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ORIGINAL RESEARCH

Advanced Atrioventricular Block in Athletes: Prevalence and Role of Anti-Ro/Sjögren Syndrome–Related Antigen A Antibodies

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BACKGROUND: Advanced atrioventricular block (AVB), that is, higher than second-degree Mobitz-1, is an abnormal finding in athletes. Despite intensive investigation, in several cases the pathogenesis remains unknown, but frequently pacemaker implantation is still indicated. Increasing evidence points to circulating anti-Ro/Sjögren syndrome–related antigen A (SSA) antibodies cross-reacting with L-type calcium channel and inhibiting the related current as an epidemiologically relevant and potentially reversible cause of isolated AVB in adults.

The aim of the study was to determine the prevalence of anti-Ro/SSA-associated advanced AVBs in a large sample of young athletes.

METHODS AND RESULTS: A total of 2536 consecutive athletes aged <40 years without a history of cardiac diseases/interventions were enrolled in a cross-sectional study. Resting and exercise electrocardiography was performed, and those presenting any AVB were further evaluated by 24-hour Holter ECG. Athletes with second-degree AVBs and their mothers underwent anti-Ro/SSA testing. Moreover, purified immunoglobulin G from subjects with anti-Ro/SSA-positive and anti-Ro/SSA-negative advanced AVB were tested on L-type calcium current and L-type-calcium channel expression using tSA201 cells. The global prevalence of advanced AVB in the overall sample was ≈0.1%, but the risk considerably increased (2%) when intensely trained postpubertal male subjects were selectively considered. While none of the athletes with advanced AVB showed heart abnormalities, in 100% of cases anti-Ro/SSA antibodies were detected. Ex vivo experiments showed that immunoglobulin G from anti-Ro/SSA-positive but not -negative subjects with advanced AVB acutely inhibit L-type calcium current and chronically downregulate L-type-calcium channel expression.

CONCLUSIONS: Our study provides evidence that advanced AVB occurs in young athletes, in most cases associated with anti-Ro/SSA antibodies blocking L-type calcium channels. These findings may open new avenues for immunomodulating therapies to reduce the risk of life-threatening events in athletes, avoiding or delaying pacemaker implantation.

Key Words: advanced atrioventricular block in athletes ■ anti-Ro/SSA antibodies ■ autoimmunity ■ L-type calcium channels

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CLINICAL PERSPECTIVE

What Is New?

- In a large sample of consecutive athletes aged <40 years and without history of structural heart disease, the global prevalence of advanced atrioventricular block (AVB) was ≈0.1%, but the risk considerably increased (2%) when intensely trained postpubertal male subjects were selectively considered.
- While none of the subjects with advanced AVB showed structural cardiac abnormalities, circulating anti-Ro/Sjögren syndrome–related antigen A antibodies were detected in all cases, always in the absence of a manifest autoimmune disease.
- Ex vivo experiments showed that purified immunoglobulin G from anti-Ro/Sjögren syndrome–related antigen A -positive but not -negative patients with advanced AVB acutely inhibit L-type calcium current and chronically downregulate L-type calcium channel protein expression.

What Are the Clinical Implications?

- Our study provides evidence that advanced AVB occurs in young athletes, in most cases associated with anti-Ro/Sjögren syndrome–related antigen A -antibodies blocking L-type calcium channels.
- The data highlight the importance of performing complete anti-Ro/Sjögren syndrome–related antigen A -antibody testing in all athletes with advanced AVB, even in the absence of clinical signs of autoimmune disease; it might open new avenues for immunomodulating therapies to reduce the risk of life-threatening events in these subjects, avoiding or delaying pacemaker implantation.

Nonstandard Abbreviations and Acronyms

AD	autoimmune disease
AVB	atrioventricular block
$Ca_v1.2$	L-type calcium channel
I°AVB	first-degree AVB
II°AVB	second-degree AVB
III°AVB	third-degree AVB
iWB	immuno-western-blot analysis
IgG	immunoglobulin G
I_{CaL}	L-type calcium current
SSA	Sjögren syndrome–related antigen A

Regular sports training is commonly associated with evident electrocardiographic changes reflecting structural and electrical remodeling of the heart (athlete's heart).[1] Nevertheless, not all the ECG abnormalities observed in athletes are mere adaptations to physical conditioning. In a number of cases these alterations may be an expression of underlying cardiac disease, structural or electric, exposing athletes to a risk of exercise-associated sudden cardiac death. Thus, discriminating physiological from potentially malignant ECG changes represents a critical issue for cardiologists and sports medicine physicians involved in the routine evaluation of young athletes.[1,2] In this scenario, while a delay in atrioventricular conduction is a common feature of the athlete's heart, particularly first-degree atrioventricular block (I°AVB) and Mobitz type I (Wenckebach) second-degree AVB (II°AVB-Mobitz 1), advanced AVB, that is, more severe II°AVBs (Mobitz 2, 2:1, and high-grade), and third-degree AVB (III°AVB) are considered abnormal findings in athletes. In this situation, further diagnostic evaluation is currently recommended, and pacemaker implantation may be indicated to prevent life-threatening events.[1,2]

Occurrence of severe AVBs requiring pacemaker implantation in subjects aged <50 years is not rarely observed in the general population.[3] By analyzing the Danish Pacemaker and ICD Register, Rudbeck-Resdal et al[4] recently reported that in a 20-year period (1996–2015) 1027 patients with severe AVB were referred for first-time pacemaker implantation before the age of 50 years, corresponding to an incidence of 17.7 per year per 1 million inhabitants. Importantly, in half of these patients (50.3%), the cause of the AVB remained unidentified,[4] and pacemaker implantation in cases of AVB of unknown origin was associated with a worse long-term prognosis due to a heightened risk of heart failure, life-threatening ventricular arrhythmias, and death.[5] Overall, these data highlight the need for a better understanding of the underlying causes and mechanisms operating in patients with isolated AVB.

Accumulating evidence points to autoimmunity as a novel, epidemiologically relevant, and potentially reversible cause of isolated AVB in young adults.[6–11] In particular, a recent translational study performed at our institution demonstrated that >50% of consecutive patients with AVB of unknown origin or their mothers (or both) showed circulating anti-Ro/Sjögren syndrome–related antigen A (SSA) antibodies cross-reacting with the L-type-calcium channel ($Ca_v1.2$) extracellular pore region and inhibiting the related current (I_{CaL}), in most cases without a history of autoimmune diseases (ADs). Specifically, anti-Ro/SSA antibodies were more frequently found in the patient alone (acquired form) but also in the patient's mother alone (late-progressive congenital form) or in both (mixed form).[11] While in the

acquired form the autoantibody-mediated arrhythmogenic effect seems to be purely electrophysiological, it is hypothesized that a minor structural damage of the fetal atrioventricular node induced by maternal anti-Ro/SSA antibodies, subclinical at birth and then progressively worsened by age (accelerated aging), is responsible for the late progressive congenital form.[8] Accordingly, immunosuppressive therapy rapidly normalized/improved atrioventricular conduction in patients with AVB with circulating anti-Ro/SSA antibodies (acquired and mixed forms) but not in those without.[11] The relevance of these findings and their epidemiological impact in the general population[12] were strongly substantiated by a recent nationwide cross-sectional study (>100 000 Israeli subjects), which demonstrated that anti-Ro/SSA seropositivity was independently associated with a double risk of AVB when compared with seronegative subjects, independent of overt AD.[13]

Based on these data, it is likely that anti-Ro/SSA antibodies play a significant role in promoting AVBs also in athletes, a population per se already predisposed to develop conduction disturbances.[1] In support of this hypothesis, we recently described the case of a 26-year-old competitive soccer player with advanced II°AVB in whom, after excluding cardiac structural abnormalities and genetic mutations, circulating anti-Ro/SSA antibodies in the mother were demonstrated.[14]

Prompted by this body of evidence, our aim was to determine the prevalence and the pathogenesis of anti-Ro/SSA-associated advanced AVBs in athletes by performing a cross-sectional study on a large sample of 2536 prospectively enrolled subjects aged <40 years.

METHODS

The authors declare that all supporting data are available within the article and its online supplementary files.

Study Population

The study sample consisted of consecutive subjects aged <40 years who underwent annual medical screening for competitive or noncompetitive sport activity at the Centre for Sports Medicine of Massa-Carrara, Italy. The evaluation comprised medical history, physical examination, and resting and exercise 12-lead electrocardiography. Prior history of cardiac diseases or heart interventions represented exclusion criteria for enrollment.

The local ethical committee (Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, Sezione Area Vasta Sud Est) approved the study, and subjects (or a parent in the case of minors) gave their informed consent.

ECG Recordings

All subjects underwent 12-lead ECG (25 mm/s and 10 mV/cm; sampling rate 1 kHz), in the supine position during quiet respiration, and then after exercise, consisting of stepping up and down on a platform at a rate of 30 steps per minute for 3 minutes (step test). PR interval was measured from the start of the P wave to that of the QRS complex. Accumulating evidence indicates that PR interval is profoundly influenced by several physiologic factors, particularly sex (men have a longer PR interval than women),[15,16] age (PR interval gradually increases during the life span),[16] and training level.[1,17] As such, we analyzed our sample as a whole and thereafter stratified by these variables. Specifically, 3 age ranges were selected, that is, 5–11 years, 12 to 15 years and 16 to 39 years, on the basis of the evidence that (1) the widely accepted upper normal limit for PR interval is 180 ms until the age of 11 (childhood), while from that age onward it increases to 200 ms[18]; (2) after age 15 years (postpubertal age), the impact of the divergent effects of sex hormones on cardiac electrophysiology becomes substantial, as already well demonstrated and accepted for the QT interval.[19] Moreover, based on the mean number of sessions (ie, sport activity ≥90 minutes) per week reported by the athlete on enrollment, 3 levels of training were defined: low (0–2 sessions/wk), medium (3–4 sessions/wk), and high (5–7 sessions/wk). This classification, practical and easy to use during the interview, shows a good correspondence with the stratification on the basis of the volume of exercise proposed by McKinney et al[20] (exerciser: up to 4 h/wk; recreational athlete: 4–6 h/wk; competitive athlete: >6 h/wk), therefore providing a reliable estimate of the activity level.

PR interval duration >200 ms (>180 ms in children aged <12 years) was considered abnormally prolonged.[3,18] Moreover, given that PR interval is also deeply influenced by day-to-day heart rate (HR) changes,[21,22] and that adjustment for the HR results in a high intrasubject stability of this parameter over time,[21] PR interval was additionally adjusted by using the Soliman–Rautaharju formula (PR interval+0.26 (HR–70) to obtain the HR-corrected PR interval.[23]

First-degree and higher AVBs were classified according to current American College of Cardiology Foundation/American Heart Association/Heart Rhythm Society guidelines,[3,24] as detailed in Data S1. The athletes in whom resting or exercise ECG showed first-degree or higher AVB underwent additional evaluation by 24-hour ECG ambulatory monitoring. The most severe degree of AVB documented in a single subject at the end of all these investigations, was taken into account for statistical analyses.

Echocardiography

To exclude the presence of significant structural heart diseases, all subjects presenting with II°AVB were further investigated by transthoracic echocardiography.

Anti-Ro/SSA Antibody Testing

All athletes showing II°AVB, and their mothers, underwent a blood withdrawal to test the presence of circulating anti-Ro/SSA-antibodies. Three different methods were employed, that is, fluoroenzyme immunoassay, immuno-western-blot analysis (iWB), and line-blot immunoassay. Additional details are provided in Data S1.

When at least 1 of these methods tested positive for anti-Ro/SSA antibodies, the diagnosis of anti-Ro/SSA-associated AVB was made, then further subclassified as acquired, late-progressive congenital, or mixed, depending on the specific pattern observed (Data S1).[8,25]

Purification of Immunoglobulin G from Sera of Patients With AVB

A detailed description of the immunoglobulin G (IgG) purification procedure is provided in Data S1.

Electrophysiological Study

Patch-clamp experiments were performed in tSA201 cells[26] transiently expressing $Ca_v1.2$ L-type calcium channel. Methodological details are described in Data S1.

Western Blot Analysis for $Ca_v1.2$ Protein Expression Studies

As described in Data S1, western blot experiments were conducted in tSA201 cells transiently expressing $Ca_v1.2$.

Statistical Analysis

Based on the Kolmogorov–Smirnov test, parametric or nonparametric analyses were performed. Statistical evaluation of the differences between 2 unpaired groups (no AVB versus AVB; no II°AVB versus II°AVB; non anti-Ro/SSA–associated II°AVB versus anti–Ro/SSA-associated II°AVB; female versus male subjects) was carried out by the 2-tailed unpaired *t* test or the 2-tailed Mann–Whitney test. Comparisons among 3 unpaired groups (5–11 versus 12–15 versus 16–39 years; low versus medium versus high training level) were evaluated by 1-way ANOVA or the Kruskal–Wallis test. Comparisons between patch-clamp experiments at baseline and after IgG incubation were performed by the paired *t* test. The 2-sided Fisher's exact test was carried out to evaluate categorical variables.

P values ≤0.05 were considered significant (InStat, GraphPad Software, La Jolla, CA).

RESULTS

Characteristics of the Study Population

Overall, 2549 consecutive subjects aged <40 years were screened. Of these, 13 subjects were excluded (Figure 1) due to history of surgical or interventional procedures on the heart (dual atrioventricular node pathway catheter ablation, n=4; surgical closure of persistent ductus arteriosus of Botallo or patent foramen ovale, n=3), congenital structural cardiac diseases (interventricular sept defect, n=2; combined interventricular septum defect, interatrial septum defect and pulmonary stenosis, n=1), or genetic diseases potentially affecting the heart (Down syndrome, n=2; Marfan syndrome, n=1).

The resulting study population consisted of 2536 subjects, 1401 male and 1121 female subjects (undeclared sex for privacy reasons, n=14). Most subjects belonged to an age range between 5 and 15 years (mean age, 13.3 years), while 20.3% were in postpubertal age (>15 years). A variety of sports disciplines were practiced, most frequently soccer, volleyball, dance, basketball, tennis, and swimming (Table S1). Mean training level was medium-low (3.2 sessions/week), while 15.3% were highly trained athletes. A history of AD was found in 10 cases only (0.4%), the most frequent diagnosis being autoimmune thyroiditis (n=5; Table 1).

ECG Findings

Resting ECG showed a median PR interval of 130.0 ms. PR interval values were significantly longer in male subjects and progressively increased with age and training level, while opposite trends were observed for HR. Nevertheless, consistent sex-, age-, and training-associated differences also persisted when HR-corrected PR interval median values were considered (Tables S2–S4; Figure S1). By combing the data obtained by resting and exercise ECG only, AVB was found in 29 athletes (1.1%), including 23 (0.9%) I°AVB and 6 (0.2%) II°AVB-Mobitz 1 (Figure 1). These findings were refined and reclassified by the additional execution, in these subjects, of 24-hour ECG ambulatory monitoring. Overall, 19 athletes (0.7%) presented I°AVB, while 10 (0.4%) showed episodes of II°AVB, comprising 7 (0.3%) with II°AVB-Mobitz 1 and 3 (0.1%) with advanced II°AVB (ie, Mobitz 2/2:1, n=2; high-grade, n=1), while no cases of III°AVB were observed (Figure 1; Table 1; Figure 2A through 2C). Specifically, the subject with high-grade II°AVB presented during the nighttime a very long pause (7.4 seconds) due to 5 consecutive nonconducted supraventricular impulses (Figure 2C). Athletes with AVBs, particularly II°AVBs, appeared to be significantly older and showed a higher training level when compared with those without (Tables 2 and 3). Moreover, focusing on the 3 subjects with advanced

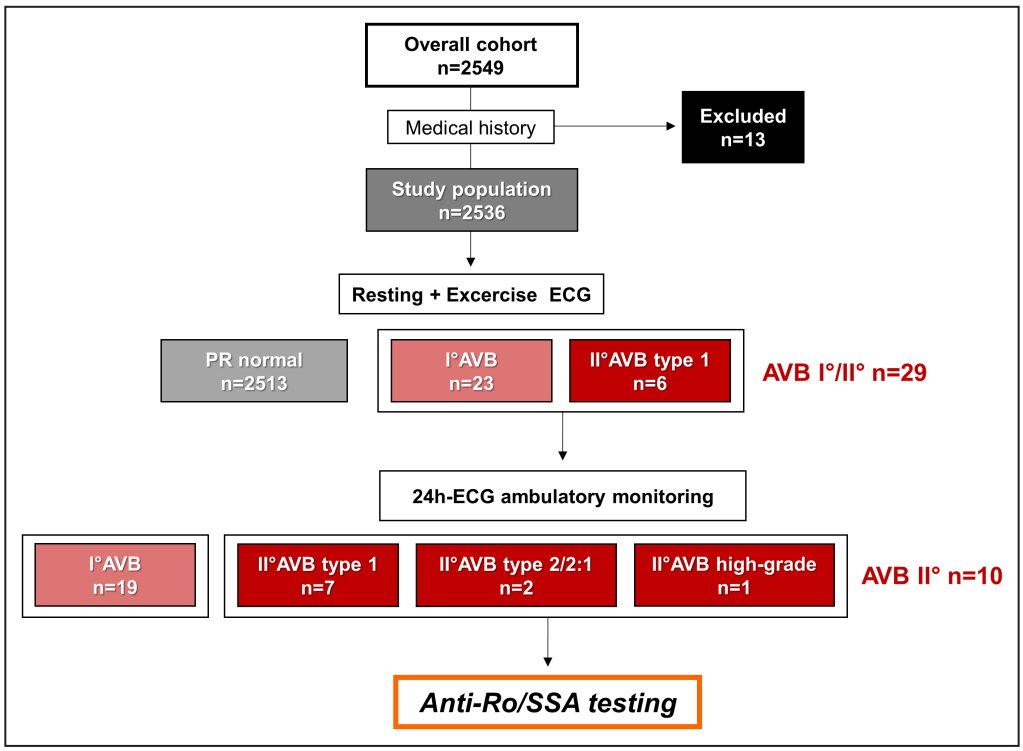

Figure 1. Flow diagram showing the overall design of the study and the key ECG findings.
Anti-Ro/SSA indicates anti-Ro/Sjögren syndrome–related antigen A antibodies; AVB, atrioventricular block; I°AVB, first-degree atrioventricular block; ; and II°AVB, second-degree atrioventricular block.

II°AVB, it should be noted that all were postpubertal male subjects with a medium-high training level. Hence, the prevalence of advanced II°AVB progressively and considerably increased from 0.1% in the overall study population up to as much as 2% in intensely trained postpubertal male subjects (Figure S2).

In agreement with current recommendations,[2] to rule out an underlying structural heart disease, all athletes with II°AVB underwent transthoracic echocardiography (and additionally cardiac magnetic resonance imaging in athletes with high-grade II°AVB), in none case showing remarkable abnormalities. Moreover, 1 of the 3 athletes with advanced II°AVB (those with II°AVB-Mobitz 2) screened for progressive cardiac conduction disease–associated genes (*SCN5A, TRPM4, SCN10A, SCN1B, SC4B, DSP, HCN4, GJC1, GJA5, KCNQ1, KCNH2, CACNA1D, CACNA1C, DES, LMNA, FLNC*) did not exhibit any pathogenic mutations.

Autoantibody Status

All 10 subjects with II°AVB and their 10 mothers underwent complete anti-Ro/SSA testing. Circulating autoantibodies were found in 2 athletes (acquired form, 20%) and in 1 mother (late progressive congenital form, 10%), always in the absence of a manifest AD. As a result, an anti-Ro/SSA-associated AVB was globally present in 30% of the athletes with II°AVB. In all 3 cases, the anti-Ro/SSA 52kD subtype was demonstrated, in the subject or the mother, invariably detected by iWB. In addition, 1 athlete also tested positive for anti-Ro/SSA 60kD antibodies, in this case revealed by fluoroenzyme immunoassay. Importantly, the 3 anti-Ro/SSA–associated cases coincided with the 3 athletes with more advanced II°AVB. This means that 100% of the athletes presenting with a II°AVB more severe than Mobitz 1 (or their mother) showed circulating anti-Ro/SSA antibodies (II°AVB-Mobitz 2, acquired form, n=1; II°AVB-2:1, late progressive congenital form, n=1; II°AVB-high grade, acquired form, n=1) versus none of those with II°AVB-Mobitz 1 (P=0.0083, Fisher exact test) (Table 4; Figure 3; Table S5).

Acute Ex Vivo Effects of Purified IgG From Patients With AVB on I_{CaL} in tSA201 Cells

To further confirm the existence of a pathogenic link between the presence of circulating anti-Ro/SSA antibodies and the atrioventricular conduction disturbances observed, tSA201 cells transiently expressing $Ca_v1.2$ protein were used to evaluate the acute effect of purified IgG from the anti-Ro/SSA–positive athlete with the most severe II°AVB (high-grade), and from a comparable control patient with isolated high-grade II°AVB but anti-Ro/SSA negative. After recording I_{CaL} under basal condition, the addition of purified IgG (75 μg/mL)

Table 1. Demography, Training Level, Electrocardiography, and Clinical Findings of the Entire Study Population

Characteristic	
Subjects, n	2536
Demography	
Male sex	1401 (55.6)
Female sex	1121 (44.4)
Age, y (range)	13.3±3.9 (5–39)
5–11	777 (30.7)
12–15	1242 (49.0)
16–39	513 (20.3)
Training level (sessions/wk)	
Low, 0–2	614 (24.2)
Medium, 3–4	1536 (60.6)
High, 5–7	386 (15.2)
Mean training level	3.2±1.3
ECG findings	
HR, bpm	74.0 (65.0–83.0)
RR, ms	810.8 (722.9–923.1)
PR, ms	130.0 (120.0–150.0)
PRc, ms	134.2 (121.0–147.8)
AVB	29 (1.1)
I°AVB	19 (0.7)
II°AVB	10 (0.4)
II°AVB Mobitz 1	7 (0.3)
II°AVB Mobitz 2/2:1	2 (0.08)
II°AVB high-grade	1 (0.04)
History of autoimmune diseases	10 (0.4)
Hashimoto thyroiditis	5 (50)
Type 1 diabetes	2 (20)
Juvenile idiopathic arthritis	1 (10)
Psoriasis	1 (10)
Graves disease	1 (10)

For continuous variables, the data are expressed as mean±SD; for categorical variables, the data are expressed as n (%). AVB indicates atrioventricular block; HR, heart rate; IAVB, first-degree atrioventricular block; IIAVB, second-degree atrioventricular block; and PRc, heart rate–corrected PR interval.

from the anti-Ro/SSA–positive athlete to the cells for 10 minutes was associated with a significant 43% mean decrease of I_{CaL} density at +10 mV (from –18.5±4.0 to –10.4±1.7 pA/pF; n=7). Conversely, no significant acute inhibitory effect on I_{CaL} was observed after adding the IgG from the anti-Ro/SSA–negative patient with AVB (Figure 4A through 4C).

Chronic Ex Vivo Effects of Purified IgG From Patients With AVB on $Ca_V1.2$ Protein Expression in tSA201 Cells

The chronic consequence of autoantibodies on the $Ca_V1.2$ channel was additionally evaluated by testing the impact of 24-hour incubation with purified IgG from the anti-Ro/SSA–positive athlete with high-grade II°AVB, or from the high-grade II°AVB anti-Ro/SSA–negative control, on $Ca_V1.2$ protein expression in tSA201 cells. Densitometric analysis of western blots demonstrated an evident decrease of $Ca_V1.2$ protein in all tSA201-Cav1.2 cells incubated with IgG from the anti-Ro/SSA–positive athlete (Figure 4Da). Conversely, no changes were observed when tSA201-Cav1.2 cells were treated with IgG from the anti-Ro/SSA–negative patient (Figure 4Da). Pooled data (experiments, n=3) demonstrated that chronic treatment with anti-Ro/SSA–positive IgG was associated with a significant ≈20% reduction of $Ca_V1.2$ protein expression when compared with both untreated (–18%) and anti-Ro/SSA–negative IgG-treated tSA201-$Ca_V1.2$ cells (–22%; Figure 4Db).

DISCUSSION

The key findings of the present study are the following: (1) In a large sample of ≈2500 consecutive athletes aged <40 years and without history of structural heart disease, the global prevalence of advanced AVB was ≈0.1% (3/2536); the risk considerably increased (2%) when intensely trained postpubertal male subjects were selectively considered; (2) while none of the subjects with advanced AVB showed structural cardiac abnormalities, circulating anti-Ro/SSA antibodies were detected in all cases, in the athlete (acquired form, n=2) or in the mother (late progressive congenital form, n=1); and (3) ex vivo experiments showed that purified IgG from anti-Ro/SSA–positive but not –negative patients with advanced AVB acutely inhibit I_{CaL} and chronically downregulate Cav1.2 protein expression.

In the general young population, advanced AVB is not a rare occurrence, and in many cases the definition of the underlying cause remains elusive. Recent nationwide registry data reported an incidence of severe AVB in subjects aged <50 years of almost 20 cases per year per 1 million inhabitants, although a definite pathogenesis was identified in only half of these patients.[4]

While specific studies in sport populations were missing, it is very likely that a similar or even higher incidence of advanced AVB is observed in athletes, given their inherent, training-induced, delayed atrioventricular conduction.[1,17] In any case, it is well recognized that advanced AVB is not a paraphysiologic finding merely due to an athlete's heart, and additional diagnostic investigations in these subjects are currently recommended.[2] However, while in a number of athletes with advanced AVB no structural heart defects or extracardiac diseases are identified, not infrequently in such cases pacemaker implantation is still indicated.[27] In this scenario, the identification of novel and potentially

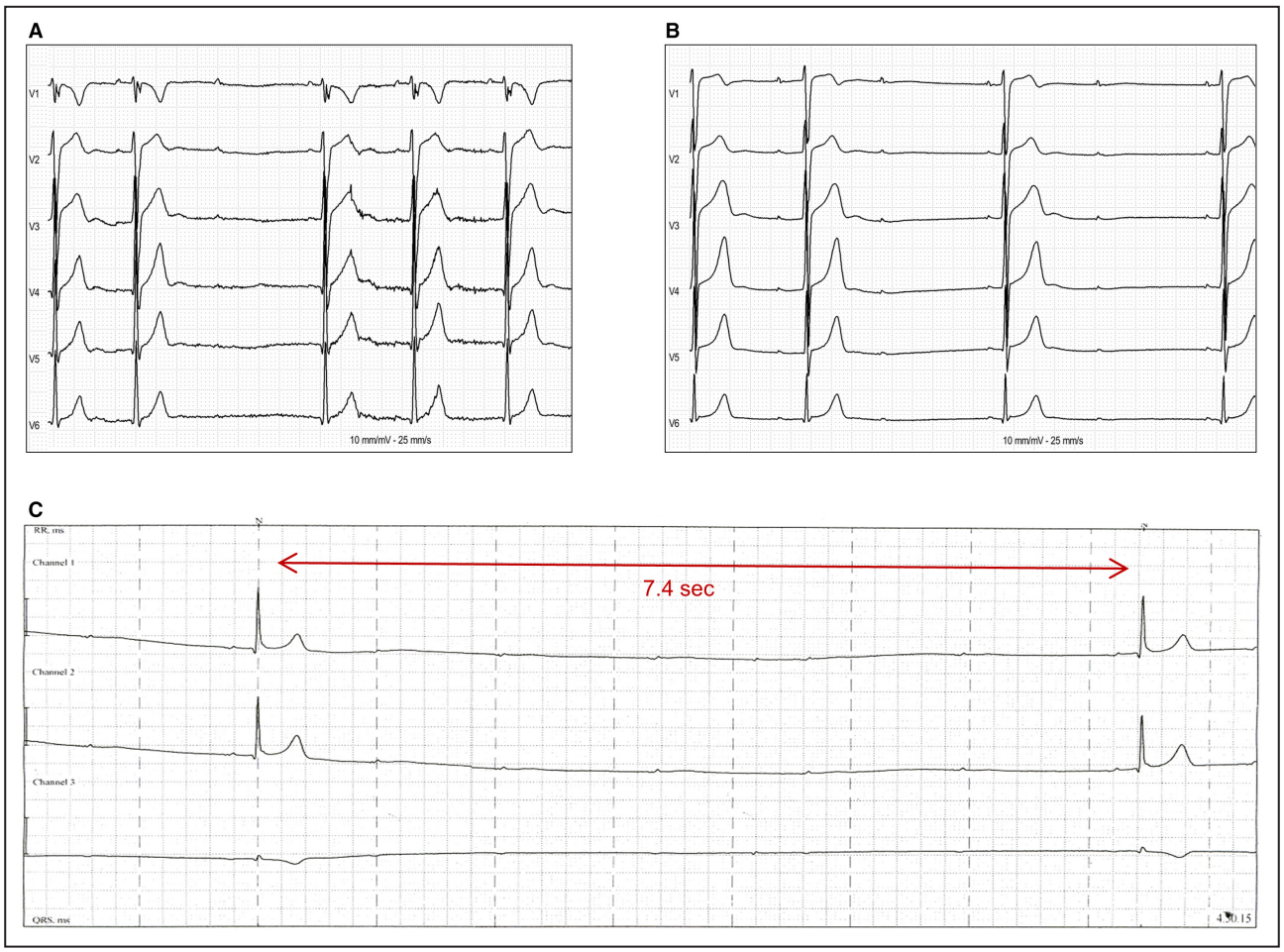

Figure 2. Representative ECG strips of the 3 athletes showing advanced II°AVB.
A, II°AVB Mobitz 2 in a 16-year-old male soccer player (high training level, 5 sessions/wk) with circulating anti-Ro/SSA 52kD antibodies (acquired form). **B**, II°AVB 2:1 in a 26-year-old male soccer player (medium training level, 4 sessions/wk), whose mother showed circulating anti-Ro/SSA 52kD antibodies (late progressive congenital form). **C**, High-grade II°AVB high-grade in a 34-year-old male cyclist (high training level, 5 sessions/wk) with circulating anti-Ro/SSA 52kD and 60kD antibodies (acquired form). anti-Ro/SSA indicates anti-Ro/Sjögren syndrome–related antigen A; AVB, atrioventricular block; and II°AVB, second-degree atrioventricular block.

reversible causes of advanced AVB in athletes represents a relevant unmet need.

Based on this background, in this article, we provide original data regarding the prevalence of advanced AVB in athletes, as well as the impact of autoimmune mechanisms in the pathogenesis of this potentially life-threatening conduction disturbance.

The first novel information coming from our study is that in a large sample of unselected young athletes, either competitive or noncompetitive, the prevalence of advanced AVB is ≈0.1% (3/2536). In fact, only few case reports/case series of severe AVB in athletes are described in the literature.[28,29] In the present study, where prior history of cardiac diseases/interventions were exclusion criteria for enrollment, none of the 3 subjects with advanced AVB showed evidence of structural heart abnormalities. Notably, all 3 athletes were post-pubertal male subjects with a medium-high training level, suggesting that these characteristics may represent putative risk factors for advanced AVB in athletes. This view, further supported by the evidence that both median PR interval and HR-corrected PR interval were significantly longer in male subjects and gradually increased with age (particularly >15 years) and training level, agrees with accumulating data demonstrating the crucial impact of sex, age, and exercise on atrioventricular conduction,[16–18] at least in part via $Ca_V1.2$ changes in nodal cells.[17,30–33] Large studies performed in the general population provided clear evidence that the PR interval is longer in the male sex,[16,18] a difference emerging from the age of 15 years onward, that is, after puberty.[18] Accordingly, experimental data demonstrated that testosterone inhibits, whereas estradiol enhances I_{CaL} in cardiomyocytes.[30,31] In addition, in both sexes the PR interval progressively increases with age,[16] while $Ca_V1.2$ expression in the heart, and specifically in

Table 2. Demography, Training Level, Electrocardiography, and Clinical Findings of the Study Population by Comparing Subjects With or Without AVB

Characteristic	No AVB	AVB	*P* value
Subjects, n	2507	29	
Demography			
Male sex	1384 (55.5)	17 (58.6)	0.88
Female sex	1109 (44.5)	12 (41.4)	
Age, y (range)	13.2±3.9 (5–39)	15.2±5.7 (10–35)	0.007*
5–11	771 (30.8)	6 (20.7)	0.27
12–15	1228 (49.1)	14 (48.3)	
16–39	504 (20.1)	9 (31.0)	
Training level (sessions/wk)			
Low, 0–2	608 (24.3)	6 (20.7)	0.69
Medium, 3–4	1519 (60.6)	17 (58.6)	
High, 5–7	380 (15.2)	6 (20.7)	
Mean training level	3.24±1.3	3.46±1.3	0.37
ECG findings			
HR, bpm	74.0 (65.0–83.0)	76.0 (64.0–84.0)	0.94
RR, ms	810.8 (722.9–923.1)	789.5 (714.3–937.5)	0.94
PR, ms	130.0 (120.0–150.0)	180.0 (180.0–200.0)	<0.001*
PRc, ms	133.6 (120.8–147.3)	187.8 (178.6–204.0)	<0.001*
I°AVB	-	19 (65.5)	
II°AVB Mobitz 1	-	7 (24.1)	
II°AVB Mobitz 2/2:1	-	2 (6.9)	
II°AVB high-grade	-	1 (3.4)	
History of autoimmune diseases	10 (0.4)	0	0.73
Hashimoto thyroiditis	5 (50)	-	
Type 1 diabetes	2 (20)	-	
Juvenile idiopathic arthritis	1 (10)	-	
Psoriasis	1 (10)	-	
Graves disease	1 (10)	-	

Most severe AVB degree documented in the single patient was considered.

For continuous variables, the data are expressed as mean±SD or median (interquartile range); for categorical variables, the data are expressed as n (%).

Differences between the 2 groups were evaluated by 2-tailed unpaired *t* test, 2-tailed Mann–Whithey test, or 2-sided Fisher exact test.

AVB indicates atrioventricular block; HR, heart rate; IAVB, first-degree atrioventricular block; IIAVB, second-degree atrioventricular block; and PRc, heart rate–corrected PR interval.

*indicates *P* values <0.05.

nodal cells, gradually decreases.[32,33] Furthermore, an elegant study recently conducted in racehorses and mice undergoing intense training showed that PR interval prolongation occurrence, which developed even in the presence of complete autonomic block, was accompanied by an evident reduction of $Ca_v1.2$ expression and I_{CaL} density at the level of the atrioventricular node.[17] Overall, these data strongly suggest that male sex hormones, aging, and training can synergistically delay atrioventricular conduction, via electric remodeling reducing $Ca_v1.2$ reserve in the atrioventricular node. Accordingly, selectively focusing on the subsample constituted by the highly trained male subjects aged >15 years, we found that the prevalence of advanced AVB markedly increased to 2% (2/100), that is, ≈20 times higher than that observed in the overall population.

Table 3. Demography, Training Level, Electrocardiography, and Clinical Findings of the Study Population by Comparing Subjects With or Without IIAVB

Characteristic	No II°AVB	II°AVB	*P* value
Subjects	2526	10	
Demography			
Male sex	1393 (55.5)	8 (80.0)	0.21
Female sex	1119 (44.5)	2 (20.0)	
Age, y (range)	13.3±3.9 (5–39)	17.1±7.5 (12–35)	0.002*
5–11	777 (30.8)	0	0.072
12–15	1236 (49.0)	6 (60.0)	
16–39	509 (20.2)	4 (40.0)	
Training level (sessions/wk)			
Low, 0–2	614 (24.3)	0	0.039*
Medium, 3–4	1530 (60.6)	6 (60.0)	
High, 5–7	382 (15.2)	4 (40.0)	
Mean training level	3.24±1.3	3.90±1.0	0.11
ECG findings			
HR, bpm	74.0 (65.0–83.0)	64.5 (60.7–76.0)	0.11
RR, ms	810.8 (722.9–923.1)	930.3 (789.5–988.1)	0.11
PR, ms	130.0 (120.0–150.0)	200.0 (172.5–217.5)	<0.001*
PRc, ms	134.1 (121.0–147.5)	200.5 (172.2–215.2)	<0.001*
II°AVB Mobitz 1	-	7 (70.0)	
II°AVB Mobitz 2/2:1	-	2 (20.0)	
II°AVB high-grade	-	1 (10.0)	
History of autoimmune diseases	10 (0.4)	0	0.84
Hashimoto thyroiditis	5 (50)	-	
Type 1 diabetes	2 (20)	-	
Juvenile idiopathic arthritis	1 (10)	-	
Psoriasis	1 (10)	-	
Graves disease	1 (10)	-	

Most severe AVB degree documented in the single patient was considered.

For continuous variables, the data are expressed as mean±SD or median (interquartile range); for categorical variables, the data are expressed as n (%).

Differences between the 2 groups were evaluated by 2-tailed unpaired *t* test, 2-tailed Mann–Whithey test, or 2-sided Fisher exact test.

AVB indicates atrioventricular block; HR, heart rate; IIAVB, second-degree atrioventricular block; and PRc, heart rate–corrected PR interval.

*indicates *P* values <0.05.

Table 4. Demography, Training Level, Electrocardiography, and Laboratory Findings of the Subjects With II AVB: Comparison Between Anti-Ro/SSA–Associated and Non–Anti-Ro/SSA–Associated Forms

Characteristic	Non anti-Ro/SSA–associated II°AVB		Anti-Ro/SSA–associated II°AVB		*P* value
Subjects	7		3		
Demography					
Male sex	5 (71.4)		3 (100)		0.86
Female sex	2 (28.6)		0		
Age, y (range)	13.4±1.6 (12–16)		25.7±9.5 (16–35)		0.007*
5–11	0		0		0.067
12–15	6 (85.7)		0		
16–39	1 (14.3)		3 (100)		
Training level (sessions/wk)					
Low, 0–2	0		0		0.67
Medium, 3–4	5 (71.4)		1 (33.3)		
High, 5–7	2 (28.6)		2 (66.7)		
Mean training level	3.57±1.0		4.67±0.6		0.11
ECG findings					
HR, bpm	76.0 (63.5–78.0)		60.0 (54.0–62.5)		0.11
RR, ms	789.5 (769.7–944.9)		1000.0 (961.5–1125.0)		0.11
PR, ms	200.0 (170.0–210.0)		210.0 (195.0–215.0)		0.49
PRc, ms	198.4 (171.6–212.9)		208.7 (191.5–213.1)		0.57
II°AVB Mobitz 1	7 (100)		0		0.007*
II°AVB Mobitz 2/2:1	0		1 (33.3)		
II°AVB high-grade	0		2 (66.7)		
Anti-Ro/SSA positivity	0		3 (100)		
Autoantibody profile	Athlete	Mother	Athlete	Mother	
Anti-Ro52kD	…	…	2/3 (67)	1/3 (33)	
iWB	…	…	*2/2 (100)*	*1/1 (100)*	
FEIA	…	…	*0*	*0*	
LIA	…	…	*0*	*0*	
Anti-Ro60kD	…	…	1/3 (33)	0	
iWB	…	…	*0*	-	
FEIA	…	…	*1/1 (100)*	-	
LIA	…	…	*0*	-	
Anti-Ro52kD + 60kD	…	…	1/2 (50)	0	

Most severe AVB degree documented in the single patient was considered.

For continuous variables, the data are expressed as mean±SD or median (interquartile range); for categorical variables, the data are expressed as n (%).

Differences between the 2 groups were evaluated by 2-tailed unpaired *t* test, 2-tailed Mann–Whitney test, or 2-sided Fisher exact test.

Anti-Ro/SSA indicates anti-Ro/Sjögren syndrome–related antigen A; AVB, atrioventricular block; FEIA, fluoroenzyme immunoassay; HR, heart rate; II AVB, second-degree atrioventricular block; iWB, immune-western-blot analysis; LIA, line immunoblotting; and PRc, heart rate–corrected PR interval. Results of specific methods for anti-Ro/SSA antibody testing are reported in italic.

*indicates *P* values <0.05.

The other key novel finding of the present study is that in athletes with II°AVB, circulating anti-Ro/SSA antibodies are frequently detected (30% of cases) in the subject or in the mother, and the prevalence markedly increases (100%) when individuals with advanced AVB are specifically considered. In all these 3 athletes, the anti-Ro/SSA 52kD subtype was detected, invariably revealed by iWB only, in 2 cases present in the serum of the subject (acquired form), while in the third case in that of the mother (late progressive congenital form). Notably, in none of these athletes a history of AD was found.

Such findings are consistent with previous data obtained in the general population.[7,34] Indeed, a recent cross-sectional study performed in 34 adults (aged <65 years) with isolated AVB, consecutively enrolled from the general population, provided evidence that circulating anti-Ro/SSA antibodies, principally anti-Ro/SSA 52kD detected by iWB, were present in ≈50% of patients or in their mothers or in both ones, most commonly an acquired form in the absence of a manifest AD.[11] Moreover, a nationwide population study recently performed in Israel on >100 000 subjects found a 2 times higher risk of AVB in anti-Ro/SSA–positive than in anti-Ro/SSA–negative subjects, regardless of the presence or absence of AD.[13] In further support of these data, the findings of the present study point to circulating anti-Ro/SSA antibodies as an epidemiologically relevant risk factor for advanced AVB also in the sports population. Moreover, given that in our athletes anti-Ro/SSA positivity was always asymptomatic for other autoimmune manifestations, our study once more underlines the need for a specific and complete autoantibody testing in all cases of advanced AVB in a structurally normal heart, even in the absence of the "red flag" of an evident AD.

The evidence that anti-Ro/SSA seropositivity is per se associated with an increased risk of AVB, further strengthens the view that the arrhythmogenic potential of these autoantibodies is due to a direct and specific interference on cardiac electrophysiology.[9,35–38] In support of this hypothesis, a recent study provided evidence that purified IgG from anti-Ro/SSA–positive patients with AVB acutely inhibit I_{CaL} and chronically decrease $Ca_v1.2$ expression by specifically cross-reacting with peptides localized in the extracellular pore region of the $Ca_v1.2$.[11] The results of the ex vivo experiments performed in the present study support the view that these mechanisms may be also operative in the advanced AVB occurring in the anti-Ro/SSA–positive athlete. Moreover, the fact that the same findings are also observed in a larger sample of subjects from the general population considerably expands their potential clinical significance.

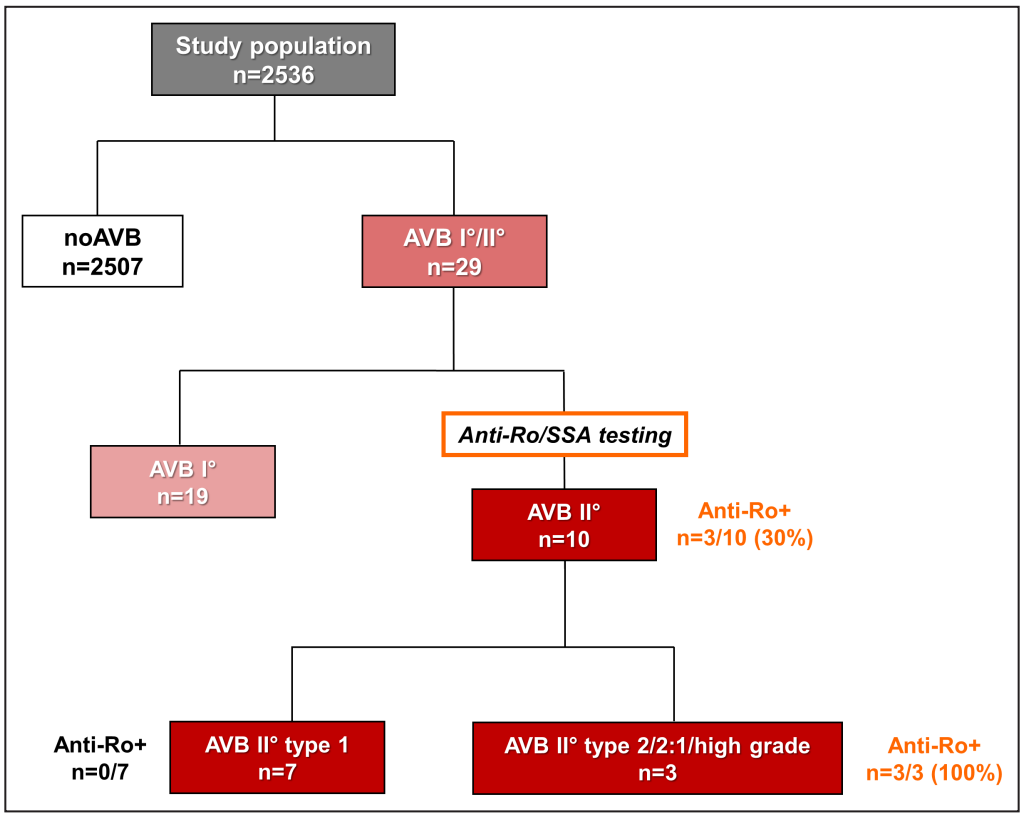

Figure 3. Flow diagram showing the results of anti-Ro/SSA testing in athletes with II°AVB. anti-Ro/SSA indicates anti-Ro/Sjögren syndrome–related antigen A; anti-Ro+/–, anti-Ro/SSA–positive/negative; AVB, atrioventricular block; I°AVB, first-degree atrioventricular block; and II°AVB, second-degree atrioventricular block.

These data suggesting the involvement of $Ca_v1.2$ in the pathogenesis of the conduction disturbances, could also help explain why male sex, postpubertal age, and high training levels represented, in our sample, risk factors for the development of advanced AVB in the presence of circulating anti-Ro/SSA antibodies. As discussed above, it can be speculated that testosterone, exercise, and age may synergistically act in reducing $Ca_v1.2$ reserve in the atrioventricular node, thereby creating a predisposed substrate critically emphasizing the negative electrophysiological impact of these autoantibodies on atrioventricular conduction. Finally, an additional potential role might also be played by genetic factors. In fact, although genetic data are available in only 1 patient with advanced AVB and no pathogenic mutations were detected, we cannot rule out that polymorphism-driven differences in calcium channel reserve might further predispose some subjects to the development anti-Ro/SSA–associated AVB. This intriguing and potentially important point warrants further investigation.

In this scenario, detecting circulating anti-Ro/SSA antibodies in athletes with advanced AVB and a structurally normal heart might have a relevant impact on patient management, because autoantibodies, like training, may represent a potentially modifiable risk factor for delayed atrioventricular conduction in these subjects. Indeed, accumulating recent data provided evidence that immunomodulatory treatments are rapidly and highly effective in improving or even completely reversing advanced AVBs in patients with circulating anti-Ro/SSA antibodies (acquired or mixed form), strongly supporting the purely electrophysiological nature of the autoimmune interference on atrioventricular conduction.[6,8,10,11,25,39] Notably, glucocorticoids and major immunosuppressants do not seem to be the sole therapeutically active agents in these patients. Preliminary data from our institution suggest that other immunomodulating drugs with fewer systemic adverse effects, specifically hydroxychloroquine, may also be safe and effective in the long term as pacemaker-sparing therapy in these patients.[10,11] This would represent a very appealing therapeutic alternative since hydroxychloroquine, unlike glucocorticoids, could be provided with a more favorable benefit/risk ratio compared with pacemaker implantation, particularly in patients with AVB of unknown origin,[5] and not being included in the list of substances prohibited by the World Anti-Doping Agency (www.globaldro.com).

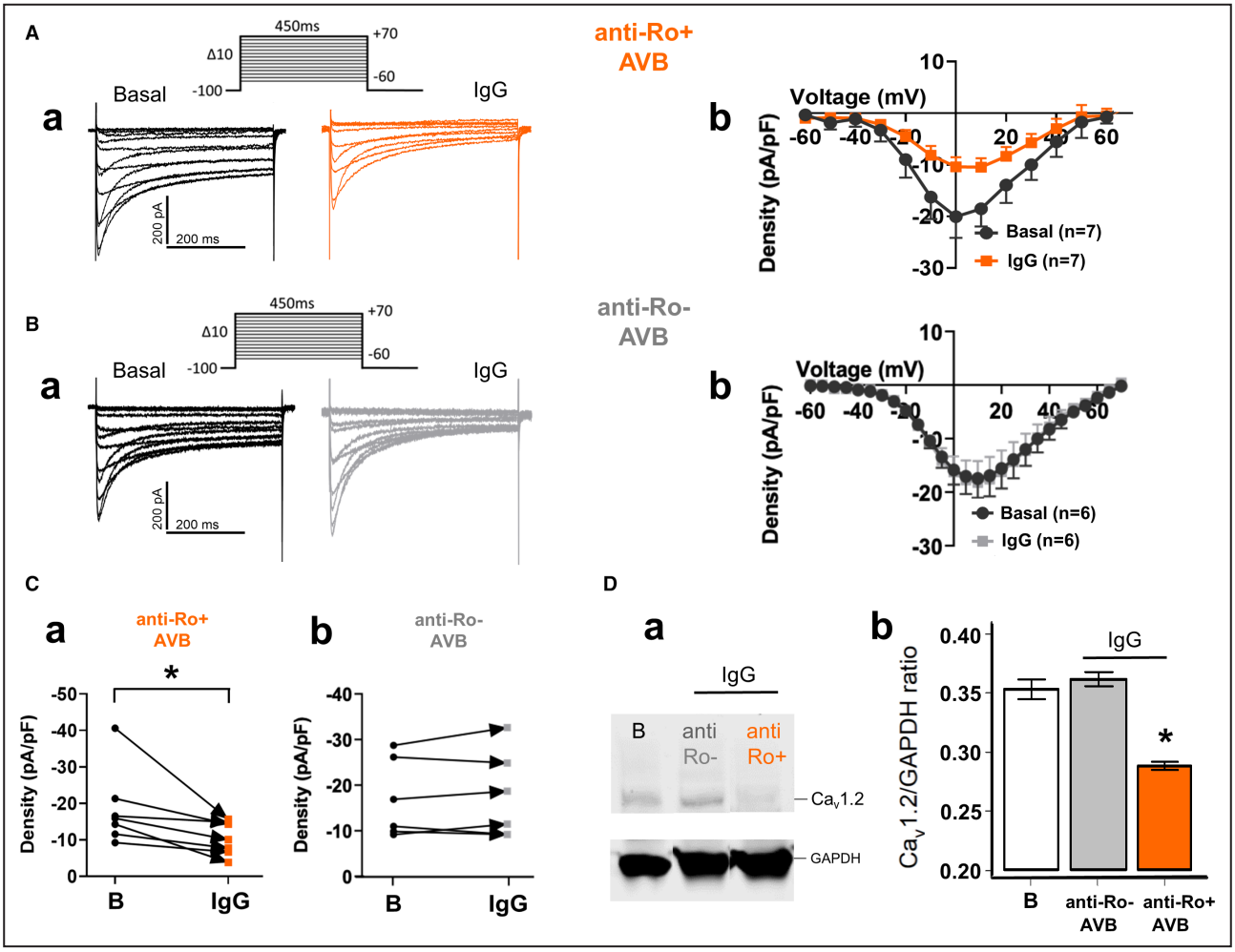

Figure 4. Acute and chronic ex vivo effects of purified IgG from patients with AVB on I_{CaL} in tSA201 cells and $Ca_v1.2$ protein expression in tSA201 cells.

A, I_{CaL} recorded from transiently transfected tsA201cells with recombinant expression of $Ca_v1.2$, before (black) and after (orange) the incubation with purified IgG from the anti-Ro/SSA–positive athlete with II°AVB high-grade (75μg/mL for 10 minutes). (a) Representative whole-cell calcium current traces. I_{CaL} currents were recorded in the presence of 2 mM $CaCl_2$ from a holding potential of –100 mV. Time scale was 200 ms and the current amplitude scale is 200 pA, as indicated. (b) Averaged current–voltage relationships before and after IgG treatment (n=7). **B**, I_{CaL} recorded from transiently transfected tsA201cells with recombinant expression of $Ca_v1.2$, before (black) and after (gray) the incubation with purified IgG from a control patient with isolated II°AVB high-grade but anti-Ro/SSA negative (75 μg/mL for 10 minutes). (a) Representative whole-cell calcium current traces. (b) Averaged current–voltage relationships before and after IgG treatment (n=6). **C**, Plots showing I_{CaL} current densities recorded at 10 mV before and after treatment with IgG. (a) Anti-Ro/SSA–positive athlete with AVB (n=7); 2-tailed paired *t* test, **P*<0.05. (b) Anti-Ro/SSA–negative control with AVB (n=6); 2-tailed paired *t* test, n.s. not significant. **D**, $Ca_v1.2$ protein expression in tSA201-Cav1.2 cells before and after 24-h treatment with 40 μg/mL purified IgG from the anti-Ro/SSA–positive athlete with II°AVB high-grade and the control anti-Ro/SSA–negative patient with isolated II°AVB high-grade. (a) Representative western blot for $Ca_v1.2$ at baseline (B, line 1) and after treatment with IgG from anti-Ro/SSA–negative (line 2) and anti-Ro/SSA–positive (line 3) patients. (b) Corresponding histograms of densitometric analysis for $Ca_v1.2$ normalized to GAPDH (n=3 each). One-way ANOVA, *P*<0.001; Tukey–Kramer post hoc multiple comparison test, **P*<0.05 vs B and anti-Ro AVB after IgG treatment. Anti-Ro+/– indicates anti-Ro/SSA–positive/negative; AVB, atrioventricular block; IgG, immunoglobulin G; and I_{CaL}, L-type calcium current.

This study has some limitations that should be acknowledged. First, the study design is able to show only association, not causation, and the small number of events limits the strength of the conclusions. Moreover, while structural heart disease in the 3 subjects with advanced AVB was ruled out by imaging, the rest of the study sample did not undergo screening echocardiograms, as baseline cardiac status was determined by history alone. Given that congenital heart disease occurs in ≈1% of the general population,[40] this fact might have affected the denominator, thereby slightly underestimating the prevalence of advanced AVB of unknown origin in the overall sample. Furthermore, while this study focused on the

interaction of anti-Ro/SSA antibodies with $Ca_v1.2$, which is expressed throughout the cardiac tissues, $Ca_v1.3$, which has been demonstrated to be expressed in the supraventricular tissue but not in the ventricles of adult normal hearts,[41] was not evaluated in this study. Specifically, the expression of $Ca_v1.3$ during development[42] as well as the expression in the ventricular versus supraventricular tissue has been established,[41–44] however, the relative expression of $Ca_v1.3$ to $Ca_v1.2$ in the atrioventricular node is controversial at best, especially in the context of relevance to an intact tissue.[45,46] Zhang et al[45] and Marger et al[46] examined the relative contributions of $Ca_v1.3$/$Ca_v1.2$ in the atrioventricular node and relied on the Cav1.3 knockout ($Ca_v1.3$–/–) mice model. Zhang et al failed to demonstrate any differences in the total I_{CaL} densities recorded from the 3 different genotypes (knockout $Ca_v1.3$–/– mice, $Ca_v1.3$+/– heterozygous, and wild-type mice), indicating a small contribution of $Ca_v1.3$ to the total I_{CaL} in the atrioventricular node and that the null mutant mice may present with compensatory changes. These limitations also apply to the paper by Marger et al, who used the same $Ca_v1.3$ knockout mice to evaluate the relative contribution of $Ca_v1.3$ and $Ca_v1.2$ in atrioventricular nodal cells. Unlike Zhang et al, Marger et al showed an 11% residual total I_{CaL} densities in the atrioventricular node of $Ca_v1.3$–/– mice compared with wild-type mice, in this case, indicating an important role of $Ca_v1.3$ in the atrioventricular node. Collectively, the exact contributions of $Ca_v1.3$ and $Ca_v1.2$ to atrioventricular node electrogenesis remains to be determined, and the proposed relationships between atrioventricular nodal cells and whole tissue behavior remain largely speculative.

In conclusion, our study provides evidence for the first time that advanced AVB is not a rare occurrence in young athletes without history of cardiac disease and that a significant percentage of these cases are associated with circulating anti-Ro/SSA antibodies cross-reacting with cardiac L-type calcium channels. The finding is epidemiologically relevant, particularly in consideration of the huge number of athletes active worldwide. In fact, based on the more recent data diffused by the Italian National Olympic Committee, in Italy only the overall sports population in 2018 included ≈12.8 million of athletes, competitive or non-competitive (www.coni.it). This leads us to estimate ≈12 800 cases of advanced AVB, among which 30% were anti-Ro/SSA associated (n≈3800), including two thirds (n≈2500) with acquired forms. Such data highlight the importance of performing complete anti-Ro/SSA antibody testing (including iWB) in all athletes with advanced AVB, even in the absence of clinical signs of AD. In fact, given that anti-Ro/SSA antibodies, in the acquired form, exert their arrhythmogenic activity through electrophysiological and thereby potentially reversible effects,[8,11] the detection of circulating autoantibodies might open to immune-modulating (and in perspective decoy peptide based)[47] treatments as novel therapeutic opportunities to reduce the risk of life-threatening events in a significant number of athletes, avoiding (or delaying) pacemaker implantation.[10]

ARTICLE INFORMATION

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Supplemental Material

Data S1
Tables S1–S5
Figures S1–S2

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