CIE, and in 52 out of 106 (49.0%) on ELISA. In our cohort, no association was seen between CHB and the simultaneous presence of both anti-Ro and anti-La Ab (p=0.563).

Anti-p200 Ab resulted positive on ELISA in 131 out of the 207 maternal samples (63.3%). Independently from maternal disease, anti-p200 Ab demonstrated a significant association with CHB and CCHB (CHB=81.0%, CCHB=82.4% *vs*. HC=59.0%; p=0.013 and p=0.020); in these subgroups, anti-p200 Ab titer was significantly higher than in HC mothers (AR_{CHB}=2.030 (0.208–4.052) and AR_{CCHB}=2.098 (0.227–3.977) *vs*. AR_{HC}=0.925 (0.200–3.816); p:0.017 and p:0.016) (Table I, Fig. 1a). No significant difference was observed between the titer of anti-p200 Ab of mothers with babies suffering from IAVB and women with babies affected by CCHB (AR_{IAVB}= 1.409 (0.197–4.156) *vs*. AR_{CCHB}=2.098 (0.227–3.977); p=0.761) (Table I, Fig. 1a). No correlation was seen between maternal anti-p200 Ab titer and neonatal PR interval on electrocardiogram at birth (r=0.22, p=0.102).

To verify whether CHB may be dependent from maternal disease, a control group composed by 42 anti-Ro Ab positive mothers with healthy children was matched for diagnosis and age at delivery with the 42 mothers of children affected by CHB. Prevalence of anti-p200 Ab was respectively 80.9% and 50% (p=0.006) in CHB and in HC mothers; in addition, sera from mothers of affected children showed a significantly higher anti-p200 Ab titer (AR_{CHB}=2.030 (0.208–4.052) *vs*. AR_{HC}=0.337 (0.200–3.549); p=0.005) (Fig. 1b).

Anti-52 kD Ro Ab resulted positive in 23 out of 33 samples tested on IB (69.7%). Due to the very small number of sera tested for this specificity, we were not able to analyse the association between anti-52 kD Ro and CHB.

Prevalence of anti-p200 Ab in maternal diseases

In SS patients, anti-p200 Ab

Table I. Antibodies profile in the enrolled mothers.

Mothers of		Anti-Ro CIE (n=206)	Anti-Ro ELISA (n=196)	Anti-p200 ELISA (n=207)	Anti-p200 Ab titer (ratio) (n=207)
HC	pos	152 (96.2%)	143 (96.0%)	94 (59.1%)	0.925
	neg	6 (3.8%)	6 (4.0%)	65 (40.9%)	(0.200-3.816)
NR	pos	6 (100%)	5 (83.3%)	3 (50.0%)	0.553
	neg	0	1 (16.7%)	3 (50.0%)	(0.230-3.250)
IAVB	pos	8 (100%)	8 (100%)	6 (75.0%)*	1.409**
	neg	0	0	2 (25.0%)	(0.197-4.156)
CCHB	pos	32 (94.1%)	33 (100%)	28 (82.4%)*	2.098**
	neg	2 (5.9%)	0	6 (17.6%)	(0.227-3.977)
CHB	pos	40 (95.2%)	41 (100%)	34 (81.0%)*	2.030**
	neg	2 (4.8%)	0	8 (19.0%)	(0.208-4.052)
SS	pos	70 (100%)	67 (97.1%)	61 (87.1%)*	2.471**
	neg	0	2 (2.9%)	9 (12.9%)	(0.331-3.989)
SLE	pos	37 (92.5%)	33 (97.1%)	18 (45.0%)	0.300
	neg	3 (7.5%)	1 (2.9%)	22 (55.0%)	(0.200-3.040)
UCTD	pos	76 (97.4%)	74 (94.9%)	40 (50.6%)	0.400
	neg	2 (2.6%)	4 (5.1%)	39 (49.4%)	(0.200-3.674)
Asymptomatic mothers	pos	15 (83.3%)	15 (100%)	12 (66.7%)	0.674
	neg	3 (16.7%)	0	6 (33.3%)	(0.200-3.914)

Relative percentage into bracket. HC: mothers of healthy children; NR: mothers of babies with neonatal rash; IAVB: mothers of children with incomplete atrium-ventricular block; CCHB: mothers of babies suffering from complete congenital heart block; CHB: mothers with children affected by CHB. SS: mothers with Sjögren's syndrome; SLE: mothers with systemic lupus erythematosus; UCTD: mothers with undifferentiated connective tissue disease. *p-value <0.05 (nominal variables); **p-value <0.05 *versus* HC/other diseases.

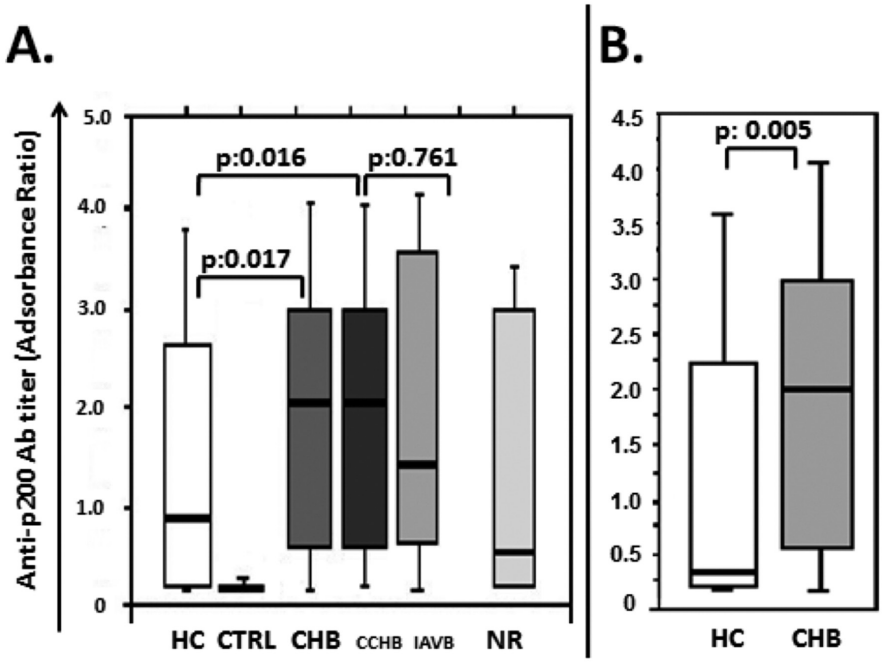

Fig. 1 A . Anti-p200 antibodies titer in mothers who resulted positive for at least one assay for anti-Ro/SSA Ab detection and in a control group;
B. comparison between anti-p200 Ab titers in mothers of children with congenital heart block (CHB) and a group of anti-Ro/SSA positive women with healthy children (HC) matched for age and disease. Titer expressed as adsorbance ratio between the optical density obtained from every single sample and the reference serum provided in the kit. Range expressed as 10th and 25th percentile, median, 75th and 90th percentile.

were detected at a higher prevalence (SS=87.1% *vs*. SLE=45.0%, UCTD=50.6% and asymptomatic mothers: 66.7%; $p<0.0001$, $p<0.0001$, $p=0.024$) and with a higher titer than in other CTD and asymptomatic mothers (AR_{SS}=2.471 (0.331–3.989) *vs*. AR_{SLE}=0.300 (0.200–3.040), AR_{UCTD}=0.400 (0.200–3.674), $AR_{a\text{-}symptomatic\ mothers}$=0.674 (0.200–3.914); $p<0.001$, $p<0.0001$, $p=0.028$) (Table I). In women suffering from SS, SLE and in asymptomatic mothers, no association was observed between anti-p200 Ab titer and CHB (SS=AR_{CHB}=2.692 (0.603–4.127) *vs*. AR_{HC}=2.470 (0.326–3.862), $p=0.415$; SLE: AR_{CHB}=0.226 (0.192–1.673) *vs*. AR_{HC}=0.300 (0.200-2.664), $p=0.507$; asymptomatic mothers: AR_{CHB}=1.072 (0.436–2.812) *vs*. AR_{HC}=1.016 (0.200–3.914), $p=0.374$). On the other hand, UCTD mothers with children affected by CHB had a higher anti-p200 Ab titer than UCTD mothers with HC (AR=2.194 (0.213–3.943) *vs*. AR=0.341 (0.199–3.538); $p=0.027$).

Transplacental passage of anti-p200 Ab

Anti-p200 Ab resulted positive in all tested children (24/24) who were born to anti-p200 positive mothers; no significant difference was observed between neonatal and maternal Ab titer (AR_{babies}=2.247 (0.758–4.681) *vs*. $AR_{mothers}$=2.664 (1.262–4.072); $p=0.503$). In 5 out of these 24 babies we observed NL, and particularly in 4 cases we observed heart involvement (2 IAVB, 2 CCHB, 1 NR). No correlation was seen between neonatal anti-p200 Ab titer and neonatal PR interval on electrocardiogram at birth (r=0.32, $p=0.222$).

Accuracy of different methods

The performance of single assays showed that anti-p200 ELISA provided the highest specificity (41.2%) and PPV (2.7%), while the highest sensitivity (100%) and NPV (100%) was obtained with anti-Ro ELISA (Table II). Combining different assays, (the first one as screening for anti-Ro Ab and the second one as confirmation test), CIE followed by anti-p200 ELISA showed the best specificity (37.3%) and PPV (2.6%) for CHB. On the contrary, the highest sensitivity (100%) and NPV

Table II. Comparison of sensitivity, specificity, positive and negative predictive value among different assays.

Laboratory assay	Sensitivity for CHB	Specificity for CHB	Positive Predictive Value for CHB	Negative Predictive Value for CHB
CIE (anti-Ro Ab)	95.2%	3.7%	2.0%	97.4%
ELISA (anti-Ro Ab)	100%	4.5%	2.1%	100%
ELISA* (anti-p200)	81.0%	41.2%	2.7%	99.1%
CIE (anti-Ro Ab) followed by ELISA (anti-Ro Ab)	100%	4.7%	2.1%	100%
ELISA (anti-Ro Ab) followed by CIE (anti-Ro Ab)	95.1%	2.7%	2.0%	96.5%
CIE (anti-Ro Ab) followed by ELISA* (anti-p200 Ab)	82.1%	37.3%	2.6%	99.0%
ELISA (anti-Ro Ab) followed by ELISA (anti-p200 Ab)	80.5%	36.1%	2.5%	98.9%

The analyses were performed using a single assay and using two methods in combination (the first for screening and the second for confirmation after a positive screening test). CIE, Counter-Immune-Electrophoresis; ELISA, Enzyme-Linked-Immune-Sorbent-Assay.
*: Chi-Square test with a p value<0.05.

Table III. Odds Ratio for CHB in children from mothers with different antibody profile and diseases.

	OR (95% CI)		
	Total	Anti-p200 Ab^{pos}	Anti-p200 Ab^{neg}
Antibodies maternal profile			
Anti-Ro Ab^{pos} (either 52 kD or 60 kD or both)	0.76 (0.15-3.91)	2.60* (1.17-5.78)	0.33* (0.14-0.79)
Anti-Ro Ab^{pos} and anti-La Ab^{pos}	1.38 (0.62-3.07)	1.52 (0.67-3.44)	0.57 (0.07-4.78)
Anti-Ro Ab^{pos} and anti-La Ab^{neg}	0.73 (0.33-1.62)	1.50 (0.67-3.35)	0.40 (0.14-1.11)
Anti-p200 Ab^{pos}	2.98* (1.3-6.83)	n.a.	n.a.
Anti-p200 Ab^{neg}	0.34* (0.15-0.77)	n.a.	n.a.
Maternal disease			
Mothers with SS	1.11 (0.55-2.26)	1.26 (0.61-2.59)	0.48 (0.06-3.94)
Mothers without SS	0.9 (0.44-1.83)	2.09* (1.05-4.17)	0.35* (0.15-0.84)
Mothers with SLE	0.50 (0.18-1.37)	0.47 (0.10-2.11)	0.59 (0.17-2.10)
Mothers without SLE	1.99 (0.73-5.45)	3.32* (1.53-7.19)	0.32* (0.12-0.86)
Mothers with UCTD	0.77 (0.38-1.57)	1.66 (0.75-3.69)	0.28* (0.08-0.94)
Mothers without UCTD	1.30 (0.64-2.65)	1.73 (0.87-3.42)	0.56 (0.20-1.54)

OR: odds ratio; CI: confidence index; SS: Sjögren's Syndrome; SLE: Systemic Lupus Erythematosus; UCTD: Undifferentiated Connective Tissue Disease; *: p value < than 0.05; ^{pos}: positivity; ^{neg}: negativity; n.a.: not applicable.

(100%) was obtained with a screening on CIE followed by anti-Ro ELISA for confirmation (Table II).

Association between age/disease/ treatment during pregnancy of the mothers and CHB occurrence

In our cohort, the occurrence of CHB was not associated to SS (p=0.771), UCTD (p=0.470) and SLE (p=0.173). As expected, a significant association with CHB was observed in children born to women that carried anti-Ro Ab without any clinical condition (asymptomatic mothers, p=0.008; OR:3.65 (CI: 1.34–9.92)).

Age at delivery did not differ in CHB and HC mothers (32 (27–37) *vs*. 32 (26–39.5); p=0.568). The occurrence of CHB was not associated to the treatment used by mothers during pregnancy (prednisone, p=0.531; hydroxychloroquine, p=0.816; low-dose aspirin: p=0.808; cyclosporine-A, p=0.410; azathioprine, p=0.823).

Evaluation of CHB risk

The odds ratio (OR) for CHB in children born to mothers with anti-Ro Ab, and with both anti-Ro and anti-La Ab was respectively 0.76 (CI: 0.15–3.91) and 1.38 (CI: 0.67–3.07) (Table III). Children born to mothers with and without anti-p200 Ab resulted with an OR for CHB of 2.98 (CI: 1.3–6.83) and 0.34 (CI: 0.15–0.77) respectively. Particularly, a medium-high titer of anti-p200 Ab (AR≥ 1.86, set on the 33rd percentile of the positivity range) provided a higher risk for CHB than a low titer (OR:2.34 (CI: 1.10–5.00) *vs*. OR:1.16 (CI: 0.52–2.59), respectively). In the presence of SS, SLE, UCTD, OR for CHB was 1.11 (CI: 0.55–2.26), 0.50 (CI: 0.18–1.37) and 0.77 (CI: 0.38–1.57), respectively (Table III). The multivariate analysis confirmed that the positivity for anti-p200 Ab and the condition of "asymptomatic mothers" with anti-Ro Ab may be independent risk factors for the development of CHB (p:0.035, OR: 2.98 (1.3–6.83) and p=0.008, OR: 3.65 (1.34–9.92), respectively).

Discussion

The evaluation performed by Strandberg is nowadays the widest collaborative study on anti-p200 Ab predictive value in CHB, thanks to a large multiethnic population composed by Finnish, Swedish and American mothers. These Authors demonstrated that high titers of anti-p200 Ab were associated with CHB development (10). Furthermore, they showed an increase of the PPV using a home made anti-p200 ELISA kit after a preliminary test for anti-52 kD Ro Ab. Differently from this study, we included all the mothers positive for anti-Ro Ab, independently from their fine specificity (either anti-52kD Ro Ab or anti 60 kD Ro Ab or both), as usually observed in our clinical practice.

First, our attention focused on different laboratory assays specificity and sensitivity. Anti-p200 ELISA provided a lower sensitivity and NPV for CHB than CIE and anti-Ro ELISA. On the

contrary, anti-p200 allowed to increase the specificity and PPV for CHB when used as confirmation assay. Therefore, anti-p200 Ab detection cannot substitute the conventional screening assays, but it may be helpful to stratify CHB risk, if used as additional method in anti-Ro positive pregnant women. Unfortunately, we were not able to assess the comparative value of anti-p200 and anti-52 kD Ro Ab, due to the small number of available data.

The analysis of anti-p200 Ab in various pathologic groups showed a higher prevalence and titer in mothers affected by SS. This observation suggests that anti-p200 Ab may be disease-related autoantibodies, and it can support the increased risk for CHB in this condition as already reported in the literature by some Authors (13-15).

As observed by Strandberg (10), we also found a significantly higher titer of anti-p200 Ab in mothers of children with CHB. The comparison between our study group (anti-Ro positive mothers with children affected from CHB) with a similar control group (anti-Ro positive mothers with HC, matched for age and disease) confirmed this observation, allowing to exclude that the higher titer observed in CHB group was dependent from maternal disease. Taken as a whole, these data suggest a possible level-dependent mechanism of these antibodies in inducing CHB. However, the lack of correlation between the neonatal PR electrocardiographic interval and maternal anti-p200 Ab titer suggests that the heart involvement severity is not directly related to such autoantibodies, remarking the great complexity of CHB pathogenic model. Therefore, the presence of anti-p200 Ab seems to be only one of the multiple variables implicated in CHB, as already reported (3-5). Since the low number of collected cases, we could not verify the association between anti-p200 Ab and neonatal rash.

The choice of different technical steps seems to be relevant for the detection of anti-p200 Ab. In particular, the peptide coating to the plate is probably determinant for specific antibodies binding on ELISA setting. As a demonstrative example, maternal sera from the American Research Registry for Neonatal Lupus gave different results as analysed either with a direct or an indirect peptide coating (9, 10). Furthermore, comparable results were obtained in our evaluation and in Strandberg's study using a similar technique (indirect binding through a streptavidin-biotin dependent system). The lack of standardisation of this method could also justify the discrepancies. In consideration of these limits, we suggest that a wide control group should be tested every time and that the cut-off should be selected on the 99th percentile of the range.

Regarding anti-p200 Ab placental passage, our analysis was performed on a small group of sera obtained from children at birth, or within the first month of life. Only 24 mothers of the 44 available children resulted positive for anti-p200 Ab; in all these 24 cases such antibodies crossed the placenta. Furthermore, anti-p200 Ab titer was very similar in mothers and children. Therefore, our findings seem to confirm previous data showing a high transplacental passage of anti-Ro Ab (16, 17). In our cohort, only few babies who resulted positive for anti-p200 Ab developed CHB; this may be explained by the large heterogeneity of factors involved in the CHB pathogenesis (3-5). Unfortunately, we were unable to verify whether the antibodies with anti-p200 specificity displayed a higher rate of transplacental passage compared to anti-Ro Ab, but other Authors recently studied in detail umbilical cord blood levels in 123 neonates, showing similar trasnplacental passage for all the Ro autoantibodies (17).

Anti-p200 Ab resulted as one of the most relevant serological risk factors in the analysis of risk for CHB (Table III). Particularly, medium-high titers of anti-p200 Ab are associated with a higher risk than low titers, whereas the absence of anti-p200 Ab seems to be protective against CHB. Previous studies reported an increase of CHB risk in children born to mothers affected by SS and UCTD (18) and in babies of women with both anti-Ro Ab and anti-La Ab (14). In our evaluation, anti-Ro plus anti-La Ab gave a lower risk for CHB than anti-p200 Ab; children of SLE women had a lower risk than others. Furthermore, we excluded that the different rate of CHB among various CTD was related to the treatment used during pregnancy. The multivariate analysis confirmed that anti-p200 Ab are an independent risk factor for CHB. Multivariate analysis showed also a significantly increased risk of CHB in children born to mothers carrying anti-Ro Ab, without any clinical symptom. This is an obvious result due to a clear selection bias, since asymptomatic mothers were recruited when the CHB was detected in foetuses.

As reported in the literature, CHB occurs in about 2% of children born to anti-Ro Ab positive women (13). Even if the real occurrence of this event can be verified only with longitudinal observations, the NPV and PPV calculated on Bayes'theorem can be helpful in estimation of CHB risk. For example, if a mother carries anti-p200 Ab, the probability for CHB raises to 2.7%, while in their absence the probability decreases to 0.9%. Our findings are in agreement with the study by Reed (17), in which maternal reactivity against the p200 epitope was significantly more frequent in CHB mothers (44/50 mothers: 88%) compared to anti Ro positive non-CHB mothers (19/33: 66%) , with a 88% sensitivity and 34% specificity for CHB, very similar to the sensitivity and specificity we calculated (81% and 41.2%). Accordingly, they concluded that "anti-p200 was the least likely of the Ro autoantibodies to be false-positive in mothers who have never had an affected child" (17).

Despite the limitations of our retrospective study, our evaluation underlines the role of anti-p200 assay in the counselling of patients with anti-Ro Ab that desire a pregnancy. In fact, the absence of this fine specificity is apparently related to a significant decrease in the risk of delivering an affected child. Given the wide prevalence of anti-Ro Ab in patients with rheumatic diseases, this could be a valuable and additive information for family planning.

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References

1. BRUCATO A, CIMAZ R, CAPORALI R, RAMONI V, BUYON J: Pregnancy outcomes in patients with autoimmune diseases and anti-Ro/SSA antibodies. *Clin Rev Allergy Immunol* 2011; 40: 27-41.
2. QU Y, XIAO GQ, CHEN L, BOUTJDIR M: Autoantibodies from mothers of children with congenital heart block downregulate cardiac L-type Ca channels. *J Mol Cell Cardiol* 2001; 33: 1153-63.
3. CLANCY RM, BACKER CB, YIN X, KAPUR RP, MOLAD Y, BUYON JP: Cytokine polymorphisms and histologic expression in autopsy studies: contribution of TNF-alpha and TGF-beta 1 to the pathogenesis of autoimmune-associated congenital heart block. *J Immunol* 2003; 171: 3253-61.
4. BUYON JP, CLANCY RM: Autoantibody-associated congenital heart block: TGFbeta and the road to scar. *Autoimmun Rev* 2005; 4: 1-7.
5. BUYON JP, CLANCY RM: Dying right to live longer: positing apoptosis as a link between maternal autoantibodies and congenital heart block. *Lupus* 2008; 17: 86-90.
6. CLANCY RM, NEUFING PJ, ZHENG P *et al*.: Impaired clearance of apoptotic cardiocytes is linked to anti-SSA/Ro and -SSB/La antibodies in the pathogenesis of congenital heart block. *J Clin Invest* 2006; 116: 2413-22.
7. SALOMONSSON S, DÖRNER T, THEANDER E, BREMME K, LARSSON P, WAHREN-HERLENIUS M: A serologic marker for fetal risk of congenital heart block. *Arthritis Rheum* 2002; 46: 1233-41.
8. SALOMONSSON S, SONESSON SE, OTTOSSON L *et al*.: Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block. *J Exp Med* 2005; 201: 11-7.
9. CLANCY RM, BUYON JP, IKEDA K *et al*.: Maternal antibody responses to the 52-kd SSA/RO p200 peptide and the development of fetal conduction defects. *Arthritis Rheum* 2005; 52: 3079-86.
10. STRANDBERG L, WINQVIST O, SONESSON SE *et al*.: Antibodies to amino acid 200-239 (p200) of Ro52 as serological markers for the risk of developing congenital heart block. *Clin Exp Immunol* 2008; 154: 30-7.
11. AMBROSI A, DZIKAITE V, PARK J *et al*.: Anti-Ro52 monoclonal antibodies specific for amino acid 200-239, but not other Ro52 epitopes, induce congenital heart block in a rat model. *Ann Rheum Dis* 2012; 71: 448-54.
12. BERNSTEIN RM, BUNN CC, HUGHES GR: Research Article: Identification of antibodies to acidic antigens by counterimmunoelectrophoresis. *Ann Rheum Dis* 1982; 41: 554-5.
13. BRUCATO A, FRASSI M, FRANCESCHINI F *et al*.: Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. *Arthritis Rheum* 2001; 44: 1832-5.
14. GORDON P, KHAMASHTA MA, ROSENTHAL E *et al*.: Anti-52 kDa Ro, anti-60 kDa Ro, and anti-La antibody profiles in neonatal lupus. *J Rheumatol* 2004; 31: 2480-7.
15. JULKUNEN H, KURKI P, KAAJA R *et al*.: Isolated congenital heart block. Long-term outcome of mothers and characterization of the immune response to SS-A/Ro and to SS-B/La. *Arthritis Rheum* 1993; 36: 1588-98.
16. MOTTA M, RODRIGUEZ-PEREZ C, TINCANI A, LOJACONO A, CHIRICO G: Outcome of infants from mothers with anti-SSA/Ro antibodies. *J Perinatol* 2007; 27: 278-83.
17. REED JH, CLANCY RM, LEE KH, SAXENA A, IZMIRLY PM, BUYON JP: Umbilical cord blood levels of maternal antibodies reactive with p200 and full-length Ro 52 in the assessment of risk for cardiac manifestations of neonatal lupus. *Arthritis Care Res* (Hoboken) 2012; 64: 1373-81.
18. JULKUNEN H, ERONEN M: Long-term outcome of mothers of children with isolated heart block in Finland. *Arthritis Rheum* 2001; 44: 647-52.