Arrhythmogenicity of Anti-Ro/SSA Antibodies in Patients With Torsades de Pointes

Pietro Enea Lazzerini, MD; Yuankun Yue, MD; Ujala Srivastava, PhD; Frank Fabris, BS; Pier Leopoldo Capecchi, MD; Iacopo Bertolozzi, MD; Maria Romana Bacarelli, Tech; Gabriella Morozzi, BS; Maurizio Acampa, MD; Mariarita Natale, PhD; Nabil El-Sherif, MD; Mauro Galeazzi, MD; Franco Laghi-Pasini, MD*; Mohamed Boutjdir, PhD*

Background—In patients with autoimmune disease, anti-Ro/SSA antibodies (anti-Ro/SSA) are responsible for a novel autoimmune-associated long-QT syndrome by targeting the hERG potassium channel and inhibiting the related current (I_{Kr}). Because anti-Ro/SSA are also present in a significant proportion of healthy subjects and may be associated with torsades de pointes (TdP) arrhythmia, we tested the hypothesis that anti-Ro/SSA may represent a silent risk factor in patients developing TdP.

Methods and Results—Twenty-five consecutive patients who experienced TdP were prospectively collected independent of ongoing therapies and concomitant diseases. Anti-Ro/SSA were detected by fluoroenzyme immunoassay, immuno–Western blotting, and line-blot immunoassay. Purified IgGs from anti-Ro/SSA-positive and anti-Ro/SSA-negative patients were tested on I_{Kr} using HEK293 cells stably expressing the hERG channel. As expected, in TdP patients, many known corrected QT interval–prolonging risk factors were simultaneously present, including hypokalemia that was the most common (52%). Anti-Ro/SSA were present in 60% of the subjects, mostly the anti-Ro/SSA-52-kD subtype detected by immuno–Western blotting only. A history of autoimmune disease was found in only 2 of anti-Ro/SSA-positive patients. Experimental data demonstrated that purified anti-Ro/SSA-positive IgGs significantly inhibited I_{Kr} and cross reacted with hERG-channel proteins. Moreover, anti-Ro/SSA-positive sera exhibited high reactivity with a peptide corresponding to the hERG-channel pore-forming region.

Conclusions—Anti-Ro/SSA may represent a clinically silent novel risk factor for TdP development via an autoimmune-mediated electrophysiological interference with the hERG channel. We propose that TdP patients may benefit from specific anti-Ro/SSA testing even in the absence of autoimmune diseases as immunomodulating therapies may be effective in shortening corrected QT interval and reducing TdP recurrence risk. (Circ Arrhythm Electrophysiol. 2016;9:e003419. DOI: 10.1161/CIRCEP.115.003419.)

Key Words: arrhythmias, cardiac ▶ autoimmunity ▶ hERG1 potassium channel ▶ immune system ▶ torsades de pointes

Anti-Ro/SSA antibodies (anti-Ro/SSA), almost exclusively belonging to the IgG class, consist of 2 subtypes (ie, anti-Ro/SSA-52 kD and anti-Ro/SSA-60 kD) and are frequently detected not only in patients with connective tissue disease (CTD) but also in a significant proportion of apparently healthy subjects.1 The pathogenic role of these antibodies on the conduction system of the immature heart is well recognized.2 Indeed, considerable evidence links the transplacental passage of anti-Ro/SSA from the mother to the fetus with the risk of developing congenital atrioventricular block.1 Conversely, the adult heart is traditionally considered invulnerable to these antibodies. However, increasing evidence suggests that anti-Ro/SSA, particularly anti-Ro/SSA-52 kD, may also be arrhythmogenic for the adults,1,4 and the prolongation of the corrected QT interval (QTC) seems to be the most frequent abnormality observed.5,6 A molecular mimicry phenomenon leading to an inhibitory cross-reaction with the cardiac potassium (K)-channel hERG, conducting the rapid component of the delayed rectifier K current, I_{Kr}, seems to be the mechanism involved. In fact, recently, our group demonstrated that anti-Ro/SSA-positive sera, purified IgGs, and affinity-purified...
WHAT IS KNOWN

- In patients with autoimmune disease, anti-Ro/SSA antibodies are responsible for a novel autoimmune-associated long-QT syndrome by targeting the hERG potassium channel and inhibiting the related current I_{Kr}.
- Anti-Ro/SSA are the most frequent autoantibodies found in the general population, and positive subjects are, in most cases, completely asymptomatic for autoimmune diseases, particularly when anti-Ro/SSA-52-kD positivity occurs alone.

WHAT THE STUDY ADDS

- Circulating anti-Ro/SSA antibodies are highly prevalent in patients with torsades de pointes arrhythmia, in most cases in the absence of any history of autoimmune disease.
- Anti-Ro/SSA antibodies from torsades de pointes patients are arrhythmogenic as a result of a direct cross-reaction with the hERG potassium channel at the pore region leading to I_{Kr} inhibition.
- Anti-Ro/SSA may represent a clinically silent novel risk factor in torsades de pointes patients: these subjects may benefit from specific anti-Ro/SSA testing even in the absence of autoimmune disease as immunomodulating therapies may be effective in shortening QTc and reducing torsades de pointes recurrence risk.

Several studies have demonstrated that anti-Ro/SSA are the most frequent autoantibodies found in the general population with a frequency ranging from 0.5% to 2.7%.

Notably, this rate increases to 15% when the laboratory method used is immuno–Western blotting. Anti-Ro/SSA-positive subjects, in most cases (2/3), completely asymptomatic for autoimmune diseases (ADs), particularly when anti-Ro/SSA positivity occurs alone.

In addition, 52-kD Ro/SSA antigen–immunized guinea pigs showed QTc prolongation on ECG after developing high titers of anti-Ro/SSA.

Anti-Ro/SSA antibodies are responsible for a novel autoimmune-associated long-QT syndrome by targeting the hERG potassium channel and inhibiting the related current I_{Kr} and prolong action potential duration by directly binding the hERG-channel protein, likely at the pore region where homology between 52-kD Ro antigen and hERG is present. Anti-Ro/SSA antibodies are responsible for novel autoimmune-associated long-QT syndrome by targeting the hERG potassium channel and inhibiting the related current I_{Kr}. In addition, 52-kD Ro/SSA antigen–immunized guinea pigs showed QTc prolongation on ECG after developing high titers of anti-Ro/SSA.

Several studies have demonstrated that anti-Ro/SSA are the most frequent autoantibodies found in the general population with a frequency ranging from 0.5% to 2.7%. Notably, this rate increases to 15% when the laboratory method used is immuno–Western blotting.

Anti-Ro/SSA-positive subjects, in most cases (2/3), completely asymptomatic for autoimmune diseases (ADs), particularly when anti-Ro/SSA-52-kD positivity occurs alone. Nakamura et al reported the case of a 42-year-old woman with a marked QTc prolongation and torsades de pointes (TdP) episodes with no known predisposing factors for QTc prolongation except for high levels of anti-Ro/SSA. Interestingly, serum and purified IgG from this patient also significantly reduced I_{Kr}. It is noteworthy that this patient was asymptomatic for AD despite the presence of anti-Ro/SSA (which were detected only because the patient was tested for these autoantibodies given the absence of other QTc prolonging conditions).

Accordingly, it seems conceivable that anti-Ro/SSA may be silently involved in a number of patients who developed “idiopathic” life-threatening QT-related arrhythmias (including drug-induced TdP) and sudden unexplained deaths. To test this hypothesis, we prospectively studied patients who experienced TdP associated with QTc prolongation with the aim of evaluating (1) the prevalence of anti-Ro/SSA positivity and (ii) the electrophysiological effects of purified IgGs from anti-Ro/SSA-positive patients, as assessed by I_{Kr} inhibition.

Patients and Methods

Study Population

We prospectively enrolled (from 2008 to 2015) 25 consecutive patients who presented with TdP-associated QTc prolongation, independent of ongoing therapies and concomitant diseases, including ADs. Demographic, clinical, and laboratory characteristics of study patients, as well as ongoing treatment with QTc-prolonging medications, are detailed in Table 1 and Table I in the Data Supplement. Local ethical committee approved the study, and patients gave their informed consent in accordance with the principles of the Declaration of Helsinki.

ECG Recordings

Diagnosis of TdP was based on the presence of at least 1 episode of polymorphic ventricular arrhythmia and a rate ranging from 160 to 240 beats per minutes, associated with QTc prolongation. The QTc measurement is detailed in Methods in the Data Supplement.

Anti-Ro/SSA Testing

All patients underwent a venous withdrawal to determine serum anti-Ro/SSA by using different methods including (1) fluoroenzyme immunoassay (FEIA) by an EliA system (Phadia, Freiburg, Germany) using recombinant 60-kD and 52-kD Ro proteins, (2) iWB analysis using a kit with human Hep-2 cells as the source of extractive 60-kD and 52-kD Ro antigens, denatured by sodium dodecyl sulfate (Marblot Hep-2; MarDx, Carlsbad, CA), and (3) line-blot immunoassay (LIA) by a kit using extractive 60-kD Ro protein purified (not denaturated) from the bovine thymus and spleen by affinity chromatography and recombinant 52-kD Ro proteins (Anti-ENA Profile Plus 1 Europline, Euroimmun, Lübeck, Germany). Moreover, all sera were also tested for antinuclear antibodies by indirect immunofluorescence on human Hep-2000 cells (Fluorescent IgG antinuclear antibody-Ro test, Immunoconcepts, Sacramento, CA).

Purification of IgG From Patients’ Sera

IgG purification is detailed in Methods in the Data Supplement.

Electrophysiological Study

Experiments were performed on HEK293 cells stably expressing the hERG channel as previously described and detailed in Methods in the Data Supplement.

Western Blot Analysis

Western blot experiments were performed as described in Methods in the Data Supplement.

Enzyme-Linked Immunosorbent Assay

ELISA was used for the detection of reactivity of patients’ sera with a 31-amino acid sequence peptide corresponding to the extracellular loop between S5 and S6 of the pore-forming region of the α1-subunit of the hERG channel (E-pore peptide). Similarly, a scrambled form of the E-pore peptide was used as an internal control. The sequences of the peptides and ELISA details are described in Methods in the Data Supplement.

Statistical Analysis

Statistical evaluation of the differences between 2 groups was performed by the 2-tail Student t test for unpaired data for continuous
variables normally distributed and the 2-tail Mann–Whitney U test for continuous variables not normally distributed. The 2-sided Fisher exact test was performed to evaluate categorical variables. For electrophysiology data, paired 2-tail Student t test was used before and after IgGs. \( P < 0.05 \) was considered significant (GraphPad-InStat, version 3.06; GraphPad, San Diego, CA; Microsoft Corp, Redmond, WA).

Results

Patient Characteristics

As expected,\(^1\) most patients in our TdP population were women (\( \approx 75\% \)) and older than 65 years (mean age, 75.2±9.6 years; Table 1). Moreover, a high prevalence of recognized QTc-prolonging risk factors of acquired origin were present. The most recurrent conditions are the presence of an underlying cardiac disease (76% of the cases), QTc-prolonging medications (68%), and electrolyte imbalances (64%; hypokalemia 52%). Among drugs, amiodarone was the most frequently used (32%). In all patients in this study, only 3 patients (12%) had a history of AD, rheumatoid arthritis in 2 cases, and celiac disease in the other. No patients were affected with CTD. Notably, in all cases, \( >1 \) known QTc-prolonging factor, including electrolyte imbalances, diseases, or drugs, was simultaneously identifiable, on average, \( >3 \) (Table 1; Table I in the Data Supplement).

Autoantibody Status

Anti-Ro/SSA were present in 15 (6 men) of 25 subjects (60% of the study population). In the large majority of cases (13/15), anti-Ro/SSA reactivity in serum was selectively detected by iWB and in all cases but one, the anti-Ro/SSA-52-kD subtype reactivity was detected. In 2 patients, anti-Ro/SSA-52-kD positivity was revealed by FEIA and/or LIA. Moreover, 4 patients were positive to antinuclear antibody testing, with a titer ranging from 1:80 to 1:320 (Table 1 in the Data Supplement). Among the patients with a history of AD, 2 of 3 were anti-Ro/SSA-52-kD positive (Table 2; Table 1 in the Data Supplement).

By comparing patients’ characteristics on the basis of autoantibody status, we did not find any significant difference between the 2 groups for mean QTc duration or prevalence of QTc-prolonging risk factors, including electrolyte imbalances, diseases, and medications. However, in anti-Ro/SSA-positive subjects, the mean age and incidence of female sex were both significantly lower when compared with anti-Ro/SSA-negative patients (Table 2).

Because the rate of anti-Ro/SSA-52-kD–positive subjects in our cohort was particularly high and detected in most cases with iWB only, the results of all the iWB assays performed in the previous year in our laboratory were retrospectively evaluated as an internal control. Among a total of 411 subjects who underwent iWB testing for the clinical suspicion of AD, only 14.1% were anti-Ro/SSA-52-kD positive (13.9% [52/374] women and 16.2% [6/37] men). By comparing the 2 cohorts, we obtained an odds ratio of 9.1 (95% confidence interval, 3.9–21.3; 2-sided Fisher exact test, \( P < 0.0001 \)), indicating an \( >9 \)-fold higher probability of having anti-Ro/SSA-52-kD positivity in the presence rather than in the absence of TdP.
To further confirm these findings, 25 patients matched for age, sex, and concomitant diseases but without QTc prolongation or history of TdP (Table II in the Data Supplement) were prospectively enrolled as an additional control group in which patients numbers 16 and 18 was used in both untransfected HEK293 cells (lanes 1 and 3) but recognized 2 bands at 155 kDa in transfected HEK293 cells (lanes 2 and 4) cor-
responding to the glycosylated and endoplasmic reticulum–re-
tained hERG channel protein, Western blot experiments were performed using proteins from HEK293 cells stably expressing hERG channels (n=4) and untransfected HEK293 cells (negative control; n=4). Figure 6A shows that anti-Ro/SSA-positive IgG from patients 6 and 135 kDa in transfected HEK293 cells (lanes 2 and 4) corre-
To assess whether the autoantibodies functionally interact with the hERG channel, the effect of purified anti-Ro/SSA-positive IgGs from patient number 6 was tested on $I_{\text{Kr}}$.

\begin{table}[h]
\centering
\caption{Demographic, Clinical, and Laboratory Characteristics in Anti-Ro/SSA-Positive vs Negative Patients}
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Characteristics} & \textbf{Positive Patients} & \textbf{Negative Patients} & \textbf{P Value} \\
\hline
Patients, n & 15 & 10 & \\
\hline
Age, y & 72.4±15.4 & 79.6±12.3 & 0.04 \\
\hline
Women, n (%) & 9 (60) & 10 (100) & 0.05 \\
\hline
Mean QTc, ms & 600.7±101.0 & 630.5±69.0 & 0.41 \\
\hline
Electrolyte imbalances, n (%) & 10 (67) & 6 (60) & \\
\hline
Hypokalemia & 7 (46) & 6 (60) & 0.68 \\
\hline
Hypocalcemia & 5 (33) & 1 (10) & 0.34 \\
\hline
Concomitant diseases, n (%) & 13 (86) & 9 (90) & 1 \\
\hline
Cardiac diseases & 10 (67) & 9 (90) & 0.34 \\
\hline
Left ventricular hypertrophy & 4 (27) & 3 (30) & 1 \\
\hline
Acute coronary syndrome & 4 (27) & 2 (20) & 1 \\
\hline
I–III degree atrioventricular block & 1 (7) & 5 (50) & 0.16 \\
\hline
Dilated cardiomyopathy/heart failure & 2 (13) & 4 (40) & 0.17 \\
\hline
Chronic coronary artery disease & 2 (13) & 0 & 0.50 \\
\hline
Extracardiac diseases & 8 (53) & 3 (30) & 0.41 \\
\hline
Chronic kidney disease & 1 (7) & 3 (20) & 0.26 \\
\hline
Diabetes mellitus type II & 3 (20) & 0 & 0.25 \\
\hline
Rheumatoid arthritis & 1 (7) & 1 (10) & 1 \\
\hline
Hypothyroidism & 1 (7) & 1 (10) & 1 \\
\hline
Celiac disease & 1 (7) & 0 & 1 \\
\hline
QTc-prolonging medications, n (%) & 10 (67) & 7 (70) & 1 \\
\hline
Amiodarone & 4 (27) & 4 (40) & 0.66 \\
\hline
Mean medication number per patient & 1.1±1.1 & 1.0±0.7 & 0.80 \\
\hline
Mean QTc-prolonging risk factor number per patient*† & 3.7±1.3 & 3.7±1.2 & 1 \\
\hline
\end{tabular}
\end{table}

*Diseases recognized to be a risk factor for QTc prolongation.14–20
†Electrolyte imbalances, diseases, and QTc-prolonging medications.

This patient was not on any medication known to affect QT interval and had normal electrolytes (Table I in the Data Supplement). Figure 1A shows the ECG from patient 6 in a sinus rhythm (a) with a QTc=620 ms and during a TdP arrhythmia (b). Anti-Ro/SSA-positive IgG (75 μg/mL) inhibited both $I_{\text{Kr}}$ peak and tail currents (Figure 1B–1F). Selected current traces are shown before (Figure 1B) and after the application of anti-Ro/SSA-positive IgG (Figure 1C). The $I$–$V$ relationships for $I_{\text{Kr}}$ peak and tail densities before and after the application of anti-Ro/SSA-positive IgGs are shown in Figure 1D (n=6) and Figure 1E (n=6), respectively. $I_{\text{Kr}}$ peak density at $−10$ mV decreased by 21% from 49.8±5.54 to 39.5±5.49 pA/pF ($P<0.001$; n=6) and $I_{\text{Kr}}$ tail density decreased by 19.6% from 69.1±8.3 to 55.5±8.2 pA/pF ($P<0.001$; n=6) on anti-Ro/SSA-positive IgG application. Figure 1G shows the voltage protocol used to record $I_{\text{Kr}}$. The inhibition of $I_{\text{Kr}}$ was time dependent and partially reversible (Figure 2). Additional anti-Ro/SSA-positive IgGs from another 7 patients (numbers 1, 2, 3, 5, 9, 11, and 15) significantly ($P<0.001$; n=6 each) inhibited $I_{\text{Kr}}$ Peak (Figure 3A) and tail densities (Figure 3B).

\begin{figure}[h]
\centering
\caption{Anti-Ro/SSA-Negative IgGs Did Not Affect $I_{\text{Kr}}$}
\end{figure}

\begin{figure}[h]
\centering
\caption{Anti-Ro/SSA-Positive but Not Anti-Ro–Negative IgG Cross-React With hERG Channels}
\end{figure}

To test whether the inhibition of $I_{\text{Kr}}$ by anti-Ro/SSA-positive IgG is because of a direct interaction with the hERG-channel protein, Western blot experiments were performed using proteins from HEK293 cells stably expressing hERG channels (n=4) and untransfected HEK293 cells (negative control; n=4). Figure 6A shows that anti-Ro/SSA-positive IgG from patients 6 and 3 did not recognize any bands in untransfected HEK293 cells (lanes 1 and 3) but recognized 2 bands at $\sim155$ and $135$ kDa in transfected HEK293 cells (lanes 2 and 4) corresponding to the glycosylated and endoplasmic reticulum–retained hERG channel consistent with previous reports.7,21,22 Figure 6B shows no bands when anti-Ro/SSA-negative IgG from patients numbers 16 and 18 was used in both untransfected HEK293 cells (lanes 1 and 3) and transfected HEK293 cells expressing hERG channels (lanes 2 and 4).
Anti-Ro/SSA-Positive Sera From Patients With TdP Showed High Reactivity to the E-Pore Peptide

Because of the previously demonstrated homology between 52-kD Ro protein (aa302-aa321) and hERG α1-subunit (aa574–aa598) at the pore region, we tested the reactivity of patients’ sera with the E-pore peptide and its scrambled form. Sera of each patient were tested in triplicates. Figure 7A shows that anti-Ro/SSA-positive sera (n=15) showed significant reactivity to the E-pore peptide compared with the scrambled peptide (n=15) and with anti-Ro/SSA-negative sera (n=10). The average reactivity reading was 1.16±0.45 optical density in anti-Ro/SSA-positive sera versus 0.25±0.13 O.D (P<0.001) in the scrambled peptide and 0.21±0.13 optical density in anti-Ro/SSA-negative sera (P<0.001). Peptide competition experiments were performed using the E-pore peptide to neutralize the anti-Ro/SSA-positive IgG. Figure 7B shows that anti-Ro/SSA-positive IgG preincubated with the E-pore peptide resulted in a lighter band (lane 1) compared with the anti-Ro/SSA-positive IgG alone (lane 2) suggesting that the E-pore peptide bound to the anti-Ro/SSA-positive IgG. Figure 7C shows the relative hERG band densities with anti-SSA/Ro-positive IgG alone and with the preincubated E-pore peptide from a total of 4 experiments. In the presence of the E-pore peptide, the hERG band density was reduced by 65% (P<0.001).

Discussion

The main findings of this study are as follows: (1) in unselected patients with TdP, anti-Ro/SSA-52-kD positivity is highly prevalent (60% of cases); (2) in these patients, iWB is the most sensitive laboratory method to reveal the presence of circulating autoantibodies and in most cases in the absence of any history of AD; (3) purified IgGs from anti-Ro/SSA-positive but not anti-Ro/SSA-negative patients presenting with TdP inhibited \( I_{Kr} \) by direct binding to the hERG channel; (4) sera from patients anti-Ro/SSA-positive exhibited reactivity to the E-pore peptide corresponding to the hERG-channel pore region. Altogether, these findings suggest that anti-Ro/SSA may represent a clinically silent novel risk factor for TdP.
development as a result of an autoimmune-mediated interference with ventricular repolarization.

TdP is a peculiar polymorphic ventricular tachycardia that occurs in patients with acquired and congenital long-QT syndrome (LQTS). It is life threatening as it can degenerate into ventricular fibrillation and cause sudden cardiac death. Although there is no threshold of QTc prolongation at which TdP is certain to occur, the risk of TdP gradually increases as the QTc prolongs, with an approximately 5% to 7% exponential increase in risk for each 10-ms prolongation of QTc. Accordingly, TdP usually develops in patients with a markedly prolonged QTc, a condition in most cases requiring the simultaneous presence of multiple QTc-prolonging factors, congenital and/or acquired, that act synergistically to impair ion channels contributing to the ventricular repolarization process. Among these currents, a particularly relevant role is played by IKr, conducted by the hERG-K channel whose inhibition represents the most frequently underlying molecular mechanism for LQTS and TdP, including both acquired (drugs and hypokalemia) and congenital ion channel defect in LQTS.

Mounting evidence indicates that the hERG-channel is also a specific molecular target for anti-Ro/SSA, thereby responsible for a novel form of acquired LQTS mediated by an autoimmune origin. Although anti-Ro/SSA-positive CTD patients frequently display QTc prolongation and complex ventricular arrhythmias, anti-Ro/SSA are also silently present in a significant proportion of asymptomatic subjects of the general population. An emblematic case of an anti-Ro/SSA-associated TdP in an apparently healthy young woman has been recently reported in which clear evidence of a direct mechanistic link between circulating anti-Ro/SSA and QTc prolongation was provided. This raises the distinct possibility that anti-Ro/SSA may represent a previously unrecognized risk factor involved in patients developing TdP.
The results of this study support this viewpoint. Indeed, we demonstrated the presence of circulating anti-Ro/SSA in 60% of TdP patients. It should be noted that, although relatively small, our study group nevertheless represents the incidence of standard risk factors usually found in the general TdP population. The majority of patients usually exhibit >1 risk factor (3.5 on average in our sample). In this regard, it is striking to note that anti-Ro/SSA positivity is the most common QTc-prolonging risk factor demonstrable, even more than the most frequently occurring classic single risk factor, that is, hypokalemia. Compared with anti-Ro/SSA-negative patients, anti-Ro/SSA-positive subjects displayed similar characteristics for mean QTc prolongation and type fine characteristics required to reveal the specific subfractionation with the highest arrhythmogenic potential. In particular, because iWB uses the native 52-kD Ro protein (extracted by the Hep-2 cells) as an antigen unlike FEIA and LIA, which could make iWB more effective in revealing anti-Ro/SSA-52 kD, particularly the specific autoantibody subtype present was the anti-Ro/SSA-52 kD even in the absence of any clinical manifestation of AD, is another important result emerging from this study. This finding is in agreement with recent clinical and experimental data indicating the anti-Ro/SSA-52 kD as the specific subtype responsible for the electrophysiological effects on the ventricular repolarization. Indeed, purified anti-Ro/SSA-52 kD from CTD patients with QTc prolongation significantly inhibited \( I_{\text{Kr}} \) in HEK293 cells stably expressing the hERG channel, and 52-kD Ro/SSA antigen–immunized guinea pigs showed QTc prolongation on the surface ECG after developing high titers of anti-Ro/SSA.7 In CTD patients, a direct correlation has been demonstrated between anti-Ro/SSA levels and QTc prolongation but for the anti-Ro/SSA-52-kD subtype only.26 These findings are consistent with the recent evidence obtained from linear homology analysis demonstrating homology between 52-kD Ro protein (aa302-aa321) and hERG-channel protein at the pore region (aa574–aa598). This homology may account for anti-Ro/SSA off target binding to the hERG channel at this epitope mimic. The data from Figure 7 demonstrating high reactivity of anti-Ro/SSA-positive sera to the E-pore region of the hERG channel compared with anti-Ro/SSA-negative sera or to the scrambled E-pore peptide lands further support to the autoantibody interaction with the hERG channel at the pore region.

The fact that iWB analysis was the most sensitive method to detect the presence of anti-Ro/SSA-52-kD antibodies is in agreement with previous studies performed in different clinical settings.11,27 The reasons of these different results are not clear. The different antigen type and laboratory procedure used could make iWB more effective in revealing anti-Ro/SSA-52 kD, particularly the specific autoantibody subfraction with the highest arrhythmogenic potential. In particular, because iWB uses the native 52-kD Ro protein (extracted by the Hep-2 cells) as an antigen unlike FEIA and LIA, which use the 52-kD Ro recombinant protein, it is plausible that only the native 52-kD Ro protein has the epitopes with the fine characteristics required to reveal the specific subfraction of anti-Ro/SSA-52 kD with pathogenic potential for the heart (ie, those able to cross-react with hERG channels). This is consistent with recent data demonstrating that many anti-Ro/SSA-52-kD FEIA-negative CTD patients with QTc prolongation actually tested positive for this antibody when assessed by iWB.26 The significance of these findings is
further confirmed by the evidence provided here that in TdP patients, the prevalence of anti-Ro/SSA-52-kD positivity as assessed by iWB was ≈4-times higher when compared with an internal reference population of >400 subjects seen at our rheumatologic clinic (60% versus 14%), as well as with a control group of 25 patients matched for age, sex, and concomitant diseases but without QTc prolongation or history of TdP, prospectively enrolled in our internal medicine ward (60% versus 16%). Notably, overlapping results have been reported by Metsküla et al11 who found a 15% iWB positivity for anti-Ro/SSA by testing sera from 200 subjects, randomly selected from a general population sample. Further support for our data is from this study where LIA and ELISA were both markedly less sensitive than iWB, testing positive for anti-Ro/SSA-52-kD in only 3.5% and 0.5% of cases, respectively. The findings from this study per se represent a validation of the iWB results as in the sera of these patients, circulating anti-Ro/SSA-52-kD antibodies are actually present (specifically the subfraction with QTc-prolonging potential) despite FEIA and LIA were negative. These findings, together with the evidence that the majority of our anti-Ro/SSA-52-kD-positive patients were clinically silent for AD, emphasize the concept that in most cases, only a specific anti-Ro/SSA testing (particularly iWB) may reveal the presence of these autoantibodies as a concurring risk factor accounting for QTc prolongation in TdP patients.

In our recent study mentioned above,7 unequivocal experimental evidence established that anti-Ro/SSA-52 kD are able to prolong cardiomyocyte action potential and QTc through a direct cross-reaction with the hERG-channel protein. In particular, purified IgGs from sera of anti-Ro/SSA-positive CTD patients with QTc prolongation directly bind the hERG channel and significantly inhibit $I_{Kr}$. This inhibitory effect was reproduced using affinity-purified anti-Ro/SSA-52 kD.7 It is important to note that the use of purified IgGs to perform the electrophysiology investigations ruled out the possible influence of other concomitant QTc-prolonging factors, such as drugs or electrolyte imbalances on $I_{Kr}$, and pinpoint to an antibody-mediated mechanism (more particularly anti-Ro/SSA-52 kD mediated, as also indicated by our previous study7). Altogether, these findings strongly support the observation that in positive TdP patients, anti-Ro/SSA-52 kD contributes synergistically with other classical risk factors concomitantly present to induce further QTc prolongation as a result of a specific electrophysiological interference with ventricular repolarization.

The potential limitations of this study include the relative small sample size and the lack of a genetic testing for concomitant LQTS-associated mutations. However, it should be underlined that TdP is an uncommon event, maybe also because it is not easily documented for the high propensity of this arrhythmia to rapidly degenerate to ventricular fibrillation and cardiac arrest. Moreover, although genetic characterization would have been useful for a more accurate definition of the total load of risk factors in the single patient (case series
identified subclinical congenital LQTS in 5%–20% of cases of drug-induced TdP14, we anticipate that the presence of anti-Ro/SSA and genetic mutations along with other classical acquired risk factors will together predispose to TdP. In particular, because anti-Ro/SSA-associated QTc prolongation is due to a specific interference with hERG, it is conceivable that subjects carrying loss-of-function mutations/polymerisms in genes encoding for hERG or other proteins regulating hERG channel function could be particularly susceptible to develop TdP in the presence of these autoantibodies. Intriguingly, in the above cited case by Nakamura et al.,13 the anti-Ro/SSA-positive patient who developed TdP also tested positive for the D85N polymorphism in the KCNE1 gene. This polymorphism results in a defective expression of the MinK protein, which represents the β-subunit of voltage-gated channel conducting the slowly activating component of the delayed rectifier K current, IKs, but it is also a key regulator of IKr by forming a stable complex with hERG.28 Accordingly, electrophysiological studies demonstrated that the D85N variant of KCNE1 either homozygously or heterozygously expressed in experimental cell systems significantly reduces IKr from ~30 to >80%, 29,30

In conclusion, our data, for the first time, provide evidence that anti-Ro/SSA-52 kD may represent a novel, silent risk factor in patients developing TdP. These findings support the recommendation to translate into the clinical practice that patients with QTc prolongation and TdP may benefit from specific anti-Ro/SSA testing even in the absence of clinical signs of AD. The anti-Ro/SSA-mediated pathogenic activity on the ventricular repolarization seems to be related to a functional and potentially reversible electrophysiological interference with hERG-channel function. As such, immunomodulating therapies, including plasmapheresis and immunoadsorption, may be effective in shortening QTc and reducing the risk of TdP recurrence in these patients by decreasing autoantibody synthesis and/or preventing epitope binding. This view is supported by recent data from single-case reports demonstrating the effectiveness of immunosuppressive therapy in reversing QTc prolongation and other anti-Ro/SSA-associated electrocardiographic abnormalities in adults31–33 and may open novel avenues in antiarrhythmic therapy.

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Disclosures
None.

References


Arrhythmogenicity of Anti-Ro/SSA Antibodies in Patients With Torsades de Pointes
Pietro Enea Lazzerini, Yuankun Yue, Ujala Srivastava, Frank Fabris, Pier Leopoldo Capecchi, Iacopo Bertolozzi, Maria Romana Bacarelli, Gabriella Morozzi, Maurizio Acampa, Mariarita Natale, Nabil El-Sherif, Mauro Galeazzi, Franco Laghi-Pasini and Mohamed Boutjdir

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SUPPLEMENTAL MATERIAL

Supplemental Methods

ECG recordings. The QTc interval was manually measured on a standard 12-lead ECG, from the
onset of the Q wave or the onset of the QRS complex to the end of the T wave, defined as the return
to the T-P baseline. When prominent U waves (>1 mm) merging into T waves were present, they
were included in QT measurement [1]. QT interval, determined as the longest hand-measured QT
interval in any lead [2], was corrected for heart rate by the Bazett’s formula to yield the QTc value.
The QTc value was calculated by dividing the QT interval by the square root of the R-R interval.
QTc was measured from 3 non-consecutive beats (mean value) by a single investigator (M.A.), who
was blinded to the patient’s antibody status. According to the American Heart
Association/American College of Cardiology (AHA/ACC) guidelines, QTc was considered
prolonged if ≥450 ms in males, or ≥460 ms in females [2].

Purification of IgG from patients’ sera. IgG purification was performed using Melon Gel IgG
spin purification Kit (Thermo scientific). Briefly, 10-100 µL of serum per 100 µL of settled gel was
used and the Melon Gel IgG purification support and purification buffer was equilibrated to room
temperature. To obtain an even suspension, bottle containing the purification support was swirled
and a 500 µL of slurry and dispensed into a spin column placed in a micro centrifuge tube. The
uncapped column/tube assembly was centrifuged at 2,000-6,000 × g for 1 minute. Purification
buffer (300 µL) was added to the column, pulse centrifuged for 10 seconds and flow through
discarded. 100-500 µL of diluted or 10-100 µL of buffer-exchanged serum was added to the
column and incubated for 5 minutes at room temperature with end-over-end mixing. Centrifugation
for 1 minute was performed to collect the purified IgGs.
Electrophysiological study. HEK293 cells stably expressing the hERG-channel were a generous gift from Dr. Craig January from the University of Wisconsin, Madison and Dr. Zhengfeng Zhou from Oregon Health & Science University, Portland, Oregon. Details of DNA constructs and the methods of stable transfection of HEK293 cells with hERG-channel were reported elsewhere [3]. These HEK293 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 400 µg/ml geneticin (G418). Cells were washed twice with standard MEM medium, and stored in this medium at room temperature for later use. Cells were superfused with HEPES-buffered Tyrode's solution containing (in mM): 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The internal pipette solution contained (in mM): 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES (pH 7.2 with KOH). I₉ was recorded using a standard protocol shown in Figure 1G and as previously reported [4]. Based on previous work by our group [4] and others [5], we carried out the study using IgGs at the concentration of 75 µg/ml. Experiments were performed at room temperature.

Western blot analysis. Transfected HEK293 cells stably expressing HERG/Kv11.1 channels were harvested and lysed in RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 minutes at 4°C and centrifuged at 14,000 rpm for 15 minutes. After a 30 minutes incubation at 4°C, the homogenate was centrifuged at 14,000 rpm for 15 minutes. The supernatant after centrifugation was resolved by SDS-PAGE on a 4-15% Tris-HCl gel (Bio-Rad) and transferred on PVDF membrane (Bio-Rad). Blots were blocked with 5% milk for an hour and probed with purified anti-SSA/Ro IgGs and GAPDH antibodies (Sigma, Inc) overnight at 4°C. It was further probed with anti-rabbit IgG HRP (Santa Cruz) and anti-Human IgG antibody (Jackson Immunolab). The signal was detected with Clarity ECL substrate (Bio-Rad) and blots were scanned in a C-Digit blot scanner (LI-COR) at high sensitivity to obtain the image. Blocking experiments were performed by preincubation of anti-Ro/SSA-positive IgG with the E-pore peptide for 1 hour and then used side by side with the anti-Ro/SSA-positive IgG alone.
ELISA. The E-pore peptide (GNMEQPHMDSRIGWLHNLGDQIGKPYNSSGL) and the scrambled peptide (NEQDRSGYHPMKWMSIILGGSGLNGPQNDLH) were synthesized at >90% purity (Genscript USA, Inc., Piscataway, NJ), coated overnight, washed in Phosphate or Buffer Saline-Tween 20 and probed in diluted serum followed by anti-human IgG alkaline phosphatase. The plates were developed with disodium p-nitrophenyl phosphate substrate. All samples were run in triplicates. Results were expressed as the optical density (O.D.) at 405 nm minus that of the reagent blank using Molecular Devices Filertmax F3 apparatus.

References:


## Supplemental Table 1. Patients’ characteristics by case

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>ANA</th>
<th>FEIA</th>
<th>iWB</th>
<th>LIA</th>
<th>Anti-RoSSA</th>
<th>QTc (msec)</th>
<th>Concomitant QTc-prolonging factors</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
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<td>♂</td>
<td>+(1:80)</td>
<td>-</td>
<td>52kD</td>
<td>-</td>
<td>660</td>
<td></td>
<td>Hypocalcemia, hypokaliemia, acute pancreatitis</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
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<td>-</td>
<td>-</td>
<td>52kD</td>
<td>60kD</td>
<td>600</td>
<td></td>
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</tr>
<tr>
<td>3</td>
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<td>♂</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>4</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
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<tr>
<td>6</td>
<td>74</td>
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<td>+(1:320)</td>
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<td>-</td>
<td>620</td>
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<tr>
<td>7</td>
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<td>-</td>
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<td>-</td>
<td>630</td>
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</tr>
<tr>
<td>8</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>Chronic coronary artery disease, hypokaliemia, hypocalcemia</td>
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<tr>
<td>9</td>
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<td>+(1:80)</td>
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</tr>
<tr>
<td>14</td>
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<td>52kD</td>
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<tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>Hypocalcemia, hypokaliemia, sinus bradycardia</td>
</tr>
<tr>
<td>17</td>
<td>84</td>
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<td>-</td>
<td>-</td>
<td>52kD</td>
<td>-</td>
<td>680</td>
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<td>Acute complete AVB, left ventricular hypertrophy</td>
</tr>
<tr>
<td>18</td>
<td>78</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>52kD</td>
<td>-</td>
<td>690</td>
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<td>Left ventricular hypertrophy, chronic kidney disease, hypokaliemia</td>
</tr>
<tr>
<td>19</td>
<td>81</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>52kD</td>
<td>-</td>
<td>660</td>
<td></td>
<td>Dilated cardiomyopathy</td>
</tr>
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**Drugs**
- Amiodarone
- Citalopram
- Promazine
- Clarithromicine
- Levofoxacin
- Fluconazole
- Sertraline
<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Gender</th>
<th>ANA</th>
<th>FEIA</th>
<th>iWB</th>
<th>LIA</th>
<th>AVB</th>
<th>HIV</th>
<th>Diagnosis</th>
<th>Treatment</th>
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<tr>
<td>20</td>
<td>85</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Acute II°degree 2:1 AVB, dilated cardiomyopathy, hypokalemia</td>
<td>Citalopram</td>
</tr>
<tr>
<td>21</td>
<td>77</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Acute complete AVB</td>
<td>Donezepil</td>
</tr>
<tr>
<td>22</td>
<td>46</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>Acute coronary syndrome, hypokalemia, HIV infection, chronic kidney disease</td>
<td>Protease inhibitors</td>
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<tr>
<td>23</td>
<td>85</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sinus bradycardia and AVBs (II-III° degree), hypokalemia</td>
<td>Amiodarone</td>
</tr>
<tr>
<td>24</td>
<td>87</td>
<td>♂</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>Heart failure, complete AVB, rheumatoid arthritis, hypothyroidism, chronic kidney disease</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>88</td>
<td>♂</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Acute coronary syndrome, left ventricular hypertrophy, hypokalemia</td>
<td>Amiodarone, Chlarythromicine</td>
</tr>
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### Supplemental table 2. Demographic, clinical and laboratory characteristics of the control patients.

<table>
<thead>
<tr>
<th>Patients,n</th>
<th>25</th>
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</thead>
<tbody>
<tr>
<td>Age,years(range)</td>
<td>76.7±15.9(22-94)</td>
</tr>
<tr>
<td>Females,n</td>
<td>18(72%)</td>
</tr>
<tr>
<td>Mean QTc,ms(range)</td>
<td>428.5±21.7(373-460)</td>
</tr>
<tr>
<td>Electrolyte imbalances,n</td>
<td>3(12%)</td>
</tr>
<tr>
<td>Hypokaliemia</td>
<td>2(8%)</td>
</tr>
<tr>
<td>Hypocalcemia</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Hypomagnesemia</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Concomitant diseases*,n</td>
<td>24(96%)</td>
</tr>
<tr>
<td><strong>Cardiac diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Left ventricular hypertrophy</td>
<td>10(40%)</td>
</tr>
<tr>
<td>Chronic coronary artery disease</td>
<td>8(32%)</td>
</tr>
<tr>
<td>Dilated cardiomyopathy/heart failure</td>
<td>7(28%)</td>
</tr>
<tr>
<td>I-III degree atrioventricular block</td>
<td>4(16%)</td>
</tr>
<tr>
<td>Acute coronary syndrome</td>
<td>4(16%)</td>
</tr>
<tr>
<td>Sinus bradycardia</td>
<td>1(4%)</td>
</tr>
<tr>
<td><strong>Extra-cardiac diseases</strong></td>
<td>10(40%)</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>7(28%)</td>
</tr>
<tr>
<td>Diabetes mellitus type II</td>
<td>2(8%)</td>
</tr>
<tr>
<td>Subarachnoid haemorrhage</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Anorexia nervosa</td>
<td>1(4%)</td>
</tr>
<tr>
<td>QTc prolonging-medications,n</td>
<td>14(56%)</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>3(12%)</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>2(8%)</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>2(8%)</td>
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<tr>
<td>Paroxetine</td>
<td>2(8%)</td>
</tr>
<tr>
<td>Sertraline</td>
<td>2(8%)</td>
</tr>
<tr>
<td>Citalopram</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Ciproflocacin</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Promazine</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Promazine</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Hydroquinidine</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Mean medication number per patient</td>
<td>0.7±0.7</td>
</tr>
<tr>
<td>Mean QTc-prolonging risk factor number per patient†</td>
<td>2.6±1.2</td>
</tr>
</tbody>
</table>

*Diseases recognized to be a risk factor for QTc prolongation14-20;†including electrolyte imbalances, diseases and QTc-prolonging medications.