

UNIVERSITÀ DEGLI STUDI DI MILANO

PhD course in Biochemical Sciences, XXXIII cycle

Department of Medical Biotechnology and Translational Medicine



DEVELOPMENT OF FUNCTIONAL ASSAYS FOR THE STUDY OF SODIUM, CALCIUM, AND POTASSIUM ION CHANNELS ACTIVITY IN BRUGADA SYNDROME

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A.Y. 2019/2020

*To my Family and all my Friends,
who always encouraged me to go on every adventure,
and without whom none of my success would be possible
with Love,
Sara*

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Abstract

Brugada Syndrome (BrS) is an autosomal dominant channelopathy that presents a specific ECG pattern characterized by an elevation of the ST-segment in the right precordial leads, and it is related to an increased risk of sudden cardiac death (SCD). The BrS ECG patterns can manifest in three different types: type 1, type 2, and type 3. Type 1 is spontaneous and easily recognized by a normal ECG, while type 2 and type 3 are often ambiguous and they require a diagnostic test for their correct identification. The test consists in the administration of a sodium channel blocking agent which, in the presence of the pathology, unmasks the ECG pattern. This test presents several risks and it has to be executed by highly specialized teams. Thus, the development of a new test based on the measurement of specific biomarkers would therefore be crucial. Nowadays, BrS patients' treatment requires an implantable cardioverter-defibrillator, followed by an epicardial ablation of the BrS arrhythmogenic substrate through a catheter radiofrequency that eliminates the electrical abnormalities in the RVOT. In about 30% of BrS cases, it is possible to determine a genetic component responsible for the onset of the disease; the most frequent cause, among these cases, is a mutation in the SCN5A gene, encoding the α -subunit of the cardiac voltage-dependent sodium channel NaV1.5. More than 300 mutations, distributed over the entire sequence, have been found in the SCN5A gene. Usually, these mutations cause a loss-of-function of the channel even if the mechanism and the extent of the dysfunction may vary, according to the mutation; it has also been shown that mutations that cause a reduction of the sodium flux >90% or a truncated protein, can cause more severe clinical phenotypes. Unfortunately, it is currently not easy to determine the direct genotype-phenotype relationship, thus, being able to experimentally define this link would be very important, as it could provide a new parameter useful in risk stratification. In addition to sodium channel dysfunction, calcium and potassium ion channels alterations have been demonstrated as a cause of the BrS manifestation. Therefore, understanding ion channels dysfunctions and their effects could improve BrS risk stratification or diagnosis, which remain clinical challenges that need to be addressed. Based on these premises, the aim of the study was firstly to develop an *in vitro* functional voltage-sensing assay to measure NaV1.5 activity, carrying BrS SCN5A mutations to ultimately correlate them to the BrS phenotypes; secondly, we optimized fluorescence functional assays for the evaluation of potassium and calcium channel activity in peripheral blood cells to find potential biomarkers for BrS. For the first part of the thesis, several novel mutations were identified through the

genetic screening of the SCN5A gene in BrS patients, and four of them were fully characterized by *in silico* and *in vitro* analysis. The bioinformatics prediction classified the four mutations as “probably damaging”, and the functional assay confirmed this result, revealing a lower sodium channel activity as compared to wild-type SCN5A. In the second part, we developed and optimized two fluorescence *in vitro* functional assay for both potassium and calcium channels on peripheral blood cells to find potential biomarkers in BrS patients. The analysis revealed that the calcium channels activity in BrS PBMCs was higher compared to their controls, and the potassium channels activity was higher in BrS T lymphocytes compared to healthy controls. In conclusion, in this Ph.D. thesis, we developed three functional assays to study sodium, calcium, and potassium channels activity. In future, the results obtained would represent the basis for the identification of BrS biomarker, the development of a new diagnostic test, and for a better understanding of BrS etiology.

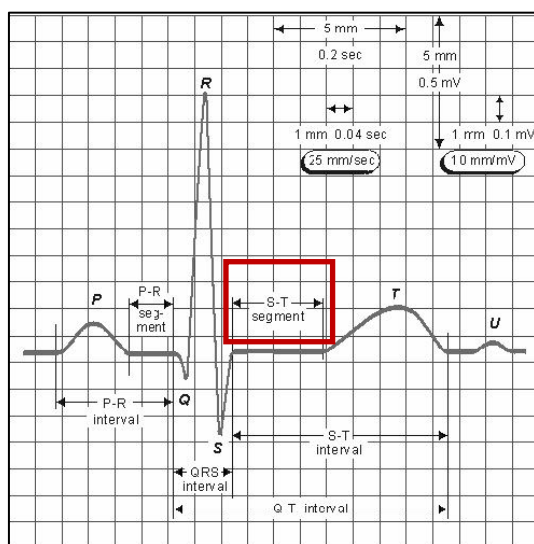
Chapter 1

Introduction

1.1 The Brugada Syndrome: definition

Brugada syndrome (BrS) is an autosomal dominant channelopathy, related to an increased risk of sudden cardiac death (SCD) [1], which was firstly described by Pedro and Joseph Brugada in 1992, as “*a novel clinical and electrocardiographic syndrome*” [2]. BrS is a hereditary arrhythmic disease characterized by a specific electrocardiographic pattern, defined by an elevation of the ST segment (Figure 1) in the right precordial leads (V1-V3) with incomplete or complete right bundle branch block, by the absence of cardiac structural changes, and by an increased risk of ventricular fibrillation and sudden death due to cardiac arrest [3][4]. The typical symptoms of BrS are cardiac arrest or syncope, especially between 30 and 50 years, but the majority of patients are asymptomatic, usually diagnosed by chance. The global prevalence of BrS varies from 5 to 20 cases per 10,000 inhabitants worldwide, and it's endemic in Asian countries [5].

A



B

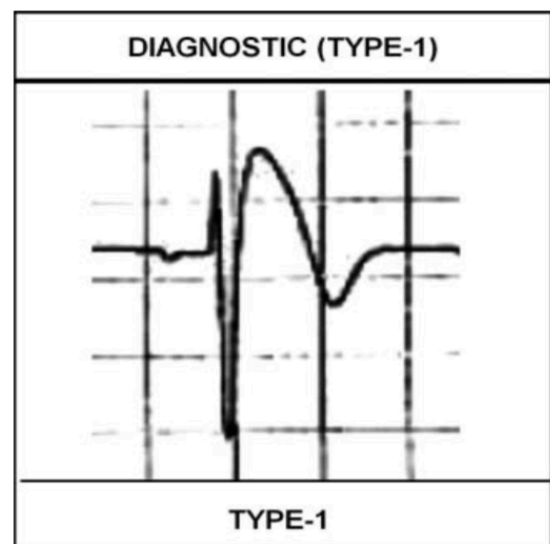


Figure 1. Schematic representation of ECG waves [6]. **A.** Representation of one cardiac cycle of ECG signal which consists in a P wave, QRS complex, and T waves. **B.** Diagnostic type 1 ECG pattern.

1.2 Brugada Diagnosis

The BrS diagnosis is based on the recognition of the specific ECG pattern that characterizes BrS, defined by a large coved ST-segment elevation ≥ 2 mm in the right precordial leads (V1-V3). However, BrS can manifest three different types of repolarization patterns in the right precordial leads (Figure 2), making the diagnosis a clinical challenge. Type 1 is the most common one and besides the ST-segment elevation, it is defined by a negative T wave inversion; type 2, beyond the ST-segment elevation, displays a saddleback showed ST-segment elevation ≥ 1 mm followed by a positive T wave with J point elevated in leads V1 through V3; type 3 shows either a saddleback or a coved ST-segment elevation of < 1 mm [7].

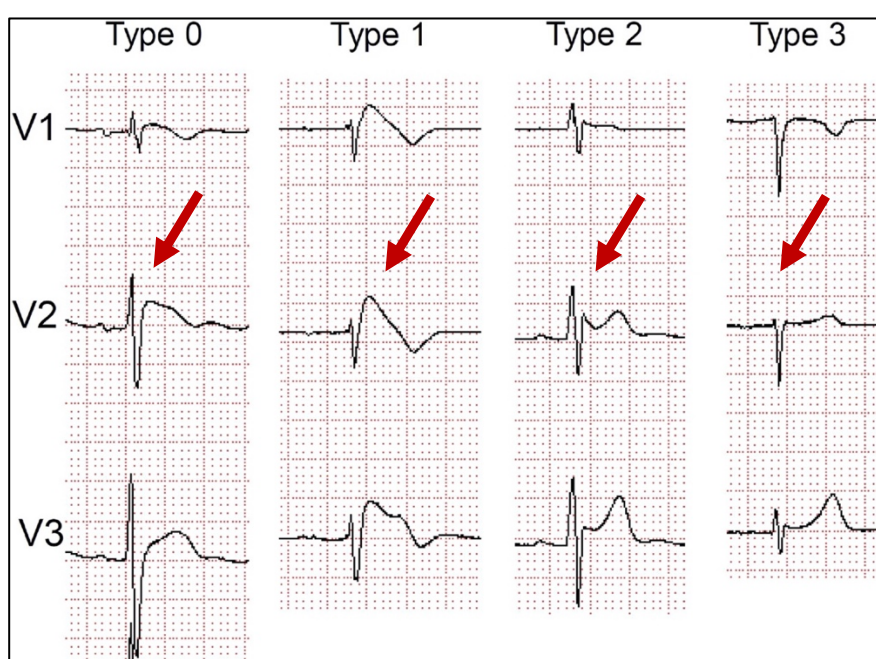


Figure 2. Electrographic patterns of Brugada syndrome. Type 0 is a healthy individual's ECG pattern; type 1 is a spontaneous ECG pattern (large coved type ST-segment elevation); type 2 (saddleback showed ST-segment elevation) and type 3 (saddleback or coved ST-segment) are unmasked by intravenous administration of sodium channel blockers.

In addition, while type 1 is considered the diagnostic hallmark of the syndrome, it is spontaneous, and it is diagnosed by a normal ECG, type 2 and 3 ECG patterns are suspicious, but not considered diagnostic [8]; indeed, the diagnosis through these two ECG patterns can only be made after drug-induced conversion to a type 1 ECG with a sodium channel blocker (i.e. 1mg/kg Ajmaline or 2mg/kg Flecainide)

[9]. In addition, type 1 ECG represents a small portion of the BrS population: as a matter of fact, Sieria et al. demonstrated that, on a study population of 400 BrS patients, only 19.5% individuals showed spontaneous type 1 ECG pattern, while the remaining 80.5% presented type 2 or type 3 BrS pattern and were diagnosed only by drug-induced procedure [10]. Another factor that makes the diagnosis more difficult is the dynamicity of the BrS ECG pattern, since it has been demonstrated that it fluctuates during the day and can change from normal (type 0) to type 1 pattern and vice versa [9][11]. Moreover, this fluctuation can be induced by various conditions as fever, hypercalcemia, diabetes, excessive consumption of food or alcohol, hyperkalemia, general drugs [12]. Furthermore, based on the 2015 Guidelines for the Management of Patients with Ventricular Arrhythmias and Prevention of Sudden Cardiac Death of the European Society of Cardiology (ESC), the diagnosis of BrS with type 1 unmasked by sodium channel blockers has to be associated with one of the following clinical episodes: ventricular fibrillation (VF), ventricular tachycardia (VT), family history of SCD or coved-type ECG, syncope, seizures, abnormal ECGs, nocturnal agonal respiration, or implanted pacemakers [3][13]. Asymptomatic BrS patients with type 1 ECG should not undergo drug-induced diagnosis because this test does not give additional diagnostic value while it could induce lethal arrhythmias [3][12].

Recently, the J. Wave Syndrome Consensus Report, based on literature and clinical studies of the Task Force Members for the Management of Patients with Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death of the European Society of Cardiology (ESC) [14] proposed a diagnostic score system for BrS named “Proposed Shanghai Score System for diagnosis of Brugada syndrome” (Figure 3). This system is based on ECG patterns, clinical history, family history, and genetic test. The spontaneous manifestation of type 1 ECG is crucial and sufficient for this system, while fever or drug-induced type 2 and 3 conversion need to be associated with other factors to diagnose BrS.

Points		Points	
I. ECG (12-Lead/Ambulatory)		III. Family History	
A. Spontaneous type 1 Brugada ECG pattern at nominal or high leads	3.5	A. First- or second-degree relative with definite BrS	2
B. Fever-induced type 1 Brugada ECG pattern at nominal or high leads	3	B. Suspicious SCD (fever, nocturnal, Brugada aggravating drugs) in a first- or second-degree relative	1
C. Type 2 or 3 Brugada ECG pattern that converts with provocative drug challenge	2	C. Unexplained SCD <45 years in first- or second- degree relative with negative autopsy	0.5
<i>*Only award points once for highest score within this category. One item from this category must apply.</i>		<i>*Only award points once for highest score within this category.</i>	
II. Clinical History*		IV. Genetic Test Result	
A. Unexplained cardiac arrest or documented VF/ polymorphic VT	3	A. Probable pathogenic mutation in BrS susceptibility gene	0.5
B. Nocturnal agonal respirations	2	Score (requires at least 1 ECG finding)	
C. Suspected arrhythmic syncope	2	≥3.5 points: Probable/definite BrS	
D. Syncope of unclear mechanism/unclear etiology	1	2–3 points: Possible BrS	
E. Atrial flutter/fibrillation in patients <30 years without alternative etiology	0.5	<2 points: Nondiagnostic	
<i>*Only award points once for highest score within this category.</i>		BrS = Brugada syndrome; SCD = sudden cardiac death; VF = ventricular fibrillation; VT = ventricular tachycardia.	

Figure 3. Proposed Shanghai Score System for diagnosis of Brugada syndrome. Probable and/or definite BrS: ≥ 3.5 points; possible BrS: 2 to 3 points; nondiagnostic: <2 points [15].

The drug challenge with sodium channel blockers is the most reliable test for BrS diagnosis. Nonetheless, this test can be associated with the occurrence of potentially life-threatening VAs, thus limiting its widespread use, besides in a few state-of-the-art facilities equipped with an “ECMO (Extracorporeal Membrane Oxygenation) [16] team” readily available in case of emergency. Nowadays the ajmaline challenge [17] is the most accurate test to-date to diagnosis BrS in patients without a spontaneous type 1 BrS ECG pattern. This test has proven to be a powerful tool in provoking the type 1 ECG pattern [18], and more accurate than flecainide, which is associated with a 36% false-negative rate [19]. However, the ajmaline drug itself can provoke VT/VF during the diagnostic procedure. As a result, ajmaline administration as a diagnostic tool is prohibited in the United States for safety reasons. Additionally, given the risk associated with the procedure, patients themselves often refuse to undergo the procedure. Additionally, the procedure requires experienced staff, in hospitals with an ECMO team. Therefore, this procedure is not widely available, implying that patients need to travel long distances for the procedure. From an economical perspective, the procedure is expensive and a considerable burden for the National Healthcare System. As a result, potential patients do not undergo the diagnostic test in a timely fashion, or at all, exposing them to considerable risk of SCD.

1.3 Epidemiology

BrS has arisen extensive interest in the recent years as it appears to be one of the major causes of SCD in apparently healthy individuals [20]. However, the estimation of the actual worldwide prevalence of BrS is difficult since the ECG pattern is very dynamic, most of the patients are asymptomatic throughout their lives, and the drug-challenge test presents difficulties, as explained before. However, based on 28 studies involving 369,069 subjects, the worldwide estimated prevalence is 0.5 – 2 cases per 1000 inhabitants, with the highest cases in Southeast Asia with 3.7 per 1000 inhabitants [21] (Figure 4). BrS is predominant in males as compared to females, probably due to the action of hormones, however, there are no conclusive experimental data to justify this aspect [21], [22]. It is well-known that healthy men, compared to healthy women, show shorter and faster repolarization and a shorter duration of QTc and JTc intervals [23]. Indeed, the interfering role of testosterone and the crucial role of androgens in ventricular repolarization has been demonstrated by two studies: two castrated men due to prostate cancer and traumatic ablation of the testes, and a study of virilization syndrome of women. Specifically, after the orchiectomy, the two men showed normalized JT intervals similar to healthy women, while the virialized women showed a normalized JT interval similar to healthy men [24]. Moreover, both neonates and before puberty children do not show sex-differences in repolarization [25] probably due to low levels of testosterone [26]. However, further studies are needed to better understand the BrS prevalence in males than female patients.

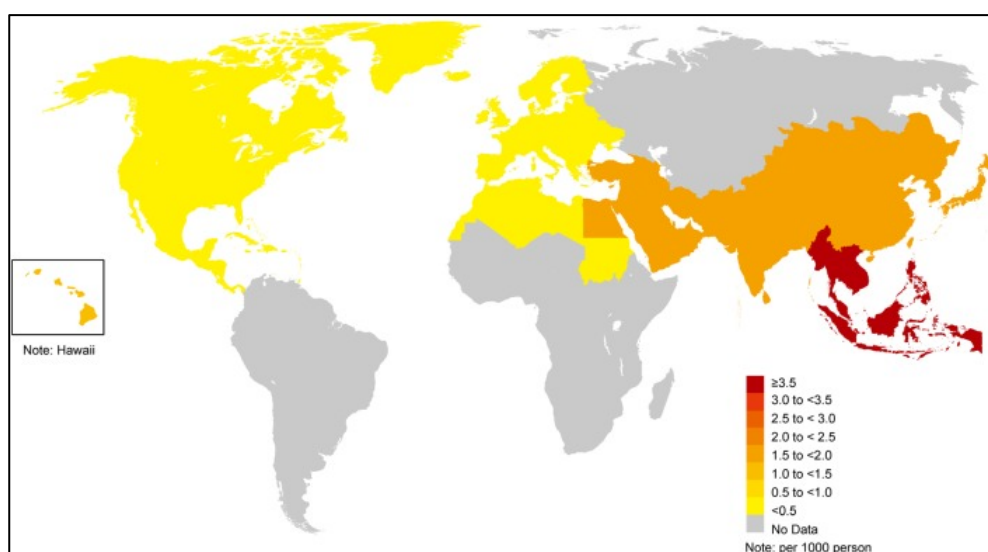


Figure 4. Worldwide map of Brugada syndrome epidemiology [21].

1.4 Therapy

To date, there are three therapeutic options to treat BrS: 1) Implantable cardioverter defibrillator (ICD); 2) drug therapy with specific anti-arrhythmic drugs (i.e. Quinidine); 3) epicardial ablation of BrS arrhythmogenic substrate [27]. It is always very important to evaluate every single patient to better optimize the therapeutic approach.

1- The ICD is recommended in BrS patients documented with ventricular tachycardia (VT) or ventricular fibrillation (VF) and BrS related-symptoms like type 1 ECG and syncope [28]. However, this approach is associated with significant risks related to the device itself since it can induce inappropriate shocks due to T-wave oversensing, sinus tachycardia, and arrhythmias [29].

2- Quinidine therapy, which is an anti-arrhythmic agent, is another approachable to block the transient outward potassium current (I_{to}). Studies showed that quinidine reduced the incidence of induced arrhythmias in BrS patients [30]. However, it has numerous side effects that prevent its prolonged and continuous use [31]. This means that this approach cannot substitute other therapies. It is only used when the patient manifests repeated arrhythmic episodes.

3- In 2011 it has been shown that the arrhythmogenic substrate of BrS was localized in the anterior aspect of the Right Ventricular Outflow Track (RVOT) epicardium therefore, VF episodes could have been prevented by radiofrequency ablation of that area [32]. This area is characterized by abnormal electrical potentials that can induce arrhythmias and, ultimately, cause cardiac arrest. Moreover, it was then demonstrated that epicardial substrate ablation of the BrS RVOT substrate by administering flecainide (2mg/kg 10 minutes) could determine the BrS ECG pattern [33]. In 2017 our group, led by Prof. Pappone, demonstrated also that the pathological substrate, characterized by delayed repolarization, could have been better identified in the presence of ajmaline (1mg/kg 5 minutes) [28].

BrS patients go through epicardial mapping (Figure 5) to identify the pathological substrate localized in the RVOT by measuring the Potential Duration to identify the cells with abnormal values. In particular, the right ventricular (RV) epicardium is mapped before (Figure 5A) and after the administration of the sodium channel blocker Ajmaline (Figure 5B), to completely unmask and detect the abnormal area. Then the normal cardiac conduction is reestablished through a catheter radiofrequency ablation that eliminates all the electrical abnormalities in the RVOT. After the procedure, epicardial mapping in presence of ajmaline is conducted again to confirm the complete elimination of the pathological substrate and type 1 BrS ECG (Figure 5C) [33].

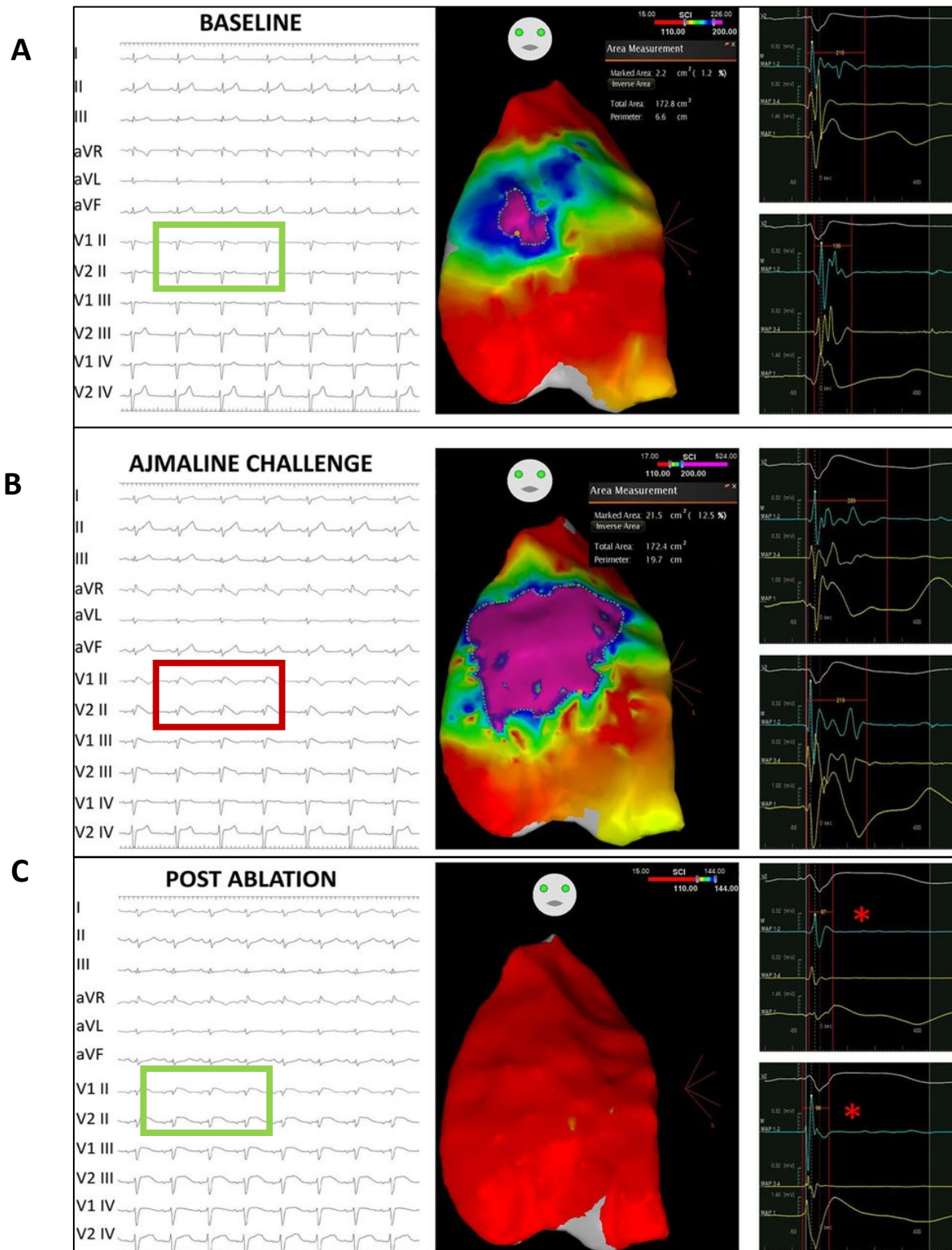


Figure 5. Epicardial substrate ablation [28]. **A.** Epicardial mapping of a baseline BrS- ECG pattern; **B.** BrS-ECG pattern after ajmaline injection; **C.** Epicardial mapping of the substrate after radiofrequency ablation. Red area identifies the area with physiological potentials; green/blue area is the border-zone area and shows a potential with relatively shorter duration; purple area underlies the abnormally prolonged potentials.

1.5 Pathogenic mechanisms

As previously stated, BrS is associated with the arrhythmogenic defects of the epicardium of RVOT and the ST elevation in the right precordial leads of the ECG; however, the causes that lead to all these abnormalities are still unclear. Probably, these mechanisms can be related to cardiac repolarization defects and sodium current reduction [34], [35]. Therefore, in order to explain its pathophysiological mechanisms, two models have been proposed: the repolarization disorder model and the depolarization disorder model.

The repolarization disorder model (Figure 6) considers that BrS is the result of a ventricular repolarization defect/alteration/abnormality. It starts from the assumption that there is a non-homogenous distribution of transient outward potassium current (I_{to}) between the epicardium substrate and the parietal myocardium, resulting in higher I_{to} expression in the epicardium than in the endocardium that generates a transmural voltage gradient. Thus in the presence of a sodium current reduction in the epicardium, a “spike and dome” action potential (AP) morphology which generates a “saddle-Back” on the BrS ECG, is visible (Figure 6B) [35]. In BrS, the T wave is negative because the epicardial AP is longer than in the endocardium (Figure 6C) [35][36]. If there is a more significant reduction of the sodium current, the I_{to} is able to repolarize the membrane beyond the transmural voltage gradients at which the calcium channel is activated; this step brings an epicardial dispersion of repolarization due to a heterogenous loss of AP plateau (Figure 6D). This dispersion causes a phase 2 reentry mechanism which could lead to VT/VF (Figure 6E) [37].

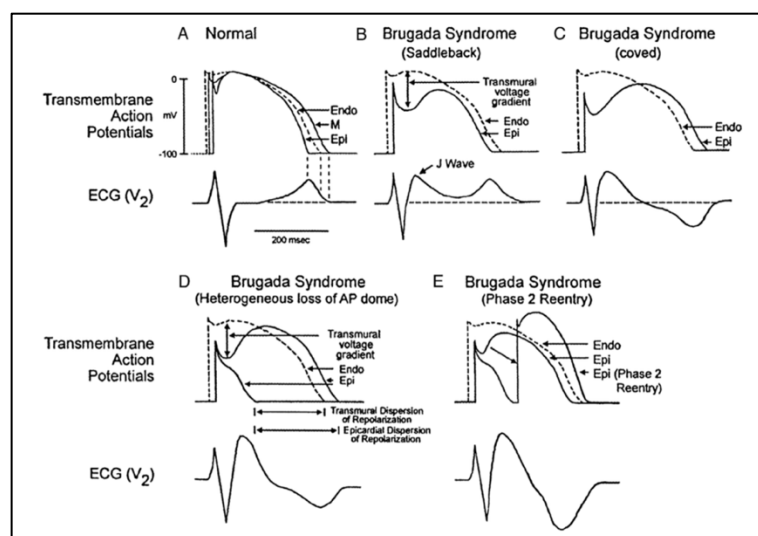


Figure 6. Representation of repolarization disorder model [35]. The BrS ECG pattern is generated by the imbalance between the inward depolarizing current and the outward repolarizing current.

On the other side, the depolarization disorder model (Figure 7) proposes that the peculiar BrS ECG pattern is related to slow conduction of P0 due to the depolarization delay of RVOT. Specifically, the characteristic of this model is that there is a conductive delay of the RVOT AP compared to the RV (Figure 7B). The temporal difference of activation of these two regions of the RV generates a gradient of extracellularly current flux from RVOT to the RV (Figure 7C), resulting in a “closed-loop circuit” and more positive membrane potential in RV compared to RVOT. Thus, the extracellular current flux into the RV causes the ST-elevation (Figure 7D); furthermore, this mechanism generates a more positive membrane potential in the RVOT compared to the RV resulting in a negative T wave (Figure 7F) [35].

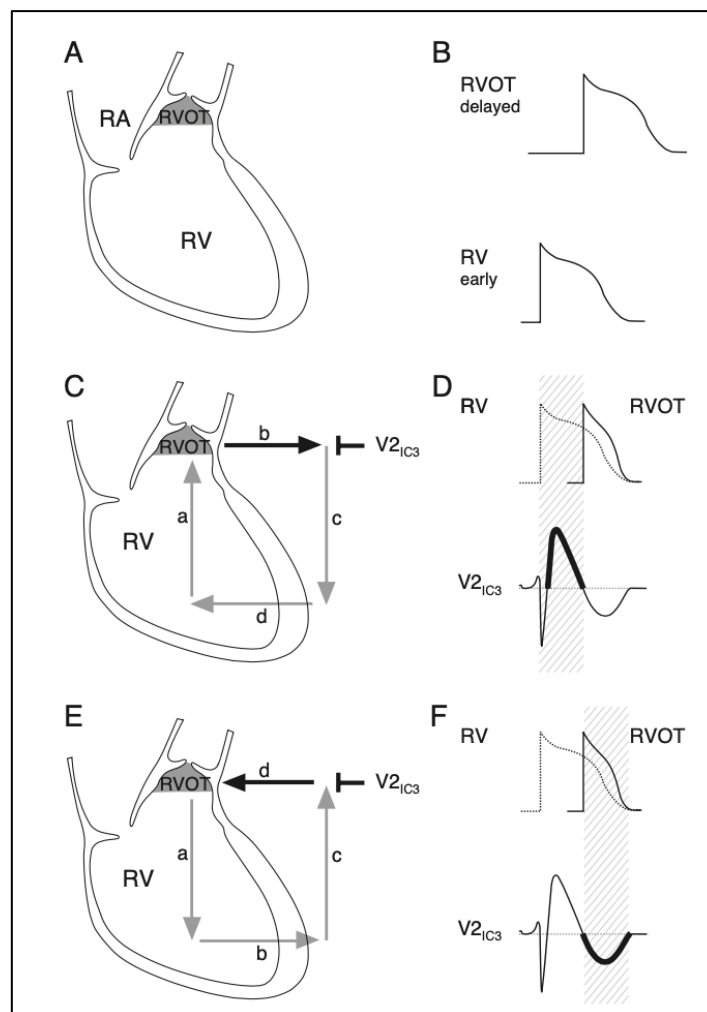


Figure 7. Representation of the depolarization disorder model [35]. The RVOT conduction delay generates current loops which are responsible for the BrS ECG.

However, based on these two divergent model disorders, the pathophysiology of BrS is still uncertain. It is likely that BrS is not a mono-factorial disease, but it is possible to perceive that RVOT can be considered the leading site of arrhythmogenesis [35].

1.6 Genetic factors of Brugada Syndrome

BrS has been considered a genetically heterogeneous channelopathy that could be inherited as an autosomal dominant trait with incomplete penetrance [38]. Since 1998 [39], 15-30 % of BrS cases have been associated with SCN5A mutations [40][41], a gene on the 3p21 chromosomal region with 28 exons which encodes for the alpha subunit of the cardiac voltage-dependent sodium channel NaV1.5 [42]. This channel is important for the maintenance of the normal function of inward and outward sodium current (Figure 8).

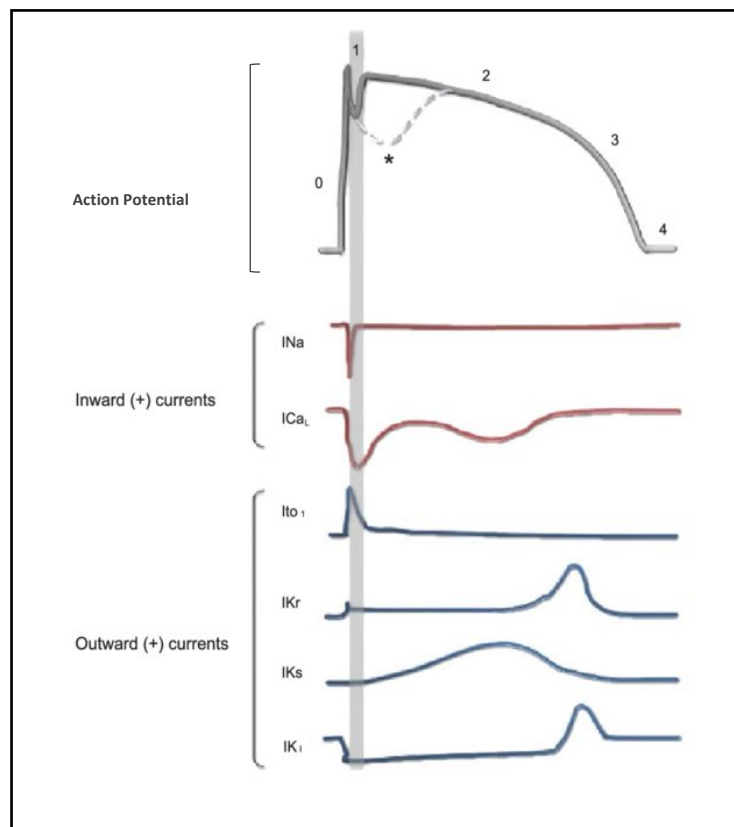


Figure 8. Ionic currents of the cardiac action potential. Inward currents are: I_{Na} and I_{CaL} ; Outward currents are: I_{to1} , I_{Kr} , I_{Ks} , I_{K1} . The grey line shows the critical phase of Brugada Syndrome [43].

The first mutations described were missense and loss-of-function mutations of SCN5A [39], followed by the identification of other variants that may alter the protein synthesis or the activation of the channel, the kinetics, the permeability, or the selectivity of sodium ions. However, it has been demonstrated that there are some mutations that do not affect the channel activity including for example, H558R, R1193Q, R620H, and R1432G mutations that do not negatively interfere with the voltage properties of NaV1.5

[44]. Specifically, H558R is a common polymorphism able to improve sodium channel activity in mutated channels by improving trafficking-deficiency, modulating the biophysical defects, and restoring normal trafficking and current [44][45]. Furthermore, the variant R1193Q transfected into HEK293T cells showed no differences in conductance density, voltage, and time dependence gating [46]. Other examples are Na_v1.5-R620H and Na_v1.5-R1432G mutations which were expressed in CHO-K1 and tsA201 cells, respectively, and by electrophysiological characterization, it was observed no significant effects on peak currents compared to the WT [47], [48]. Indeed, these variants are not considered monogenic factors able to trigger arrhythmic events.

Nevertheless, more than 500 potentially harmful mutations located in 43 genes have been associated with BrS. Beyond the SCN5A gene, four low prevalence genes – SLMAP, SEMA3A, SCNN1A, and SCN2B – have been considered causative of BrS, and 22 additional genes being potentially pathogenic variants [49], [49] (Figure 9), including also potassium and calcium ion channel encoding-genes. In addition to these mutations, the genetic heterogeneity of the syndrome, showing overlapping of genetic factors like short QT syndrome [38] and non-genetic factors like syncope, could interfere with the manifestation of the disease.

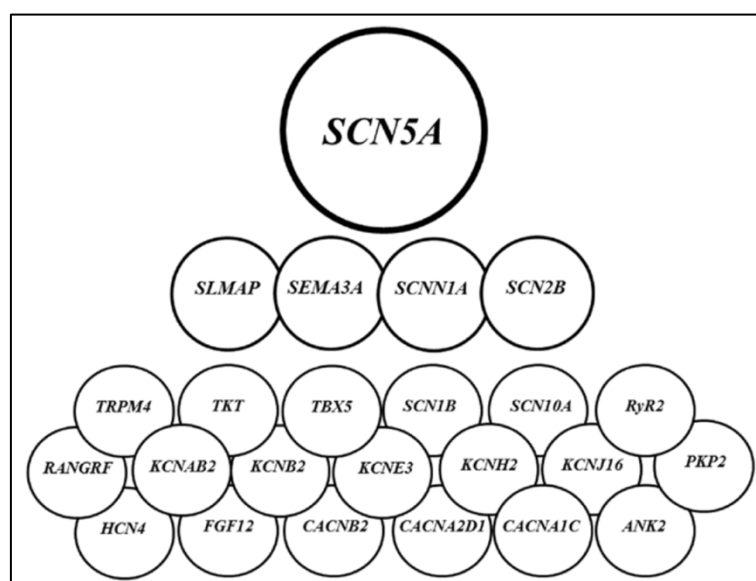


Figure 9. Genes associated to BrS. [49].

1.7 Structure of voltage-gated sodium channel

The voltage-gated sodium channel (Na_v) is constituted by α -subunit of ~260kDa, associated with one or more β subunits of ~40kDa. Nine different α -subunits (NaV1.1-NaV1.9) have been identified. They form

the core of the channel, control the gating, and allow the Na^+ ions to go through the membrane. Furthermore, four β subunits ($\beta 1$ - $\beta 4$) have been characterized and they have been shown to be employed for voltage sensitivity, ions trafficking, expression regulation, gating kinetics, and increase in sodium channel expression at the cell surface [50][51]. The α -subunits (Figure 10) have four repeated homologous domains (DI-DIV), each bearing six α -helical transmembrane segments (S1-S6) [52] wrapped around a central ion-selective pore loop (P-loop) located in the transmembrane region of the protein between the S5 and S6 segments [53], which determine the ion selectivity of the channel. All four domains are connected by intracellular peptide chains with N-terminal and C-end [54]. Moreover, the helical transmembrane segment 4 (S4) of each domain is characterized by a high density of positively charged amino acids residues (Arginine and Cysteine), which include the voltage sensor [55][56]; studies show that this segment moves inside the membrane in response to voltage transmembrane changes, from resting potential -60mV to $+30\text{mV}$, allowing the sodium ion flux to generate sodium current, therefore it is considered the initial point of activation of the channel in response to depolarization [53][57]. The short loop connecting the III and IV domain is characterized by hydrophobic isoleucine-phenylalanine-methionine (IFM) motif, and it holds the role of inactivating gate which is able to block the pore from inside the protein when the membrane is at its depolarization state [57].

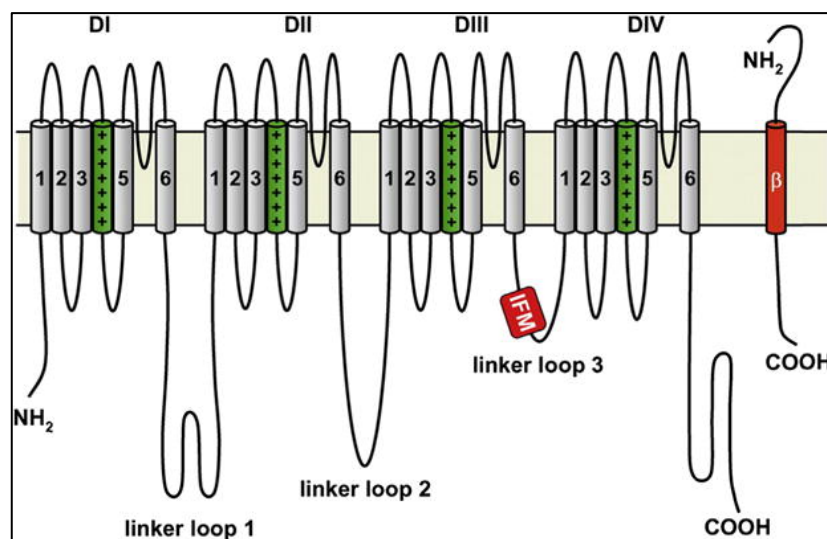


Figure 10. *NaV 1.5 protein structure. NaV1.5 is constituted by four homologous domains (DI-DIV) which constitute the α -subunit associated with β subunit. The transmembrane segments (S1-S6) are represented in grey with the exception of S4 in green. S5 and S6 segments form the pore domain. The short loop between DIII and DIV, the inactivating loop, is shown with its hydrophobic isoleucine, phenylalanine, and methionine (IFM) sequence. N-terminal and C-terminal of α and β subunits are labeled.*

Only four genes constitute the NaV1.5 β subunits (SCN1B-SCN4B) [58]. These subunits have all in common a predicted protein topology: one extracellular N-terminal Immunoglobulin (Ig) domain, one intracellular C-terminal domain, and one transmembrane segment devoted to connecting the N-domain and the C-terminus[59][60].

To date, the structure of the human NaV1.5 has not been experimentally defined yet. However, the human NaV1.4- β 1 complex structure is available [61], thus the 3D structure of NaV1.5 can be computed by predictive software (i.e. Swiss Pro) (Figure 11A). Moreover, recently the rat NaV1.5 has been structurally defined by cryogenic electron microscopy (Cryo-EM) with a resolution of 3.2-3.5 Å [62]. The high-quality electron density map granted the ability to create the atomic model of the α -subunit of the channel (Figure 11B). Thus, considering that the human and the rat NaV1.5 shows 94% of protein homology, the rat NaV1.5 structure could be considered a reliable model also for the human protein.

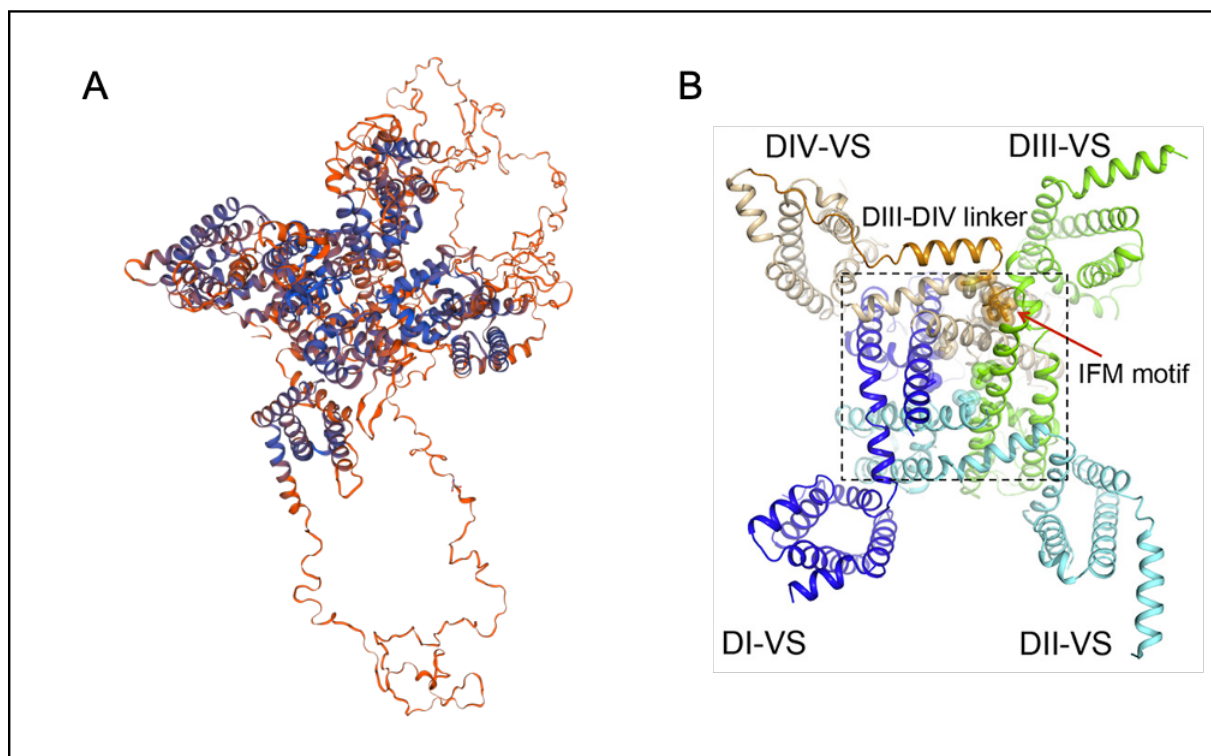


Figure 11. **A.** 3D model of hNaV1.5 [61]. **B.** Cryo-EM structure of rNaV1.5c [62].

1.8 Cardiac voltage-gated sodium channel: selectivity, activation, inactivation, and sodium current

Voltage-dependent activation of sodium channels was firstly described by Hodgkin and Huxley, studying the squid giant axon [63]. These studies brought attention to the voltage-dependent biophysical properties of sodium channel activation which relies on the changes of membrane potential due to the movement of electrical charges. NaV1.5 channels are in closed state when the transmembrane potential is about -85mV (diastole); once, the membrane depolarizes they open in only 1 millisecond (ms), allowing the sodium to enter in the cell [64]. This mechanism is mediated by the S4 segment which is characterized by repeated positively charged motifs followed by two hydrophobic residues, which creates gating charges forming ion pairs with negatively charges residues and allows the inward conductance of the ions [64][65]. Therefore, in the cardiac tissue, the sodium channel is responsible for inducing the rising phase of the AP- Phase 0 (P0) (Figure 12), which is the fast depolarization of the membrane. Right after depolarization, NaV1.5 becomes inactivated after the opening state due to a fast repolarization of the membrane (fast inactivation) [56]. Meanwhile, the IFM motif has been demonstrated to play a crucial role during the fast inactivation due to the hydrophobic interactions between the inactivation gate and both of the transmembrane S6 of domain IV and the intracellular loop which links the S4 and S5 of domain IV [56][66][67].

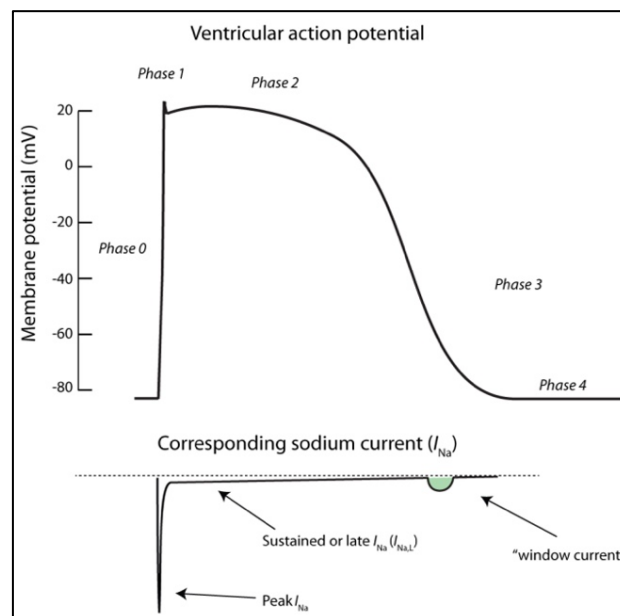


Figure 12. Ventricular action potential and its corresponding sodium current [63].

The activation and the inactivation state of NaV1.5 channels, which are voltage-dependent, are shown in Figure 13. Under normal conditions, the outward movement of the S4 segment in response to membrane depolarization brings the sodium channel to the open state (activation) within a millisecond. This mechanism leads to the initiation of the action potential [68]. The channel remains inactivated (closed state) until the repolarization of the membrane; during this time, NaV1.5 goes through different conformational changes that affect the myocardium action potential duration and frequency: fast, intermediate, and slow inactivated state [69][70][71]. Studies show that during the action potential, sodium channels reach the complete inactivated state only after 60 seconds [69]; the intermediate state is reached after ~50ms and the slow-inactivation happens after 5 seconds. The binding between the cytoplasmatic loop region that links DIII and DIV of the alpha-subunit and the hydrophobic core takes place during the fast inactivation of the NaV1.5 channel and it is considered a time and voltage-dependent step [72][73]. Slow inactivation occurs due to repeated depolarization of the channel [74], and it is independent by the fast inactivation mechanism [70]. However, all these inactivation mechanisms are not fully understood yet.

During the AP, a small fraction of sodium current can still be recorded. This continuous current linked with the slow inactivated state has been called “*persistent sodium current*” or “*late current*” [75][76][77]. This current is the result of a continuous opening and closing of the channel during depolarization and it could play a crucial role in genetic diseases [75].

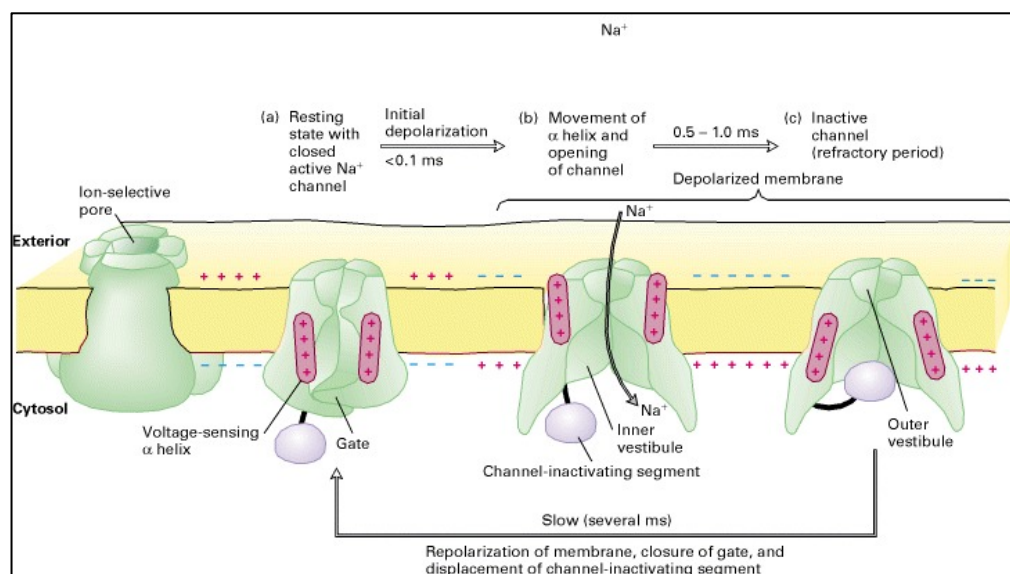


Figure 13. Function of the voltage-gated sodium channel NaV1.5.

Lodish H, Berk A, Zipursky SL, et al., *Molecular Cell Biology*. 4th edition, New York: W. H. Freeman; 2000. Section 21.2, The Action Potential and Conduction of Electric Impulses.

1.9 SCN5A mutation in BrS and other cardiac disorders

As stated before, SCN5A mutations are the most common mutations in BrS. Indeed, the first reported mutations linked to BrS were discovered in the SCN5A gene in 1998 [39]. To date, 485 variants have been associated with BrS phenotype, including missense, nonsense mutations, insertion/deletion, and splice site mutations [78]. Specifically, based on the 2015 American College of Medical Genetics and Genomics and the Association of Molecular Pathology (ACMG-AMP) guideline, 76% of 485 were coding variants and 24% of the coding variants were “null variants” [78]. Furthermore, 37% of the coding mutations were classified as pathogenic and likely pathogenic, 3% as benign or likely benign, and the remaining 60% were classified as “variant of unknown significance” (VUS) (Figure 14) [78]. The mutations are distributed on the entire sequence of NaV1.5, thus it is not possible to define a common pathogenic mechanism.

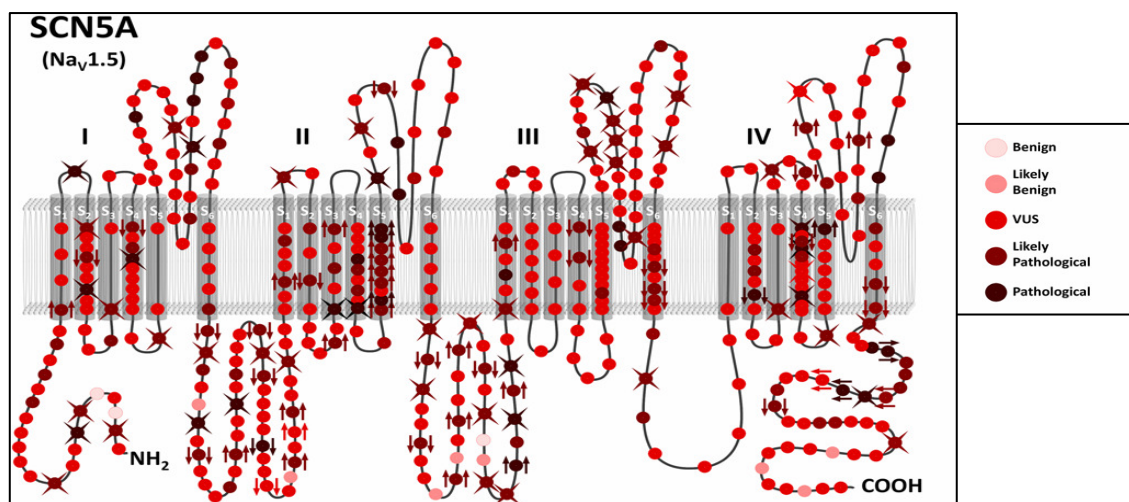


Figure 14. Location of SCN5A mutations. Each circle corresponds to a mutation at a specific region of the gene and the color is correlated to the severity of pathogenicity. Premature truncation of the protein is represented with crossed circles while frameshift mutations are represented with flanked circles. Variants with unknown significant are labeled as VUS [78].

The relationship between genotype and SCN5A channel dysfunction can sometimes be very difficult to be defined due to different disease manifestations among patients [79][80]. Functional studies of SCN5A mutations demonstrated that the variants interfering with the open reading frame (ORF) or the splice site can cause different phenotypes [42]. For example, mutations that cause premature truncation of the proteins or a reduction of the sodium flux >90%, induce a more severe phenotype [81]. Moreover,

the phenotype of NaV1.5 variants could also be modified during mRNA processing. Specifically, it has been shown that angiotensin II and hypoxia are able to interfere with SCN5A mRNA splicing and cause a truncated version of sodium channel in human heart [82]. Furthermore, the R1232W variant resulted in a small shift in channel availability but it was described as a benign polymorphism [39], and the T1620M variant was reported to change the gating properties of voltage-gated sodium channels [83], but no sodium current was observed with the co-expression of the two variants [84], indicating the detrimental effect of the combination of the two mutations. Whereas, variants like H558R could positively influence the phenotype associated with other variants and restore defective sodium channel functions of specific mutations like R282H [85]. Specifically, the mutation R282H expressed in heterologous cells showed no sodium current while when it was co-expressed with the H558R polymorphism, it showed a significant increase of the sodium current demonstrating the ability of the polymorphism to rescue the mutation [86].

It has been demonstrated that SCN5A mutations are related to other cardiac diseases, not only to BrS and that these variants can cause a loss-of-function or a gain-of-function of the sodium channel [87]. These diseases comprehend other arrhythmic syndromes and structural cardiac disorders [87].

The congenital long-QT syndrome type 3 (LQT3) is the third of the 15 forms of Long-QT syndrome (LQTS), It is an inherited autosomal ventricular arrhythmia characterized by prolonged QT/QTc interval with a QT dispersion, prolonged ST segment, late onset of T wave and a frequent prominent U wave caused by a variant of SCN5A [87]. Nowadays, more than 300 SCN5A mutations are linked to LQT3 and they are located in the S4 segment and C-terminal region and the intracellular junction between DIII and DIV [87]. These mutations can influence gating kinetics [41] and it has been shown that gain-of-function mutations in the SCN5A gene are responsible of 10% of LQT3 patients [87].

Atrial arrhythmias, in particular familial atrial standstill (AS) and familial atrial fibrillation (AF), are other disorders associated with SCN5A mutations [88]. Atrial standstill is characterized by the absence of electrical and mechanical activity in the atria of the heart. The diagnosis of this arrhythmogenic condition depends on bradycardia, no P waves, and escape rhythm [89]. Missense- and frameshift-SCN5A mutations studies showed that they can induce gain-of-function in sodium channel and electrophysiological disorders [90], [91]. Atrial fibrillation is characterized by an abnormal rhythm in the atria of the heart [88]. To date, there are 32 genes associated with AF and both loss-of-function and gain-of-function mutations of SCN5A have been associated with it [88], [92], [93].

Sick sinus syndrome (SSS), known also as sinus node disease, is an arrhythmic disorder where the natural heart's pace marker does not work properly due to a dysfunction of the sinus node [94]. Based on ClinVar

2018, 128 SCN5A variants have been associated with SSS and they can induce both loss-of-function or gain-of-function of the channel [87]. In the first case, it is possible to observe a reduction of electric coupling between the sinus node and the atrial cells; otherwise, in the gain-of-function these mutations can induce a persistent sodium current in the systolic phase which brings an increase in AP duration, reducing the sinus rate [95], [96].

In addition, evidence underlined that some mutations of NaV1.5 can be associated not only to arrhythmic disorders but also with the development of structural abnormalities, as dilatated cardiomyopathy (DCM) [97]–[99], a disease characterized by a dilatation of the ventricular chambers and decreased myocardial contractility and systolic functions [100]. For example, the mutation SCN5A-R219H found in DCM patient, causes a selective proton leak current, suggesting that the acidification of the cardiac myocytes might cause both the electrical problem and the DCM phenotype [101].

However, as stated before, mutations in NaV1.5 channel can show different clinical syndromes. Therefore, it has been proposed that this different phenotypic manifestation can be due to secondary modifiers of NaV1.5 channel, which could be considered additional genetic variants of the gene [102]. Specifically, these modifiers have been divided in two major groups: 1. Modifiers intrinsic to the lifecycle of NaV1.5, which could interfere with the expression or functionality of the mutated channel; 2. Modifiers extrinsic to NaV1.5, which could interfere with the expression of other genes by modifying the overall electrical activity of the mutated SCN5A gene. These modifiers can mitigate or aggravate the phenotype manifestation, based on their effect of the sodium current or the overall cell currents [102].

1.10 Risk stratification

Risk stratification in BrS is still a clinical challenge that needs to be addressed to improve BrS patients' management. Indeed, only spontaneous type I ECG pattern and/or aborted sudden cardiac arrest were used as the most reliable indicators of future arrhythmic events, as reported in most of the studies [8, 9]. However, more recently, other factors have been considered for risk stratification, *e.g.* age, gender, SCD familiarity, ECG variables, and SCN5A mutations [103][104]. For example, the analysis of daily fluctuations in ECG and signal-average electrocardiogram (SAECG) features could be helpful to predict high/low risk BrS patients [85]. Similarly, it has been shown that the combination of a history of syncope episodes, BrS ECG type 1, and VF during programmed electrical stimulation (PES) could be significant risk factors for BrS [86]. Furthermore, other ECG variables has been proposed as possible predictor

factors by Kawazoe et al., as he found correlations in multivariate logistic regression analysis for VF in BrS patients risk stratification using the following parameters: history of syncope, r-J interval V1, QRS (which is a series of deflections in an ECG) duration is V6, and T-peak-T-end dispersion (which is the “*difference between the maximum and the minimum T-peak-T-end interval in the V1-V6 precordial leads*” [87]) [88]. Finally, also SCN5A mutations could determine the BrS phenotype, thus representing a risk factor for the development of arrhythmic events. For example, experimental studies on NaV1.5-R1193Q, which is a common polymorphism linked to an increased risk of arrhythmic events [105, pag. 5], showed no significant sodium currents abnormalities between R1193Q and WT baseline [106]. However, this polymorphism is considered a genetic marker of cardiac conduction defects and it has been found in patients with type 1 ECG pattern, and various SCN5A-associated channelopathies [105, pag. 5], [107]. Thus, the relationship between genotype and phenotype is still a challenge due to different manifestations of the symptoms in patients that needs to be addressed in order to determine a new reliable variable for the risk stratification in BrS.

1.11 Calcium and Potassium Voltage Gated Channels

Among genes related to BrS, there are also those encoding for calcium and potassium voltage-gated channels. In particular, mutations in CACNA1C, CACNB2b, encoding the $\alpha 1$ - and β - subunits of Cav1.2 or L-type calcium channel (LTCC), respectively [108] and in KCND3, encoding the Kv4.3 potassium channel were described [109]. Voltage-gated L-type calcium (Ca^{2+}) channels (LTCC) (Figure 15), are transmembrane proteins that regulate Ca^{2+} influx in response to changes in membrane potential during depolarization. The intracellular Ca^{2+} concentration is tightly regulated. Most of them are constituted by five-subunits with a central pore-forming $\alpha 1$ subunit, a glycoprotein $\alpha 2$ and δ , a transmembrane γ subunits, and an intracellular β subunit [110][111]. The amino acid sequence of the $\alpha 1$ subunit is characterized by four repeated domains (I-IV), containing six transmembrane segments (S1-S6) each, and a single loop between transmembrane segment S5 and S6 [112]. The β and the γ subunits are constituted by α helices and four transmembrane segments, respectively. The $\alpha 2$ subunit is attached to the membrane by disulfide bonds to the δ subunit [113] and determines the principal biophysical properties of the channel [108]. The $\alpha 2$, δ , and β subunits are responsible for the acceleration of the activation and deactivation of the channel [114]. The mechanism of the γ subunit has not been sufficiently studied.

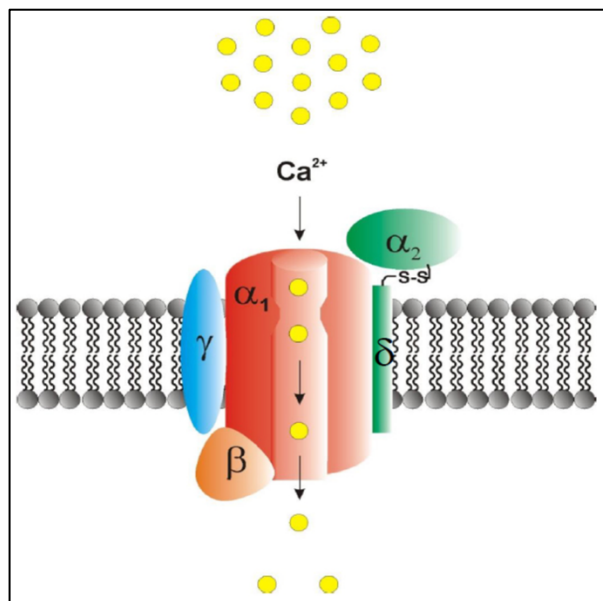


Figure 15. Voltage-gated calcium channel structure [115].

BrS-related genes causing a reduction of calcium inward current were also found among calcium channels. Specifically, loss-of-function missense mutations of CACNA1C, CACNB2b, encoding Cav1.2 or the regulatory subunits $\beta 2$ and $\alpha 1c$ have been discovered to affect the channel kinetics.

For example, three missense mutations Cav1.2- CACNA1C- G3343A, Cav1.2- CACNA1C - G5639A, Cav1.2- CACNA1C- G6388A has been shown to regulate the calcium current density and activation and inactivation kinetics of the calcium channel [116][117].

Voltage-gated potassium (K_V) channel plays a key role during the generation and propagation of the AP. They are highly selective channels and they are evolutionarily conserved [118]. The K_V family can be divided into different subfamilies. Specifically, K_V 1, K_V 2, K_V 3, and K_V 4 are constituted by four pore-forming α subunits and β subunits (Figure 16). The pore-forming α subunit contains six transmembrane segments (S1-S6) that are connected by loop regions. The first four segments constitute the voltage sensor while the last two segments are the pore domain [119]. Moreover, the voltage sensor and the pore domain are linked by S4-S5 loop which contributes to the ion-selective pore of the channel [120]. The repolarization mechanism is principally driven by outward potassium current through K_V , therefore, the principal role of the channel is to regulate the initial phase of repolarization of the AP [121]. Indeed, the regulatory subunits and gain-of-function mutations of different genes encoding potassium channels have been linked with BrS [122]. For example, the heterozygous mutations KCND3-G1292A [123],

KCND3-L450F, and KCND3-G600R [124] resulted in a gain-of-function phenotype by increasing the current density of the channel.

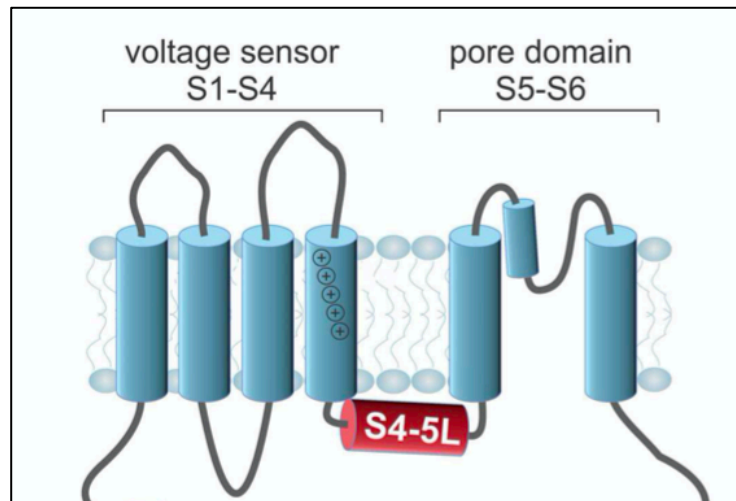


Figure 16. Voltage-gated potassium channel structure [125].

Therefore, at the cardiac level, calcium and potassium voltage-gated channels are important for the correct generation and propagation of the AP. However, they are expressed in many other tissues, including peripheral blood mononuclear cells (PBMCs) [126][127][128][129] and it has been demonstrated that they could control different cellular aspects, including proliferation and differentiation, gene transcription, cytokine secretion, migration, and so forth [127], [130]. For example, the membrane potential and the calcium influx during activation of B and T lymphocytes are mainly regulated by two potassium channels: Kv1.3 and calcium-activated $K_{CA}3.1$ channels [131]. Moreover, ion channels may interfere with the cell microenvironment by contributing to self-renewal capacity, arrest cellular differentiation, and enhance cellular motility [132]. Furthermore, P2X receptors, which are non-selective ion channels activated by ATP and responsible for sodium and calcium influx, regulate IL-2 production and proliferation of T and B cells [133]. CRAC channels in T lymphocytes play a fundamental role during “autoimmune inflammation, allotransplant rejection, and T-cell mediated hypersensitivity” [133].

The Ca^{2+} flux into cells plays a fundamental role in secretion, cell motility, and gene transcription. Specifically, Ca^{2+} plays a critical role in immune cell regulation [134]. Indeed, several studies

characterized the “*biophysical and regulatory aspects of lymphocytes calcium and potassium channels*” [135]. For example, the electrophysiology characterization of CD4⁺CD28^{null} T cells revealed an increase of the potassium current during the acute coronary syndrome, due to an increase in the Kv1.3 channel expression, thus probably contributing to the disease progression [136].

1.12 PBMCs and Cardiovascular diseases

Biomarkers could help revealing the risk or the presence of cardiac pathologies and overall improve the evaluation and the management of patients. Moreover, tissue collection for diagnostic and prognostic analysis of cardiovascular diseases is not feasible like other disease entities due to practical limitations of the procedure [137] such as bleeding, blood clots, aggravation of an existing arrhythmia and possible puncture of the heart or lungs [138]. Therefore, a series of studies aimed to identify new easily accessible tissues for biomarker discovery and mechanistic studies in cardiovascular disease [139]. Among the different cells and tissues tested, PBMCs have been identified as a barely invasive reliable tool for specific markers for cardiovascular diseases [140]. Indeed, PBMCs represent an attractive source for biological tests due to their accessibility and straightforward preparation. Furthermore, they are composed of several circulating cell types such as peripheral blood lymphocytes, granulocytes, monocytes, platelets, and circulating stem cells associated with cardiac disease processes [141].

During the last decades, abnormalities gene and protein expression of PBMCs have been associated with cardiovascular diseases. For example, reactive oxygen species (ROS) and mitochondrial dysfunction in PBMCs have been associated with heart failure in patients with single ventricle congenital heart disease (SV) [137]. Moreover, a microarray assay was able to identify three potentially prognostic biomarkers related to the development of heart failure (HF) and acute myocardial infarction (AMI) [142]. Furthermore, a gene expression signature in PBMCs has been related to thoracic aortic aneurism (TAA). This disease is usually associated with high mortality and difficulties in early detection [143]. In addition, type-1 ryanodine receptors (RyR1) and the decrease of the endoplasmic-reticulum calcium stores in B lymphocyte cells have been identified as possible biomarkers to supervise the evolution of the disease and the therapeutic response in congestive heart failure (CHF) [144]. Furthermore, the intracellular alteration of SENCER (Smooth muscle and endothelial cell enriched migration/differentiation-associated long non-coding RNA), CD31, CD146, CD45, and CD14 in mononuclear cell fractions was linked to coronary artery disease [145]. Moreover, the up-regulation of SOCS3 and FAM20 genes in PBMCs have

been linked to myocardial infarction [146]. The ongoing discovery of genomic information in PBMCs may be a new approach for further interesting therapeutic developments in cardiovascular diseases.

Aim of the study

BrS is an inherited arrhythmic disease whose the exact pathogenetic mechanisms are still unclear and under debate [147]. It has been established that the functionality of the ion channels, and especially of the cardiac sodium channel, plays a fundamental role in the disease onset [148]. The correct flow of ions inside the cell in the heart, regulated by the channels, is essential for the correct generation and propagation of the action potential and, ultimately, of cardiac contraction. The prominent role of channels in BrS is demonstrated by the fact that mutations in their coding genes have been found in BrS patients and, in particular, SCN5A mutations account for about 20% of total BrS cases [18]. Furthermore, up to now, the principle of the unique BrS diagnostic test is based on the correct functionality of the cardiac sodium channel NaV1.5 [33]. In fact, the use of a sodium channel blocking agent, i.e. ajmaline, can unmask the presence of the ECG pattern typical of BrS, when the cardiac sodium channel functionality is altered. However, this test presents risks in the execution linked to the possible development of ventricular fibrillation, and for this reason, it is carried out only in specialized centers. This represents an obstacle to a large-scale screening of the population, thus making it necessary to identify new circulating biomarkers, even in different organs than the heart, which may be the basis for the development of new diagnostic tests. Furthermore, channel mutations are also linked to the different manifestations of the disease. In fact, the various SCN5A mutations, in addition to having altered effects on the functionality of the NaV1.5 channel, can also translate into different clinical phenotypes that are associated with different degrees of severity of the disease. This aspect is very important as it suggests that being able to identify the effects of SCN5A mutations on NaV1.5 activity could also be helpful in clinical practice, providing a new parameter for risk stratification, a process that presents critical points and it is still not well defined yet.

Based on these premises, the objective of the thesis was to develop new functional assays for the study of the activity of sodium, calcium, and potassium ion channels in an High-throughput screening (HTS) manner. In particular, the functional test for the sodium channel, developed in an *in vitro* model through the overexpression of the wild type or mutated NaV1.5 channel in HEK293A cells, aimed to characterize the effects of the mutations on the functionality of the NaV1.5 channel. Therefore, this test could provide a new parameter to be integrated into the risk stratification process to improve the management of the BrS patient.

On the other hand, functional tests for calcium and potassium channels have been developed on circulating cells (PBMCs and T lymphocytes) to identify cellular alterations in easily accessible cells that

may represent candidates for new BrS biomarkers discovery. It has been shown that blood cells can present cellular alterations useful both for the definition of any biomarkers and for the identification of new pathogenetic processes, cardiac pathologies included [139]. Therefore, the information obtained from this analysis could be useful both for the development of a possible diagnostic test and for the elucidation of the etiology of BrS which at the moment remains to be fully defined.

Chapter 2

Materials and Methods

2.1 Cell culture

HEK293A cells were obtained from Sigma-Aldrich and maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM, Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO₂, 95% air-humidified atmosphere.

2.2 PBMCs isolation and T Lymphocytes activation

Peripheral mononuclear cells (PBMCs) were obtained from patients who underwent ablation procedure or ajmaline-diagnostic test at the Arrhythmology Department of the IRCCS Policlinico San Donato. All patients, who respected the inclusion and exclusion criteria, gave their informed consent before being enrolled in the study, which was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Ethics Committee.

PBMCs were isolated by density-gradient centrifugation using the Ficoll-Hypaque technique (Amersham Biosciences; Buckinghamshire, UK). They were cultured in RPMI-1640 medium (Sigma-Aldrich) with 2mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin at 37°C in 5% CO₂, 95% air-humidified atmosphere.

Non-manipulated peripheral blood T cells were activated from the PBMCs, as previously described by Katz et al. [149]. Briefly, 2×10^6 PBMCs were collected in a 6-well plate in complete RPMI-1640 medium. T cells were then activated adding directly to the well 1 µg/ml of anti-CD3 and anti-CD28 antibodies (1:1,000 dilution from 1 mg/ml stock). Cells were then incubated in at 37°C in 5% CO₂ for 3 days. At 72 hours post activation, cells were washed twice with PBS, and recombinant human IL-2 was added to the

cells at a final concentration of 100 IU/ml. Cells were allowed to grow for 7 days. The cells' properties have been assessed by evaluating the expression of the CD19 marker through flow cytometry.

2.3 NaV1.5 vector cloning

RNA was extracted from a non-mutated SCN5A BrS patients with ReliaPrep RNA Cell Miniprep System (Promega) and then retro transcribed with iScript synthesis kit (Biorad). Oligonucleotide primers were designed using the known sequence of human SCN5A (GenBank: NM_198056) and with M13 tail-sequences. Real-Time PCR was performed with 1ng of cDNA template, 0.2 μ m primers, and 1 $\times$ GoTaq[®] qPCR Master Mix (Promega) in 20 μ l of the final volume, using a StepOnePlus[®] Real-Time PCR system (Applied Biosystem). The SCN5A vector was amplified with an initial 2 min at 95 °C, followed by 40 cycles of 95 °C for 5 seconds, 30 seconds at 57 °C, 30 seconds at 72 °C, and a final stage at 72 °C for 2 minutes. Moreover, the purified PCR product and the pcDNA3.1 vector was digested by the HindIII and XbaI restriction enzymes (Fermentas, UK). The reaction mixture contained 20 μ l of hNav1.5 DNA (25 ng/ μ l), 5 μ l H buffer, 2 μ l HindIII (10 u/ μ l), 2 μ l XbaI (10 u/ μ l), and 31 μ l nuclease-free distill water. The digested product was run on the gel and purified by gel extraction kit (Promega). Furthermore, in order to insert the hNav1.5 gene into pcDNA3.1 vector, ligation was performed with T4 DNA ligase (Promega). The ligation reaction included: 6 μ l (100 ng/ μ l) of pcDNA3.1, 12 μ l of hNav1.5, 2.5 μ l of T4 DNA ligase buffer, 2 μ l of 5 u/ μ l T4 DNA ligase, 2 μ l of polyethylene glycol, (PEG 4000), and 0.5 μ l of distilled water.

Then, One Shot TOP10 chemically competent *E. coli* cells were transformed with the ligated plasmid. Briefly, one vial of One Shot[®] competent *E. coli* cells was thawed and 5 μ l of the ligation reaction was added into the competent cells; the vial was incubated in ice for 30 min, in a 42°C water bath for 30 seconds, and placed again on ice; 250 μ l of super optimal broth with catabolite repression (SOC media) was added and then the vial was incubated at 37°C for 1 hour at 225 rpm in a shaking incubator; 200 μ l of the transformation mixture was spread on LB agar plate at incubated at 37°C overnight; finally, an isolated colony was analyzed. We purified the plasmid and the cloning was confirmed by Sanger sequencing (Figure 23).

2.4 Site-directed SCN5A mutagenesis

PCR-based method was employed to generate the following mutant constructs from the SCN5A plasmid: SCN5A 86_87delinsTG, SCN5A 5089 T>C, SCN5A 3214_3215delGainsTC, and SCN5A 1045G>A. They were generated using the QuickChange Lightning Site-directed Mutagenesis Kit (Agilent), according to the manufacturer's protocol. The primers used are listed in Table 1. The reaction mixture was composed by 5 µl of 10X reaction buffer, 100 ng of DNA template, 125 ng of forward primer, 125 ng of reverse primer, 1 µl of dNTP mix, 1.5 µl of QuickSolution reagent, and 1 µl of QuickChange Lightning enzyme in a final volume of 50 µl. The reaction was initiated with a denaturing step at 95°C for 30 seconds, followed by PCR amplification with denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, extension at 68°C for 6 minutes for a total of 18 cycles. Furthermore, the reaction was placed on ice for 10 minutes to cool down and then 1 µl of Dpn I was added directly to the amplification reaction and incubated for 5 minutes 37°C to digest the parental supercoiled dsDNA. The Dpn I is a restriction endonuclease enzyme specific for methylated and hemimethylated DNA that is able to digest the parental DNA template, thus selecting the DNA that contains the mutation.

The amplification product was then transformed into XL-10 Gold ultracompetent cells. Briefly, 2 µl of Dpn I-treated DNA from each reaction was transferred to separate aliquots of the ultracompetent cells XL10-Gold. After an incubation of 10 minutes in ice, cells were heat-pulsed in 42°C water bath for 30 seconds. Then, 500 µl of preheated SOC media was added to each reaction which was incubated at 37°C for 1 hour with shaking 225 rpm. Finally, each transformation reaction was plated on LB agar plates containing 100 µg/mL ampicillin antibiotic and incubated at 37°C overnight.

The following day we continued with the preparation of starter culture following the NucleoBond® XtraMaxi protocol (Macherey-Nagel, Germany). We inoculated 300 µl of starter culture (1/1000) of LB media and 100 µg/ml ampicillin antibiotic with a single transformed bacterial colony picked from the LB agar plate and we shacked it at 37°C at 250 rpm for 16h. The following day we harvest the bacterial cells at 4,500 x g for 15 min at 4°C and we discarded the supernatant completely. We resuspended the bacterial cells in 12ml of Resuspension Buffer + RNase A, we added 12ml of cell lysis buffer and we incubated it at RT for 5min. In the meantime, we equilibrated the NucleoBond® Xtra Column Filter with 25ml of EQU Buffer. Then, we added 12ml of neutralization buffer to the suspension, we immediately mixed the lysate and we applied it into the equilibrated NucleoBond® Xtra Column Filter. We allowed the column to empty by gravity flow and then we performed the first wash of the column filter and filter with 15ml of EQU buffer and a second wash of the column only with 25ml of Wash Buffer. Then we elute

the plasmid DNA with 15ml Elution Buffer (ELU) and we collected the elute in a 50ml centrifuge tube. We let precipitated the eluted plasmid DNA with room-temperature isopropanol at 4,500 x g for 20 min at RT. We then discarded the supernatant and we washed the pellet with room-temperature 70% ethanol. We centrifuged the pellet at 4,500 x g for 5 min at RT. Finally, we dissolved the DNA pellet in 1ml of ddH_2O . The DNA purity and concentration were analyzed through Nanodrop 2000®.

Variant	Forward	Reverse
SCN5A 5089 T>C	5'-GTTCAACTTCAGACCTCCGCCAACAGCATG-3'	5'-CATGCTGTTGGCGGAGGTCTGGAAGTTGAAC-3'
SCN5A 86_87delinsTG	5'-CGAGAAGCGCATGGTGGAGAAGCAAGCCCG-3'	5'-CGGGCTTGCTTCTCCACCATGCGCTTCTCG-3'
SCN5A 3214_3215delinsTC	5'-GGGCACGGAGGAGTCGTCCAGCAAGCAG-3'	5'-CTGCTTGCTGGACGACTCCTCCGTGCCC-3'
SCN5A 1045G>A	5'-CAGGCGAGAACCCCAACCACGGCTACACC-3'	5'-GGTGTAGCCGTGGTTGGGGTTCTCGCCTG-3'

Table 1. Mutagenesis primers for SCN5A mutations. These cDNA fragment containing the mutation were fully sequenced in both forward and reverse directions.

2.5 Cell culture transfection

At day 0, 120,000 HEK293A cells were plated to reach 70-80% of confluency in a 12-well plate at day 1. After 24 hours, for the homozygous experiments, we transfected the cells with 500 ng of wild-type and mutated SCN5A vectors individually, while for the heterozygous experiments we co-transfected 250 ng of wild-type and mutated SCN5A vectors together, both with ViaFect™ Transfection Reagent (Promega), following manufacturer's instruction. After 24 hours, cells were replated at a density of 10,000 cells per well on black-walled 96-well imaging plates and cultured for other 24 hours.

2.6 Real Time qPCR

Cells were collected 24 hours after transfection and RNA was extracted with ReliaPrep™ RNA Cell Miniprep System (Promega) following the manufacturer's protocol. Then, 1 µg of RNA was reverse

transcribed into cDNA by iScript cDNA synthesis kit (BIO-RAD), according to manufacturer's instructions. The reaction mixture was composed by 4 µl of 5X iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase, and 1 µg of RNA, up to a total volume of 20µl.

Real Time qPCR analysis was carried out using GoTaq® qPCR Master Mix (Promega) on a StepOnePlus® Real-Time PCR instrument (Applied Biosystem). The reaction system contained 10 ng of cDNA template, 1X GoTaq® qPCR Master Mix (Promega), 0.2 µM of forward primer, and 0.2 µM of reverse primer, in 20 µl of total volume. The amplification protocol was: 95°C for 2 min, 40 cycles of 5 seconds each at 95°C, 30 seconds at 57°C and 30 seconds at 72°C, and a final stage at 72°C for 2 min. The primers for RT-PCR analyses are listed in Table 2. The PCR products were verified by melting curve analysis and the results were calculated by the equation $2^{-\Delta\Delta C_t}$ using the human UBC gene as a housekeeper. Each RT-qPCR assay was performed in triplicate and repeated at least three times.

Gene	Forward	Reverse
SCN5A	5'-CGTGTGTAGATGGCTTCGAG-3'	5'-GAGGTCAGTAAACGGGTCCA-3'
UBC	5'-CTGGAAGATGGTCGTACCCTG-3'	5'-GGTCTTGCCAGTGAGTGTCT-3'

Table 2. qPCR primers sequences.

2.8 Immunofluorescence staining

HEK293A cells transfected with SCN5A vectors were plated in 6-well plate (150,000cells/well) for 24 hours. Subsequently, cells were washed 3 times in PBS and fixed with 4% paraformaldehyde at RT for 30 minutes. For permeabilization and blocking, cells were incubated for 1 hour with PBS 0.1% Triton X-100 and 5% FBS at RT. Then, cells were incubated with a polyclonal rabbit anti-NaV1.5 antibody (Alomone) (1:100 dilution) in PBS 0.1% Triton X-100 overnight at 4°C in a humified chamber. Then, cells were washed three times with PBS solution and then incubated with the secondary fluorescent anti-rabbit FITC-conjugated secondary antibody (1:200 dilution; Jackson ImmunoResearch) for 1 hour at RT in the dark. Cells were washed 3 times with PBS solution. Cells were then stained with 1 µg/ml Hoescht dye

3328 (1:500 dilution) for 10 min. Cells were then analyzed under a fluorescent microscope (Axiovert, Zeiss) equipped with an acquisition camera with magnification 10X.

2.9 Disbac in vitro assay

Fluorescence Resonance Energy Fret Transfer (FRET)-based voltage-sensing assay technology was used to measure the ions current. This assay is composed of an anionic fluorescent trimethine oxonol acceptor bis-(1,3-diethylthiobarbituric acid) [DiSBAC2(3)], and a fluorescent membrane-bound coumarin phospholipid FRET donor *N*-(6-chloro-7-hydroxycoumarin-3-carbonyl)-dimyristoylphosphatidyl-ethanolamine [CC2-DMPE]. Briefly, the phospholipid-anchored FRET donor (emission: 460nm) is localized in the plasma membrane, which guarantees the measurement at the cell membrane, and it interplays with the FRET acceptor (emission: 570nm) which drive the fluorescent signal to change according to the membrane potential. Cells at a resting state have almost a negative potential, therefore the interaction of the two probes at the outside side of the cell membrane results in an efficient FRET signal. Right after depolarization, the FRET signal reduces due to the detachment of the acceptor from the donor and it moves to the positively charged inner surface of the plasma membrane generating a blue fluorescence signal (Figure 17).

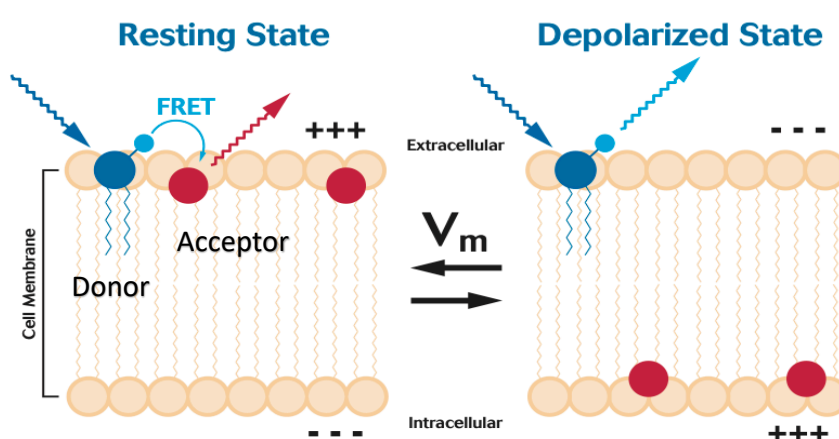


Figure 17. FRET-based voltage-sensing principles.

The transfected cells were plated in a 96-well imaging plates (10,000 cells/well) and cultured for 24 hours. Then, two dyes solution were prepared from concentrated stock solutions: CC2-DMPE loading solution (CC2-DMPE 20 μ M, 100 mg/mL Pluronic[®] F-127, Probenecid 2.5mM, 160mM NaCl, 4.5 mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM glucose, 10mM HEPES, pH7.4), and Disbac solution (Disbac 10 μ M, 100

mg/mL Pluronic® F-127, Probenecid conc, 160mM NaCl, 4.5 mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM glucose, 10mM HEPES, pH7.4).

Cultured media was removed, cells were washed with PBS, then CC2-DMPE solution (100 µL/well) was added, and cells were incubated in the dark at RT for 45 minutes. Furthermore, cells were washed with VSP-1 saline buffer, and then the cells were incubated with DiSBAC₂(3) solution (100 µL/well) in the dark at RT for 45 minutes. Then, cells were washed with VSP-1 saline buffer and replaced again with VSP-1 buffer (100 µL/well) before the reading (Figure 18).

The fluorescence recording was performed with a microplate fluorescence reader (Varioskan), equipped with 1 injector and a 96-well plate holder. The fluorescence signal was recorded with optics orientated above the plate. After the initial baseline recording (7 time points), 20 µL of stimulus buffer (160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 15 mM Veratridine, pH7.4), was added to induce the opening of the sodium channels, then a continue reading was recorded. Data were analyzed by doing the ratio of donor (460nm) to acceptor (580nm) with an excitation wavelength of 405nm normalized on the baseline of both the emissions (Figure 18).

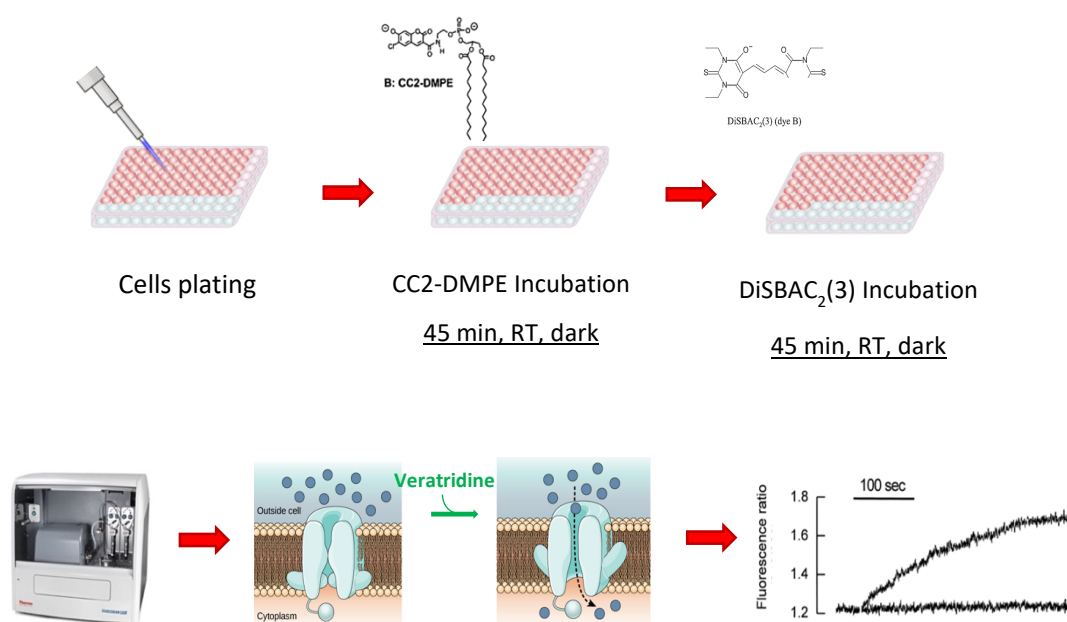


Figure 18. Disbac assay workflow. Cells were plated in a 96-well plate, and after two dyes incubation, the plate was loaded in a multiplate reader. After the baseline fluorescence recording with closed channels, a stimulus containing Veratridine was added to open the channels, allowing the measurement of the incoming sodium.

2.10 FluxOR™ II Green Potassium

FluxOR™ II Green Potassium Ion Channel technology (Thermo Scientific) is a cell-based assay specific for high-throughput screening measurements of potassium channel activity (Figure 19). Specifically, this assay is characterized by a thallium (Tl^+) sensitive fluorophore able to enter the cell through previously stimulated potassium channels. The channel activity is measured through a pre-loaded FluxOR™ II Green indicator dye into the cells that increases in cytosolic fluorescence in the presence of thallium. Therefore, the measured fluorescence is the indicator of ion channel activity that allows thallium to go into cells.

The assay was performed, following manufacturer's instructions. Briefly, PBMCs (50,000cells/well) were plated the same day of the experiment in a 96-well plate. Then, the cells were firstly incubated for 30 minutes at 37°C with 1X Loading Buffer containing the following components: 1 μ l of 100X PowerLoad™, 1 μ l FluxOR™ II Reagent (reconstituted in DMSO, as indicated), 10 μ l of 10X FluxOR™ II Assay Buffer, 88 μ l of Deionized water, and 1 μ l of Probenecid (reconstituted in deionized water, as indicated), for a total volume of 100 μ l in each well. 1X Loading Buffer was removed, wells were washed with Assay Buffer containing the following components: 89 μ l of deionized water, 10 μ l of 10X FluxOR Assay Buffer, 1 μ l of Probenecid, for a total volume of 100 μ l in each well. Then, the plate was analyzed in the Varioskan. After an initial baseline recording, the stimulus buffer containing Chloride-free Stimulus buffer, Potassium Sulfate (K_2SO_4), Thallium Sulfate (Tl_2SO_4), and deionized water at pH 7.4 was added to induce the opening of the potassium channels, then a continue reading was recorded. The FluxOR™ II Green fluorescence was measured at excitation wavelengths of 490 nm with emission at 525 nm.

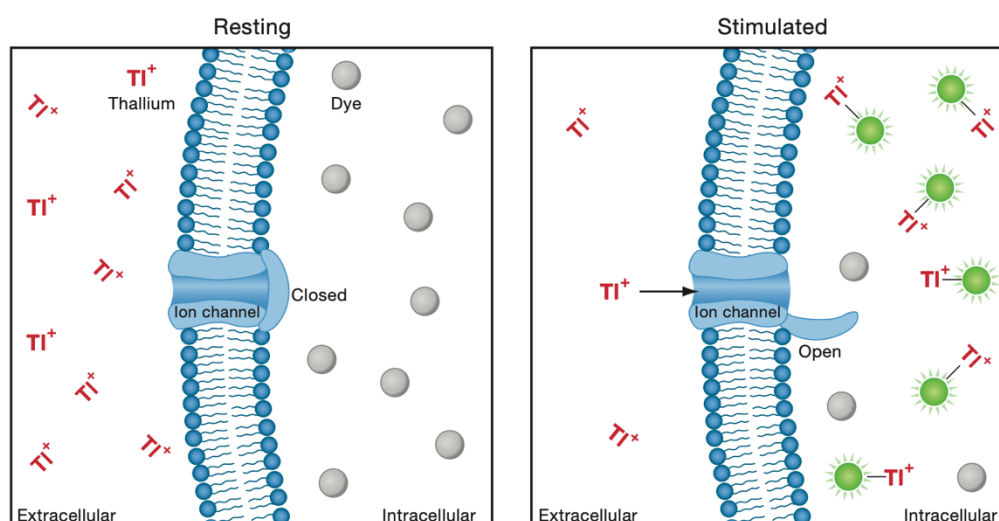


Figure 19. FluxOR assay principle. Closed potassium channel emits basal fluorescence. When thallium is added to the stimulus buffer, the thallium flows into the cells activating the dye.

2.11 Fura-2 AM calcium flux assay

Fura-2 AM (Figure 20) is a ratiometric intracellular calcium dye used to measure cellular calcium concentration through fluorescence. The channel activity is measured through a pre-loaded Fura-2 AM indicator dye where its fluorescence excitation spectrum shifts toward shorter wavelengths upon Ca^{2+} binding. The Fura-2 AM fluorescence was measured at excitation wavelengths of 340 nm and 380 nm with emission at 505 nm in response to calcium binding. The ratio of 340/380 fluorescence intensity is directly proportional to cytoplasmatic Ca^{2+} levels.

Briefly, PBMCs (50,000cells/well) were plated the same day of the experiment in a 96-well plate. Cells were incubated for 30 minutes at 37°C with the loading buffer, containing the following components: 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Glucose, and 10 mM Hepes at pH 7.4. 1 μM Fura-2 AM, probenecid at a final concentration of 2.5mM and 100X PowerLoad™ were added to the loading buffer. Cells were then washed and incubated with the loading buffer without the dye for 30 min at 37°C. Then, the plate was analyzed in the Varioskan. After an initial baseline recording, the stimulus buffer containing 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Glucose, and 10 mM Hepes and 5 μM of Ionomycin were added to induce the calcium flux, then a continue reading was recorded.

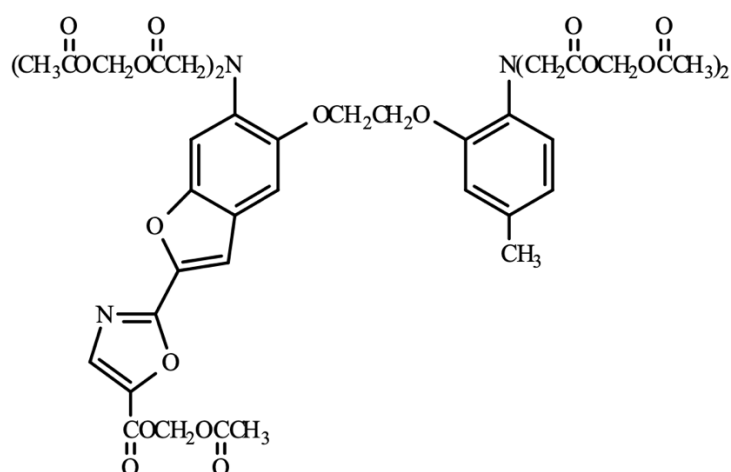


Figure 20. Fura-2 AM structure.

2.12 Bioinformatic SCN5A mutation screening

Bioinformatic prediction of the SCN5A mutation effects was performed with Panther (<http://www.pantherdb.org>) software and PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/index.shtml>). These data analysis tools are pathogenicity predictors used to predict the functional significance of the genetic variation of a gene. The probability of the mutation to cause a functional impact on the protein is based on the alignment of multi-species evolutionarily related proteins.

Specifically, Panther software score PSEP (position-specific evolutionary preservation) is based on how long (length of time: millions of years (my)) the variant of the protein last in time. The deleterious effect is directly proportional to how long the variant is preserved. The threshold is: "probably damaging" with a score higher than 450my"; possibly damaging" with a score between 450my and 200my; "probably benign" with a score lower than 200my.

Furthermore, the PolyPhen-2 score not only predicts the consequences that an amino acid substitution can have on the structure and function of a human protein but also shows how damaging a variant can be. The threshold is: "predicted to be benign" variants with a score between 0.0 and 0.15; "possibly damaging" variants with a score between 0.15 and 0.85; "predicted to be damaging" variants with a score between 0.85 and 1.0.

2.13 Statistical analysis

All statistical analysis was performed using GraphPad Prism 8. All results were evaluated with multiple t-test and p values. P values of less than 0.05 were considered to be significant. Data are presented as mean $\pm$ Standard Deviation (SD). Histograms represent the mean values and bars indicate the SD of the mean. Each experiment was carried out at least three times. Linear regression was calculated with Pearson's correlation test.

Chapter 3

Results

3.1 hNav 1.5 channel and mutations

DNA screening for SCN5A mutations is systematically performed on the BrS probands enrolled at the Arrhythmology Department of IRCCS Policlinico San Donato and a total of 54 different SCN5A mutations in 106 patients was detected (Table 1). VarSome prediction of the clinical significance of these mutations, indicated that one was classified as Benign, six were classified as Variant of Uncertain Significance (VUS), thirty-four were classified as Likely Pathogenic, and twelve were classified as Pathogenic. We focused our attention for the subsequent Nav1.5 functional study on four *de novo* mutations:

- 1- The missense variant c.86_86delinsTG (mut86), classified as VUS, that lead to the amino-acid substitution of alanine by valine in position 29 (p. Ala29Val). This mutation is located in the N-terminal of the cytoplasmic region [150].
- 2- The missense variant c.1045G>A (mut1045), classified as VUS, that lead to the amino-acid substitution of aspartic acid by asparagine in position 349 (p. Asp349Asn). This mutation is located in extracellular region between S5 D1 domain and pore-forming region [150].
- 3- The missense variant c.3214_3215delinsTC (mut3214), classified as VUS, that lead to the amino-acid substitution of glutamic acid by serine in position 1072 (p. Glu1072Ser). This mutation is located in the cytoplasmic region between S6 D2 domain and S1 D3 domain [150].
- 4- The missense variant c.5089T>C (mut5089), classified as Likely Pathogenic, that lead to the amino-acid substitution of phenylalanine by serine in position 1697 (p. Phe1697Ser). This mutation is located in the pore-forming region S4-S5 D4 domain [150].

Gene	Mutation	Varsome Prediction	Gene	Mutation	Varsome Prediction	Gene	Mutation	Varsome Prediction	Gene	Mutation	Varsome Prediction
SCN5A	c.4G>A	3	SCN5A	c.1111C>T	5	SCN5A	c.3214_3215delinsTC	3	SCN5A	c.4700_4701del	5
SCN5A	c.86_87delinsTG	3	SCN5A	c.1144C>T	4	SCN5A	c.3673G>A	4	SCN5A	c.4720G>A	4
SCN5A	c.422T>A	4	SCN5A	c.1673A>G	1	SCN5A	c.3697A>T	4	SCN5A	c.4813G>T	5
SCN5A	c.481G>A	4	SCN5A	c.1890G>A	4	SCN5A	c.3917G>A	4	SCN5A	c.4867C>T	5
SCN5A	c.535C>T	5	SCN5A	c.2039G>A	4	SCN5A	c.3946C>T	5	SCN5A	c.4894C>T	4
SCN5A	c.5863A>T	4	SCN5A	c.2077C>T	3	SCN5A	c.4057G>A	4	SCN5A	c.5000T>A	4
SCN5A	c.589G>T	4	SCN5A	c.2091G>A	5	SCN5A	c.4140C>G	4	SCN5A	c.5089T>C	4
SCN5A	c.655C>T	4	SCN5A	c.2182G>A	4	SCN5A	c.4216G>C	5	SCN5A	c.5092G>A	4
SCN5A	c.692C>T	4	SCN5A	c.2314G>A	4	SCN5A	c.4285G>A	4	SCN5A	c.5102T>G	4
SCN5A	c.733C>A	4	SCN5A	c.2414C>T	4	SCN5A	c.4294delA	4	SCN5A	c.5129C>T	5
SCN5A	c.809_812dup	5	SCN5A	c.2441G>A	4	SCN5A	c.4501C>G	4	SCN5A	c.5129C>T	5
SCN5A	c.880G>A	4	SCN5A	c.2542A>G	4	SCN5A	c.4534C>T	4	SCN5A	c.5863A>T	5
SCN5A	c.1041C>A	3	SCN5A	c.2729C>T	5	SCN5A	c.4535G>A	4	SCN5A	c.6004G>A	3
SCN5A	c.1045G>A	3	SCN5A	c.2893C>T	4	SCN5A	c.4536_4537delGClinsTT	4			

Table 1. BrS probands enrolled at the Arrhythmology Department of IRCCS Policlinico San Donato. Varsome prediction score. The American Collage of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology guidelines: 1 = benign 2= likely benign 3= uncertain significance 4= likely pathogenic 5 = pathogenic [151].

3.2 SCN5A mutations *in silico* analysis

Initially, additional *in silico* evaluation of the functional effect of the SCN5A mutation on NaV1.5 protein was performed with a structural modeling approach. Since the tridimensional structure of the channel has yet to be experimentally defined the structure of the ortholog rat protein (94% sequence homology) was employed. As regards to mut86 and mut3214, the amino-acid residues were not present in the defined structure, thus it was not possible to predict their effect on the channel structure. Mut1045 caused the substitution of a negative-charged amino-acid (Asp) with a positive-charged one (Asn) in the DI domain; in addition, this residue is surrounded by several charges, both negatives, and positives (Figure 1A – 1B). Mut5089 caused the substitution of phenylalanine with leucine with a reduction of the steric hindrance and a possible consequence on the geometry of the protein (Figure 1).

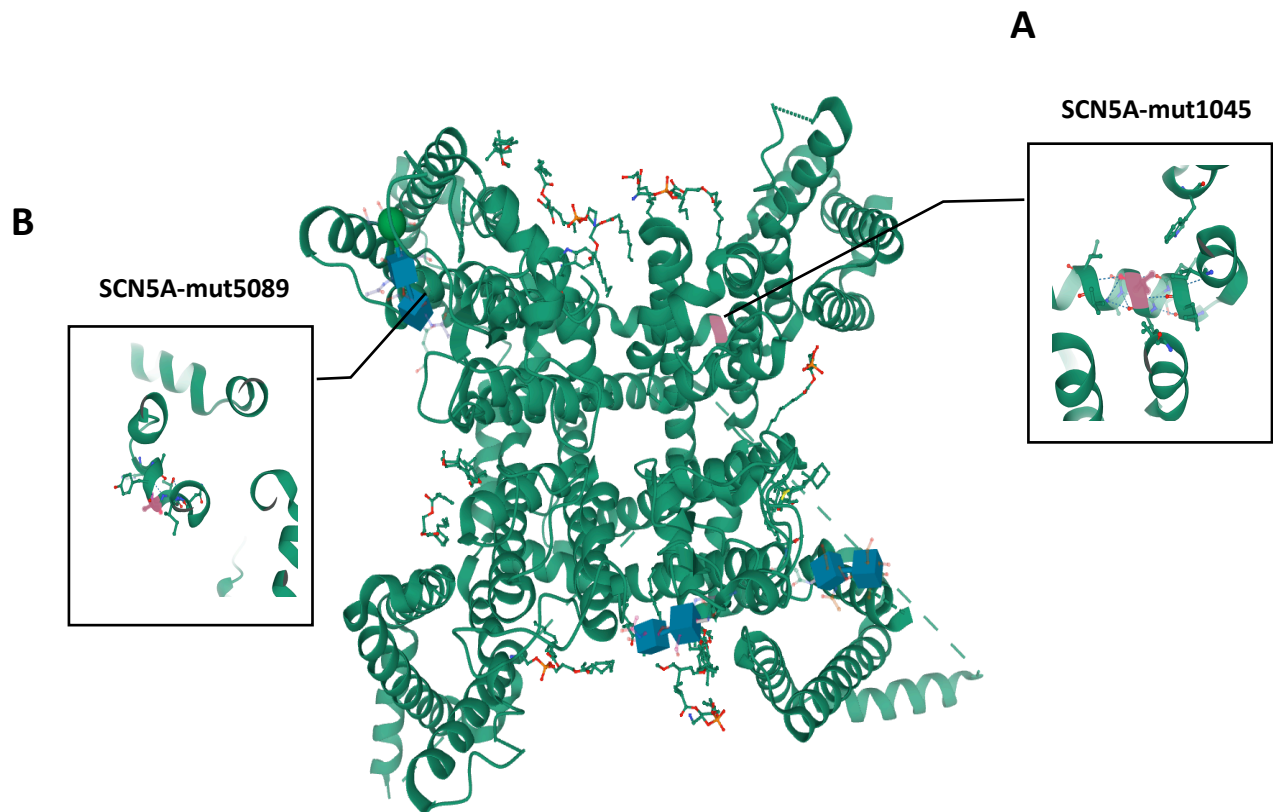


Figure 1. 3D prediction structure of NaV1.5. **A.** Mut1045 **B.** Mut5089

To further investigate the effects of SCN5A variants on NaV1.5 protein we compared paralog and ortholog conservation of the substituted amino acids. Then, for each mutation we compared how many times the amino acid was conserved in orthologous and paralogous proteins (Figure 2). Specifically, mut86 (Figure 2A), mut1045 (Figure 2B), and mut5089 (Figure 2D) were highly conserved in both paralog and ortholog alignment of SCN5A variants, while mut1045 (Figure 2C) was not highly conserved in paralog alignment.

A**SCN5A-mut86 Ala29Val**

SCN5A_HUMAN	ESLAAIEKRM A EKQARGSTTL
SCN1A_HUMAN	ESLAAIERRI A EKAK---N
SCN2A_HUMAN	ESLAAIEQRI A EKAK---R
SCN3A_HUMAN	ESLAAIEKRA A EKAK---K
SCN4A_HUMAN	ESLAAIEQRA V EEEAR---L
SCN7A_HUMAN	ESFELIKQHI A KTHNE---DH
SCN8A_HUMAN	ESLANIERRI A ESKLK---K-
SCN9A_HUMAN	QSLALIEQRI A ERKSK---E-
SCNAA_HUMAN	ESLVEIEKQI A AKQGT---KK
	:
SCN5A_HUMAN	-SLAAIEKRM A EKQARGSTTL
F6V2S7_XENTR	ESLEAIEKRI A EKAKKPK--
A0A2U4CAT9	ESLVEIEKRI A AKQEAKKT-K
Q20JQ5_DANRE	ESLAAIEQRI S EERARNT---
F1N8I1_CHICK	ESLAAIEKRI A EKLARNA--K
F1PGU3_CANLF	ESLAAIEKRM A EKQARGSAAS
SCN5A_MOUSE	ESLAAIEKRM A EKQARGSATS
	:

B**SCN5A-mut1045 Asp349Asn**

SCN5A_HUMAN	YRCLKAGEN D HGYTSFDSFA
SCN1A_HUMAN	YMCVKAGRNP N YGYTSFDTFs
SCN2A_HUMAN	YICVKAGRNP N YGYTSFDTFs
SCN3A_HUMAN	YICVKAGRNP N YGYTSFDTFs
SCN4A_HUMAN	YECIKTGRNP N YGYTSYDTFS
SCN7A_HUMAN	YVCVKAGINP D QGFTNFDSFG
SCN8A_HUMAN	YQCMKAGRNP N YGYTSFDTFs
SCN9A_HUMAN	YTCVKIGRNP D YGYTSFDTFs
SCNAA_HUMAN	YICLKTSNPN D FNYSFDSFA
	:
F6V2S7_XENTR	YICVKTGKNP N YGYTSFDTFs
A0A2U4CAT9	YFCLKTSNPN D FNYSFDSFA
Q20JQ5_DANRE	FDCLKTSRNP N YGYTSFDTFG
F1N8I1_CHICK	YICLKAGEN D HGYTSFDTFG
F1PGU3_CANLF	YRCLKAGEN D HGYTSFDSFA
SCN5A_HUMAN	YRCLKAGEN D HGYTSFDSFA
SCN5A_MOUSE	YRCLKAGEN D HGYTSFDSFA
	:

C**SCN5A-mut 3214 Glu1072Ser**

SCN5A_HUMAN	-EENSLGTEE E SSKQ---QES
SCN1A_HUMAN	-EDFSSESDL E ESKEK---LN
SCN2A_HUMAN	-EEFSSESDE E ESKEK---LN
SCN3A_HUMAN	-EEFSSESE E ESKEK---LN
SCN4A_HUMAN	-EETDTFSEP E DSKKPPQPLY
SCN7A_HUMAN	-KEIQSKSGD G SKKEK---IK
SCN8A_HUMAN	-EDVSSESDP E GSKDK---LD
SCN9A_HUMAN	-EELSSSDSD E YSKVR---LN
SCNAA_HUMAN	SSEDLAPSL E TWKDESVPQV
	*
F6V2S7_XENTR	EDFSSESLE E -----
A0A2U4CAT9	DGEEDAQSTQ Q EVIPQGQQEQ
Q20JQ5_DANRE	EEEEEDTESS E D-----
F1N8I1_CHICK	EQSTFTTEMEQ - DYFQQ---EK
F1PGU3_CANLF	DEENSLGTEE E SSKQQ---ES
SCN5A_HUMAN	DEENSLGTEE E SSKQQ---ES
SCN5A_MOUSE	DEENSLGTEE E SSKQQ---ES
	*

D**SCN5A-mut5089 Phe1697Leu**

SCN5A_HUMAN	GIDDMFNFQT F ANSMLCLFQI
SCN1A_HUMAN	GIDDMFNFET F GNSMICLFQI
SCN2A_HUMAN	GIDDMFNFET F GNSMICLFQI
SCN3A_HUMAN	GIDDMFNFET F GNSMICLFQI
SCN4A_HUMAN	GIDDMFNFET F GNSIICLFQI
SCN7A_HUMAN	GINDVSNFET F GNSMLCLFQV
SCN8A_HUMAN	GIDDMFNFET F GNSMICLFQI
SCN9A_HUMAN	GINDMNFET F GNSMICLFQI
SCNAA_HUMAN	GIDDMFNFQT F ANSMLCLFQI
	*
F6V2S7_XENTR	GIDDMFNFET F GNSMICLFQI
A0A2U4CAT9	GIDDMFNFQT F ANSMLCLFQI
Q20JQ5_DANRE	GIDDMFNFET F GNSMICLFQI
F1N8I1_CHICK	GIDDMFNFQT F ANSMLCLFQI
F1PGU3_CANLF	GIDDMFNFQT F ANSMLCLFQI
SCN5A_HUMAN	GIDDMFNFQT F ANSMLCLFQI
SCN5A_MOUSE	GIDDMFNFQT F ANSMLCLFQI
	*

Figure 2. Paralog and orthologs alignment of SCN5A variants. **A.** SCN5A-mut86 **B.** SCN5A-mut1045 **C.** SCN5A-mut3214 **D.** SCN5A-mut5089.

Moreover, two pathogenicity predictor software were employed: Polyphen2 and Panther. The likelihood of the mutation to cause a functional impact on the protein is based on the alignment of evolutionarily

related proteins. The pathogenicity predictor software evidenced the scores listed in Table 2, suggesting highly harmful mutations.

Mutation	Score Polyphen2	Score Panther
SCN5A – mut86	0.997	750my
SCN5A – mut1045	0.999	361my
SCN5A – mut3214	0.400	750my
SCN5A – mut5089	0.998	1238my

Table 2. Polyphen2 and Panther software scores.

3.3 SCN5A mutated plasmids generation

To study the functional effect of the SCN5A mutations on Nav1.5, a plasmid vector pcDNA3.1 containing the coding region of the human SCN5A sodium channel α subunit was successfully constructed. The Nav1.5 expression was driven by a CMV promoter (Figure 3) to ensure high expression levels.

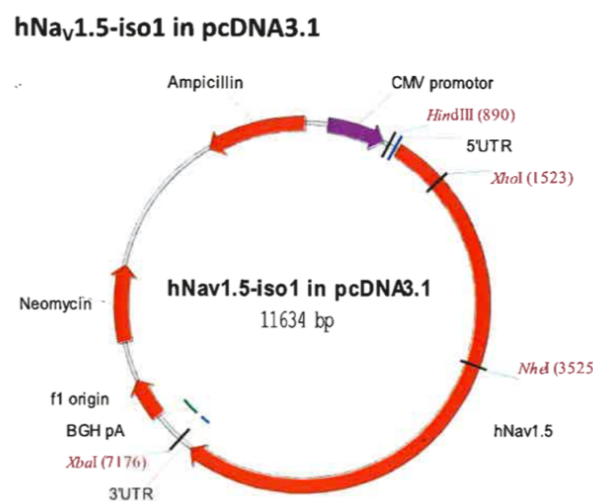


Figure 3. hNav1.5-iso1 in pcDNA3.1 with CMV promoter. It is a 11634bp vector which contains the hH1 an isoform of SCN5A. The digestion enzymes as HindIII and XhoI.

Then the four mutated plasmids were generated through a site-directed mutagenesis approach and the correct insertion of each mutation was confirmed by region-specific Sanger sequencing.

3.4 NaV1.5 functional test to evaluate the effects of SCN5A mutations

NaV1.5 functional test was carried out on HEK293A cells. These cells were chosen since they express very low levels of NaV1.5 and they do not present inward currents [152]. Cells were transiently transfected with an empty pcDNA3.1 vector (3.1 Empty) used as a negative control, with the wild type SCN5A (SCN5A WT) as a positive control, and with each mutation analyzed (SCN5A-mut86, SCN5A-mut5089, SCN5A-mut3214, SCN5A-mut1045). The plasmid expression was confirmed by Real-Time PCR (Figure 4): in particular, SCN5A-WT showed a 25.73 fold-increase, SCN5A-mut86 with a fold of 25.72, SCN5A-mut1045 with a fold of 15.57, SCN5A-mut with a fold of 25.75, SCN5A-mut5089 with a fold of 25.71, as compared to pcDNA3.1 Empty.

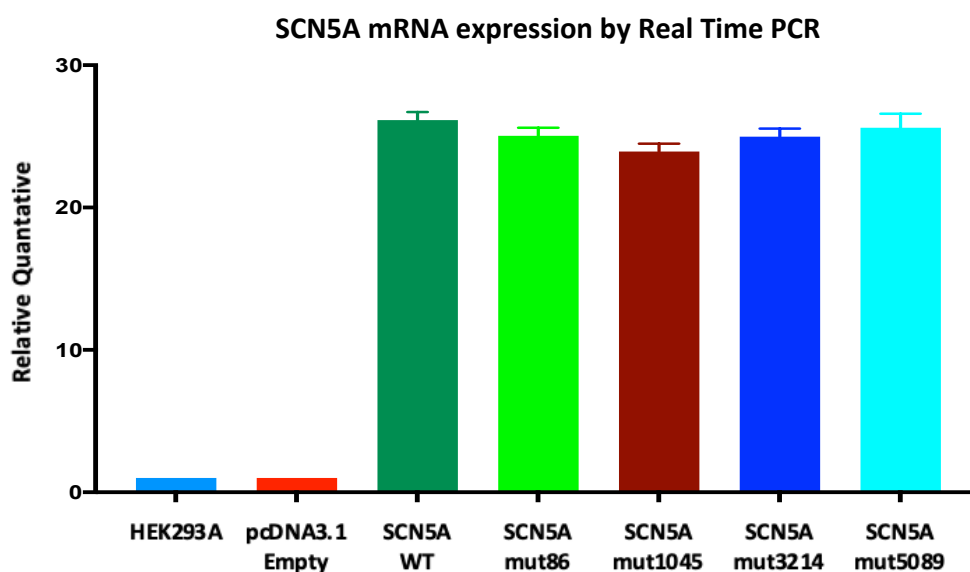


Figure 4. mRNA expression of SCN5A vectors. Data represent mean $\pm$ SD of 3 independent experiments.

Since NaV1.5 is active when localized in the plasma membrane, immunofluorescence analysis was performed on transfected cells. This technique indicated that the NaV1.5 reached the plasma membrane in all the transfected cell lines. (Figure 5).

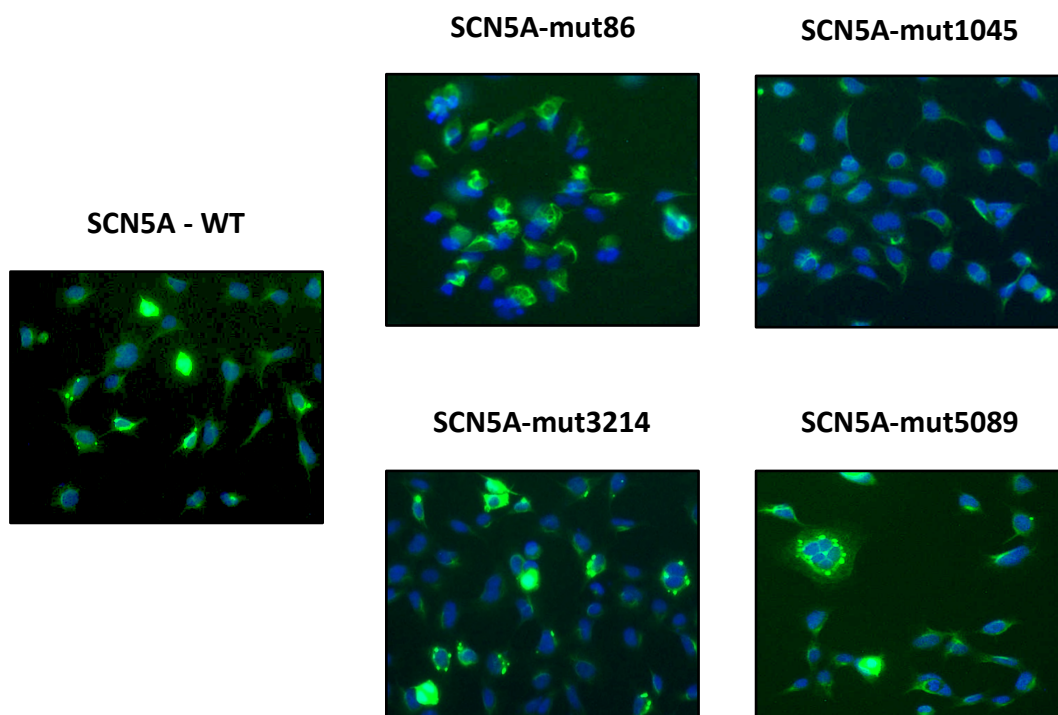
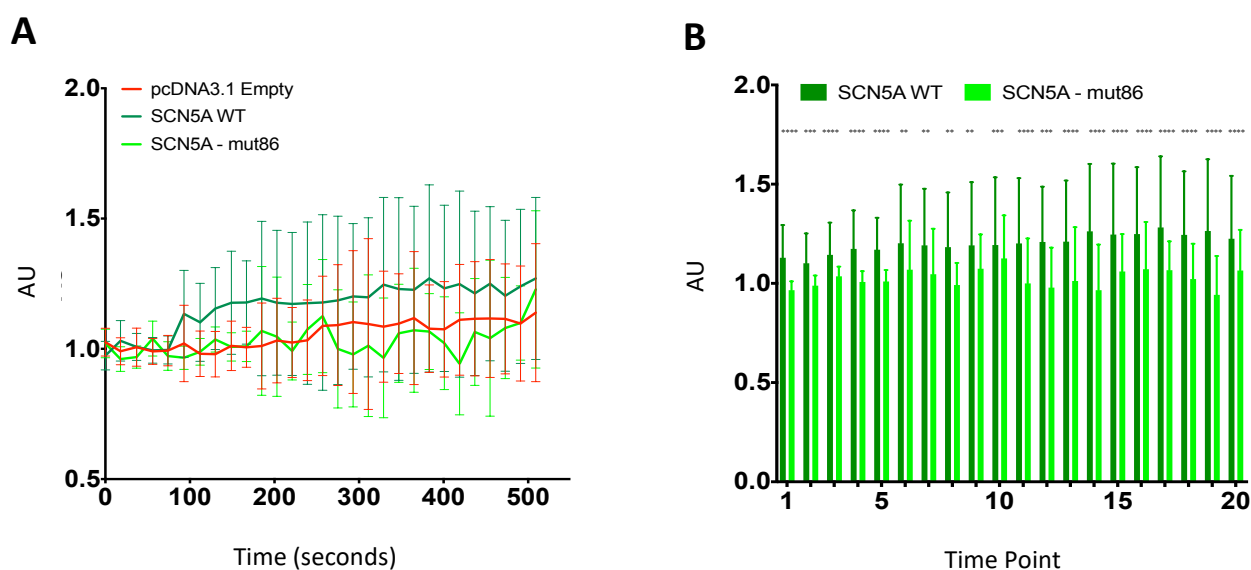


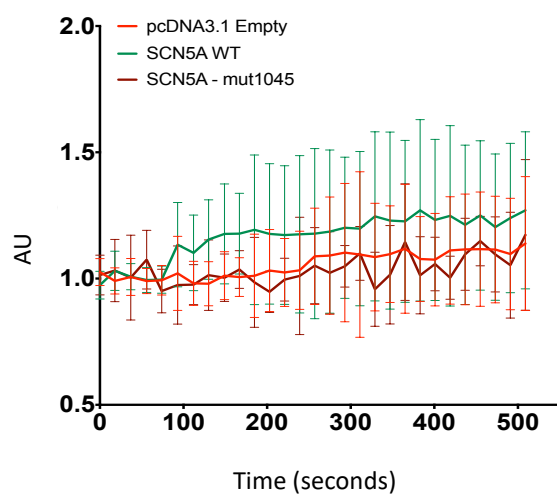
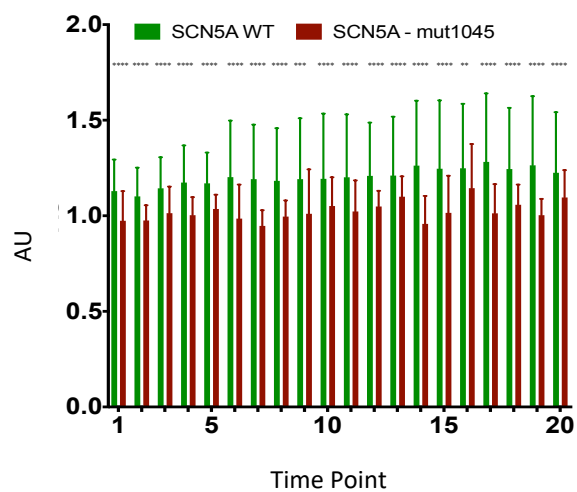
Figure 5. Immunofluorescence analysis of homozygous SCN5A vectors.

Then, the NaV1.5 functional test was performed. All the different mutations showed a significant reduction of the inward sodium flux as compared to WT-SCN5A, at all the time-points analyzed. In particular, mut-86 showed a 1.10-fold reduction (Figure 6A and 6B), mut-1045 showed a 1.13-fold reduction (Figure 6C and 6D), mut-3214 showed a 1.24-fold reduction (Figure 6E and 6F), mut5089 showed a 1.29-fold reduction (Figure 6G and 6H), as compared to the WT.

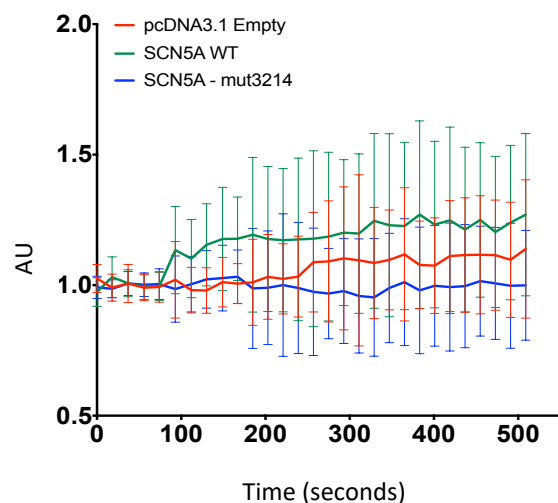
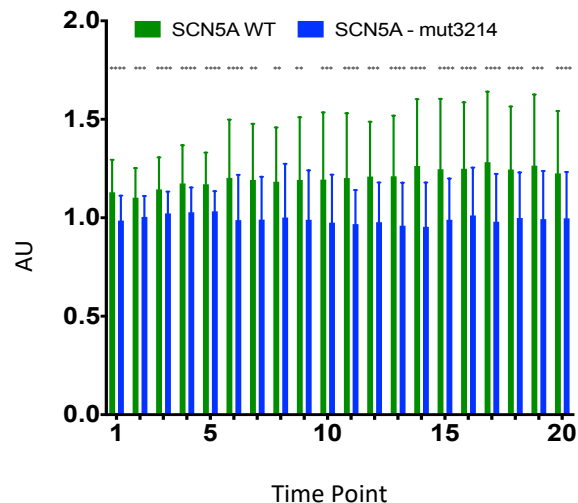
SCN5A-mut86 functional assay



SCN5A-mut1045 functional assay

C**D**

SCN5A-mut3214 functional assay

E**F**

SCN5A-mut5089 functional assay

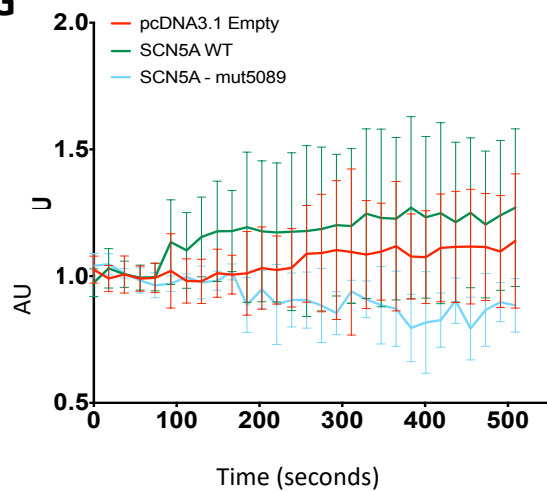
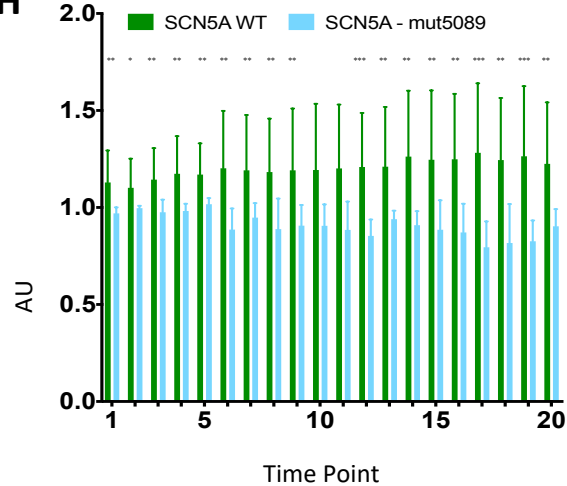
G**H**

Figure 6. Functional evaluation of homozygous SCN5A mutations. **A.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut86. **B.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut86. **C.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut1045. **D.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut1045. **E.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut3214. **F.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut3214. **G.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut5089. **H.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut5089. $n=5$. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$

Since BrS patients usually show heterozygous SCN5A mutations, to study the effects of SCN5A mutations in this situation, HEK293A cells were co-transfected with both the wild-type and each of the four mutant forms of the SCN5A. Immunofluorescence analysis showed the correct localization of NaV1.5 at the plasma membrane in all the transfected cell lines (Figure 7).

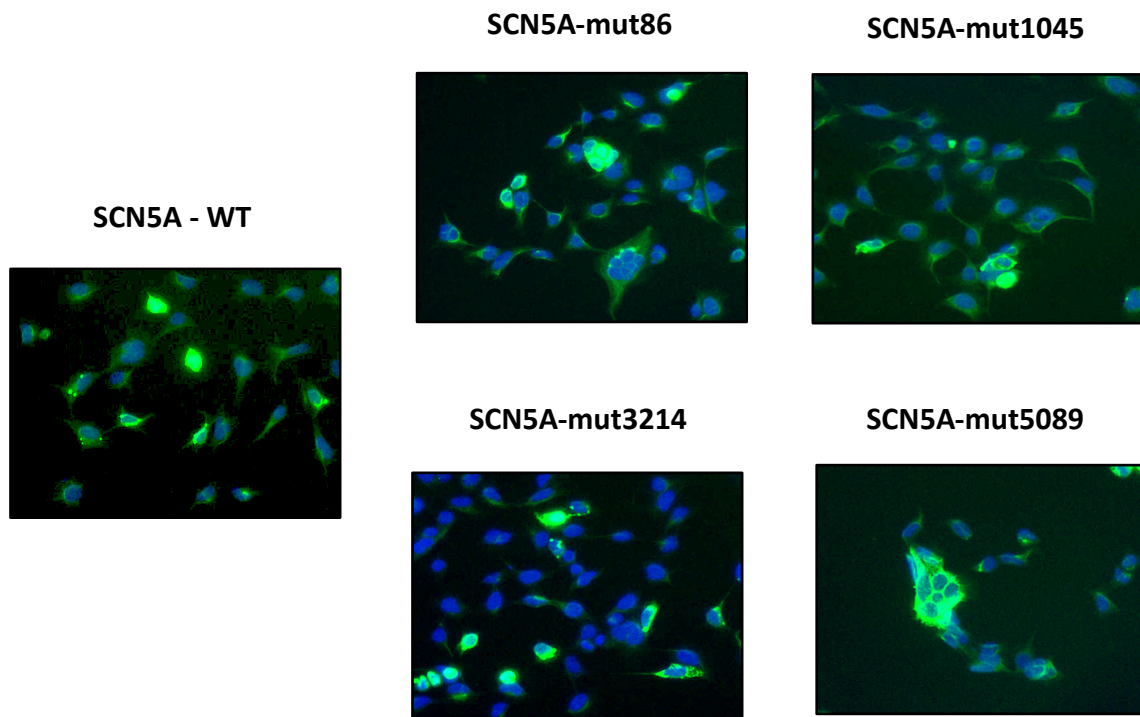
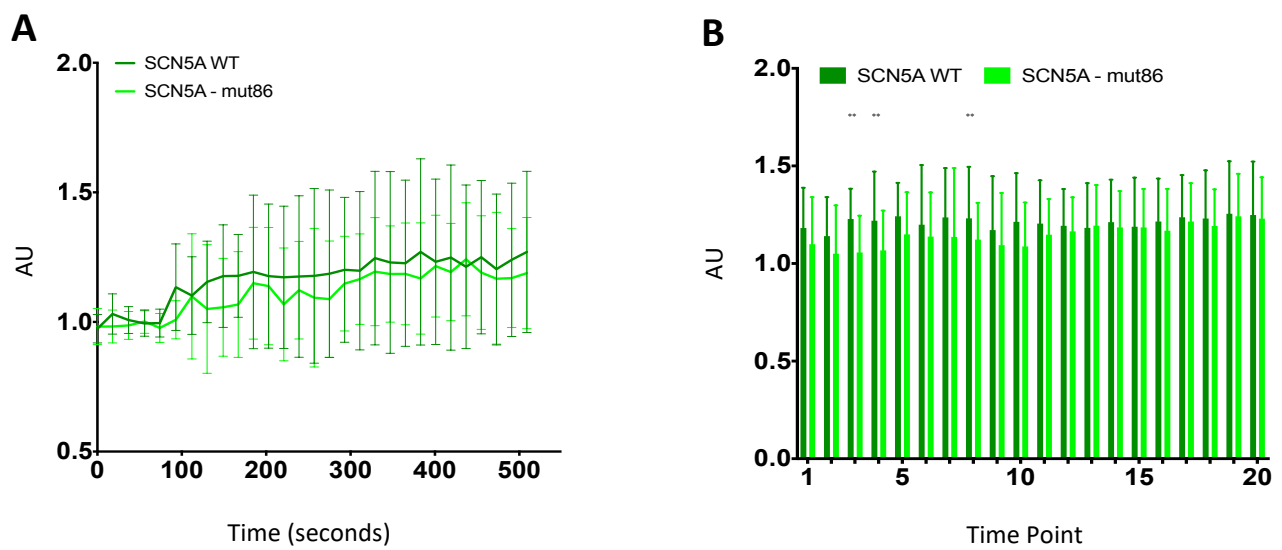


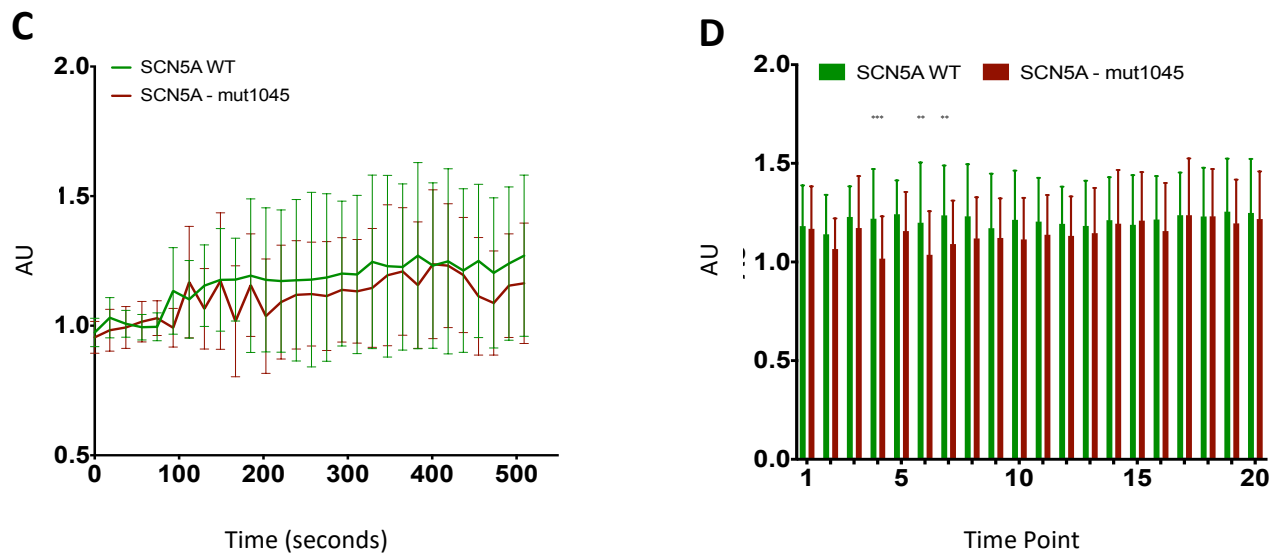
Figure 7. Immunofluorescence analysis of heterozygous SCN5A expression.

Then the NaV1.5 functional test was performed. Interestingly, SCN5A-mut86 and SCN5A-mut1045 retained the functions of the wild-type channel, with a total inward flux of sodium not different from SCN5A-WT (Figure. 8A, 8B, 8C, and 8D). SCN5A-mut3214 showed partial restoration of the inward sodium flux as compared to SCN5A-WT, with significant differences at the majority of the time-points (Figure 8E and 8F). Finally, SCN5A-mut5089 showed a significant 1.23-fold decrease in the sodium inward flux as compared to SCN5A-WT (Figure 8G and 8F).

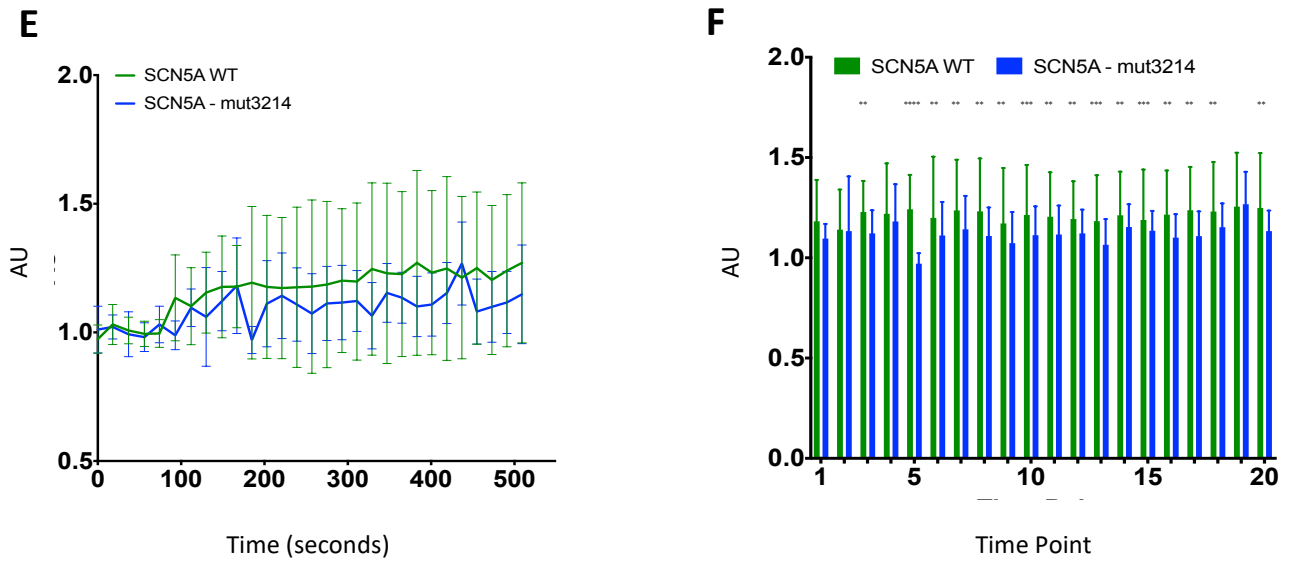
SCN5A-mut86 functional assay



SCN5A-mut1045 functional assay



SCN5A-mut3214 functional assay



SCN5A-mut5089 functional assay

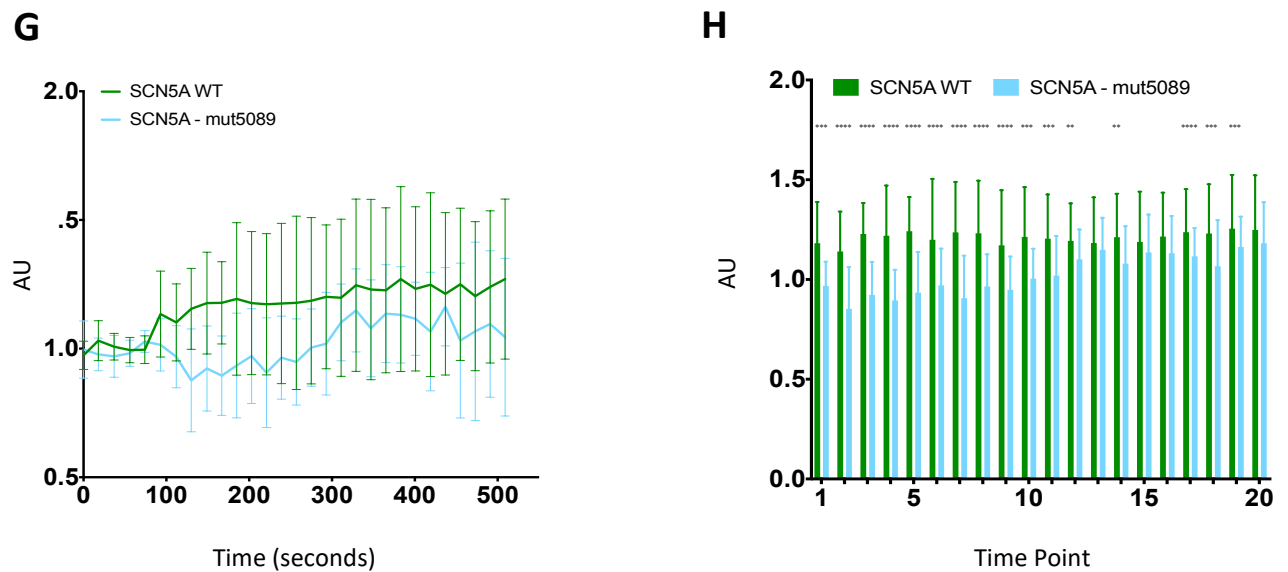


Figure 8. Functional evaluation of heterozygous SCN5A mutations. **A.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut86. **B.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut86. **C.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut1045. **D.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut1045. **E.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut3214. **F.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut3214. **G.** Sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut5089. **H.** Statistical analysis of sodium functional test on pcDNA3.1 empty, SCN5A WT, and SCN5A-mut5089. $n=5$. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$

3.5 Predictive model

To predict the effects of sodium mutation on the NaV1.5 functionality and, thus, on the BrS phenotype, a predictive model was conceived. Since the functional characterization of the SCN5A detected mutations is still ongoing, we used the score of the predictive models (Panther software), as an index of the detrimental effect of SCN5A mutations on NaV1.5. A linear multivariate regression model was established, in which the predictive scores were correlated with a series of BrS patients' clinical features. Among the covariates, the NaV1.5 domain localization of the mutation, the age and sex of the patients, the symptoms, Panther's values (Figure 9A), the presence of other mutations, and the area of the pathological BrS substrate, before and after the injection of ajmaline were included. After the evaluation of different models, a significant direct correlation between the Panther's values and the substrate area after ajmaline was highlighted (Figure 9B) with a Pearson's value of 0.1050 and a p-value of 0.034.

A

Mutation	Polyphen	Gene	Mutation	Polyphen	Gene	Mutation	Polyphen	Gene	Mutation	Polyphen
SCN5A c.4G>A	0.997	SCN5A	SCN5A c.1111C>T	1.000	SCN5A	c.3214_3215delinsTC	1.000	SCN5A	c.4700_4701del	1.000
SCN5A c.86_87delinsTG	1.000	SCN5A	SCN5A c.1144C>T	1.000	SCN5A	c.3673G>A	1.000	SCN5A	c.4720G>A	1.000
SCN5A c.422T>A	0.996	SCN5A	SCN5A c.1673A>G	1.000	SCN5A	c.3697A>T	0.021	SCN5A	c.4813G>T	1.000
SCN5A c.481G>A	0.999	SCN5A	SCN5A c.1890G>A	1.000	SCN5A	c.3917G>A	1.000	SCN5A	c.4867C>T	0.974
SCN5A c.535C>T	0.998	SCN5A	SCN5A c.2039G>A	1.000	SCN5A	c.3946C>T	1.000	SCN5A	c.4894C>T	1.000
SCN5A c.5863A>T	0.009	SCN5A	SCN5A c.2077C>T	0.999	SCN5A	c.4057G>A	0.999	SCN5A	c.5000T>A	1.000
SCN5A c.589G>T	0.660	SCN5A	SCN5A c.2091G>A	0.953	SCN5A	c.4140C>G	0.982	SCN5A	c.5089T>C	0.998
SCN5A c.655C>T	0.906	SCN5A	SCN5A c.2182G>A	1.000	SCN5A	c.4216G>C	1.000	SCN5A	c.5092G>A	1.000
SCN5A c.692C>T	0.276	SCN5A	SCN5A c.2314G>A	0.924	SCN5A	c.4285G>A	1.000	SCN5A	c.5102T>G	1.000
SCN5A c.733C>A	0.999	SCN5A	SCN5A c.2414C>T	0.126	SCN5A	c.4294delA	0.083	SCN5A	c.5129C>T	0.999
SCN5A c.809_812dup	1.000	SCN5A	SCN5A c.2441G>A	0.999	SCN5A	c.4501C>G	0.317	SCN5A	c.5863A>T	0.009
SCN5A c.880G>A	0.861	SCN5A	SCN5A c.2542A>G	0.993	SCN5A	c.4534C>T	1.000	SCN5A	c.6004G>A	0.317
SCN5A c.1041C>A	0.999	SCN5A	SCN5A c.2729C>T	0.982	SCN5A	c.4535G>A	1.000			
SCN5A c.1045G>A	1.000	SCN5A	SCN5A c.2893C>T	0.993	SCN5A	c.4536_4537delGCinsTT	0.906			

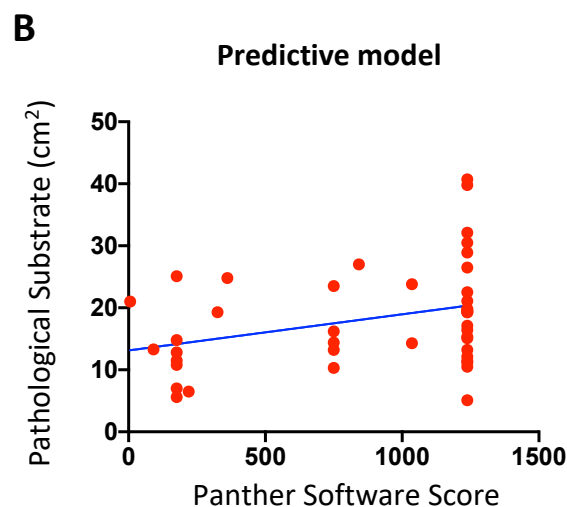


Figure 9. Predictive model. **A.** Polyphen prediction score. score between 0.0 and 0.15: “predicted to be benign”; score between 0.15 and 0.85: “possibly damaging”; score between 0.85 and 1.0: “predicted to be damaging”. **B.** Correlation between Panther scores and pathological area.

3.6 Optimization of the calcium flux assay on PBMCs

Calcium channel assay is based on the use of a ratiometric calcium-specific dye, which alters its excitation spectra upon the binding with calcium ions.

To induce the activity of the calcium channel, the ionophore ionomycin was employed. Ionomycin induces the increase of intracellular calcium in three ways. First, by the activation of the calcium influx through the native calcium channels; second, by the phospholipase C-dependent mobilization of calcium directly correlated to the intracellular stores; third, by a calcium store-regulated mechanism [153].

Thus, we used two different approaches to study the calcium release from the intracellular storages and to analyze the calcium flux from the extracellular space, using a calcium-free assay buffer and a calcium assay buffer, respectively. Firstly, we set the best ionomycin concentration and, in both cases, we found that the 5 μ M concentration was the best one, as it produced a better signal magnitude (Figure 10).

Calcium Channel Assay

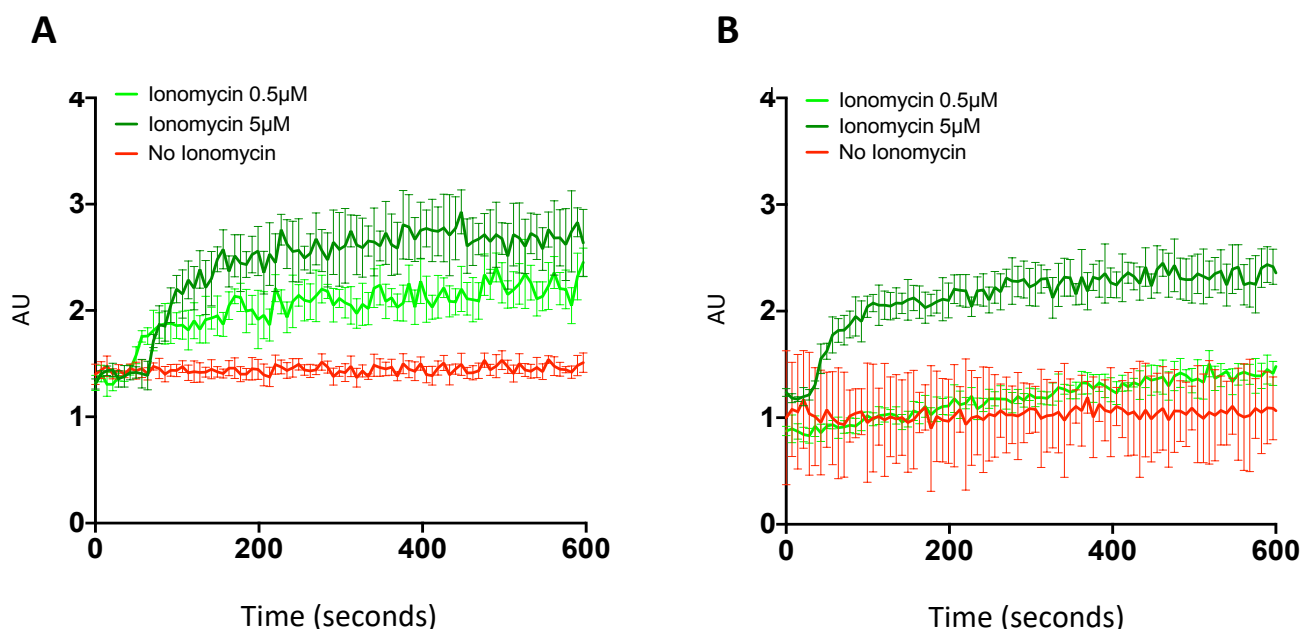


Figure 10. PBMCs stimulation with Ionomycin. **A.** PBMCs stimulated with 0.5 μ M Ionomycin and CaCl_2 Saline Buffer in light green; PBMCs stimulated with 5 μ M Ionomycin and CaCl_2 Saline Buffer in dark green. PBMCs stimulated only with CaCl_2 Saline Buffer in red. **B.** PBMCs stimulated with 0.5 μ M Ionomycin and Saline Buffer with no CaCl_2 in

light green; PBMCs stimulated with 5 μ M Ionomycin and Saline Buffer with no CaCl₂ in dark green. PBMCs stimulated only with and Saline Buffer with no CaCl₂ in red. Number of samples: 20 for each condition.

Then, we performed the Ca²⁺ flux assay on BrS patients and controls PBMCs. Firstly, we tested the calcium release from the intracellular storages, but no significant differences between BrS patients and healthy controls were detected (Figure 11).

Calcium Channel Assay in PBMCs

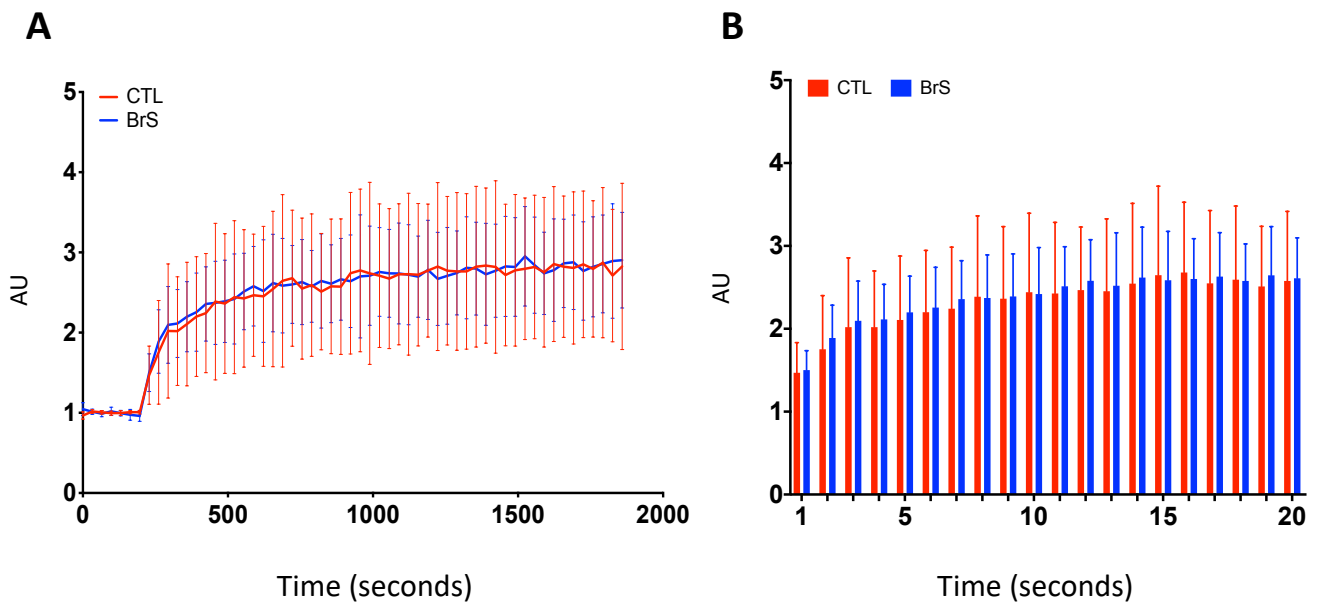


Figure 11. Fura-2 AM in vitro assay on BrS patients and controls (extracellular calcium). **A.** BrS patients and healthy controls extracellular calcium storage. **B.** Statistical analysis of BrS patients and healthy controls extracellular calcium storage. Number of samples: 20 for each condition.

However, when we analyzed the extracellular flux of calcium, we detected a statistically significant 1.3-fold increase in BrS patients, as compared to controls (Figure 12).

Calcium Channel Assay in PBMCs

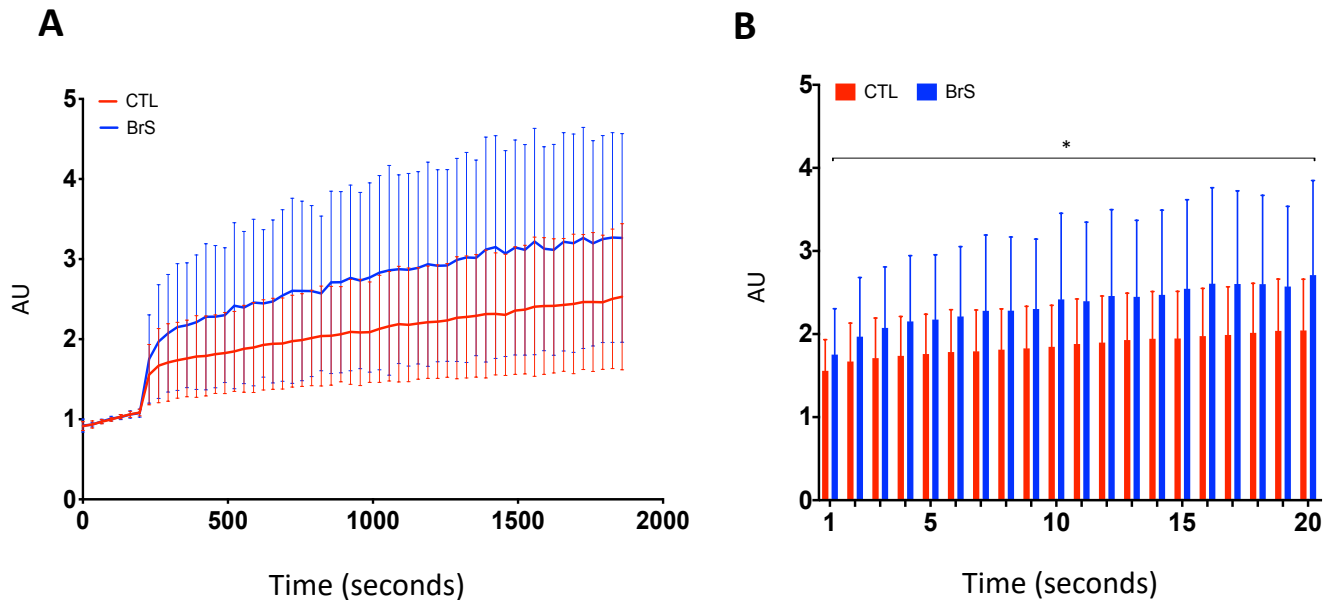


Figure 12. Fura-2 AM in vitro assay on BrS patients and controls (intracellular calcium flux). **A.** BrS patients and healthy controls intracellular calcium storage. **B.** Statistical analysis of BrS patients and healthy controls extracellular calcium storage. Number of samples: 20 for each condition. * $p < 0.05$

To further investigate Ca^{2+} flux, a gender-based comparison was performed. As it is evident in Figure 13A and 13B, there were no differences between male and female controls, while we observed a 1,28-fold increase in the calcium flux of male BrS patients, as compared to female BrS patients (Figure 13C and 13D).

Calcium Channel Assay in PBMCs

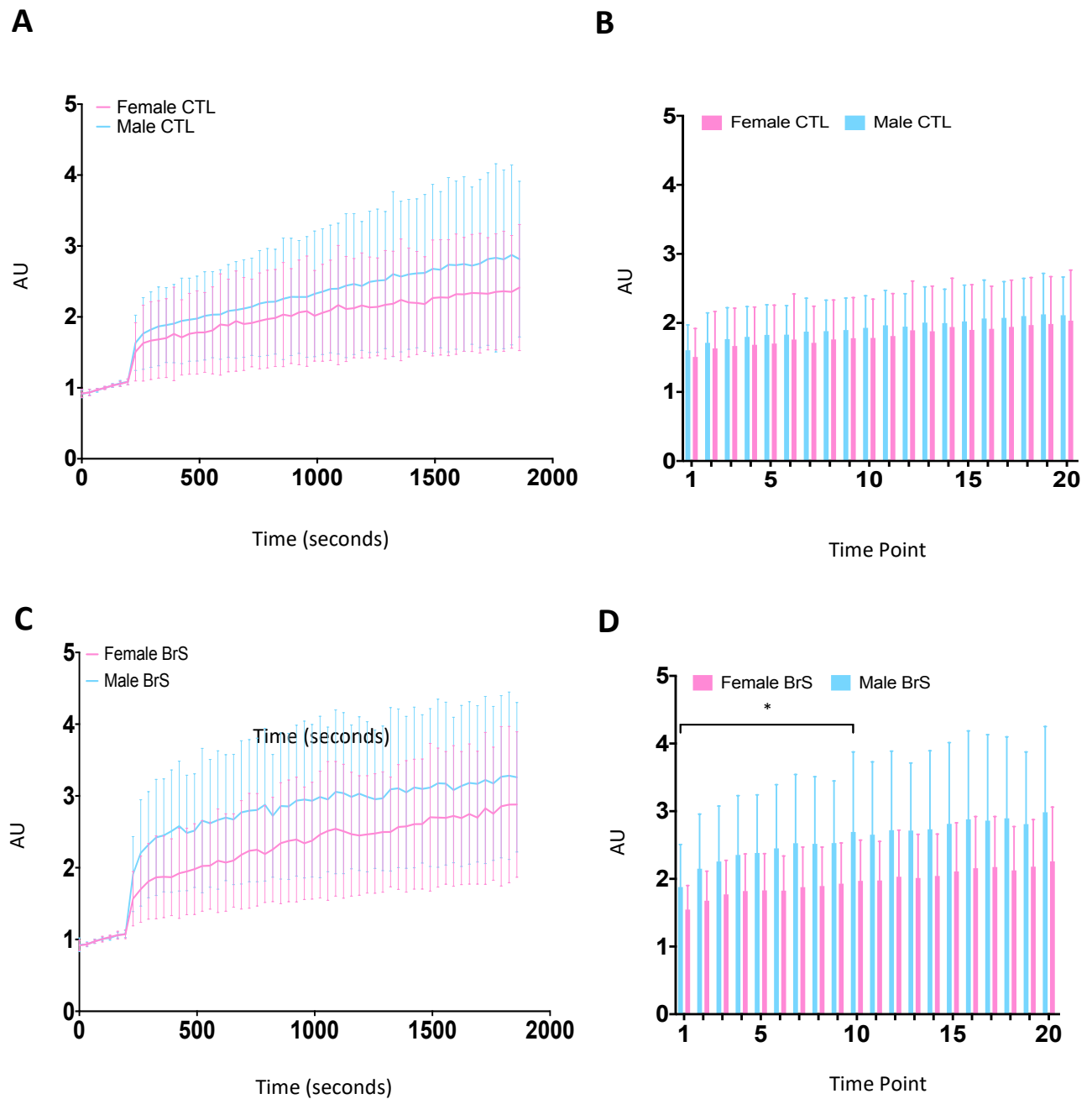


Figure 13. Fura-2 AM in vitro assay in PBMCs. **A.** Female healthy controls vs Male healthy controls. **B.** Statistical analysis of female healthy controls vs Male healthy controls. **C.** Female BrS patients vs Male BrS patients. **D.** Statistical analysis of female BrS patients vs Male BrS patients. Number of samples: 20 for each condition. Number of samples: 20 for each condition. * $p < 0.05$

Then, we made a gender-based comparison between BrS patients and controls and we detected a significant 1,35-fold increase in the calcium flux of male BrS patients, as compared to male controls (Figure 14A and 14B), while no differences were observed between females (FIG. 14C and 14D).

Calcium Channel Assay in PBMCs

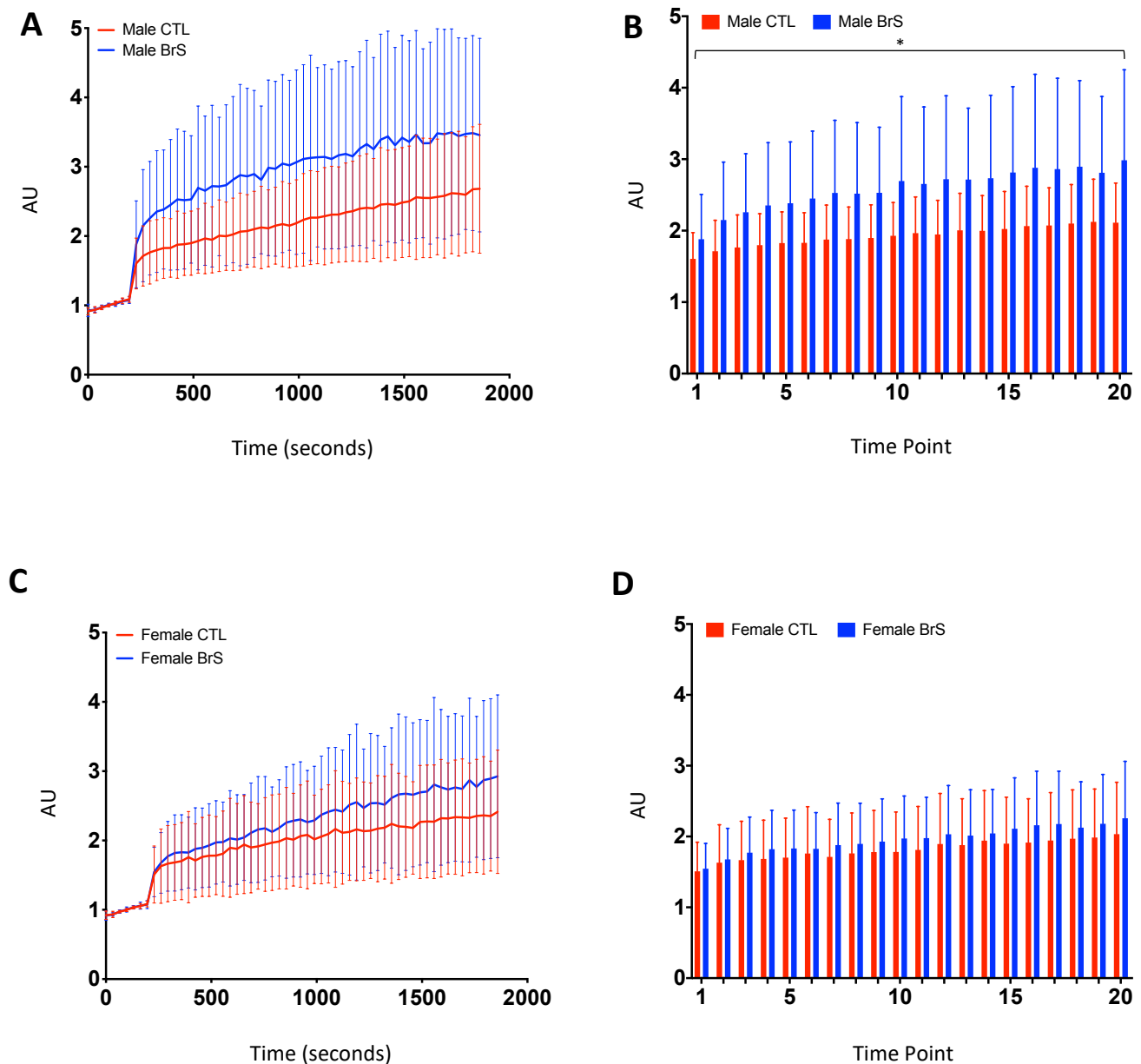


Figure 14. Fura-2 AM in vitro assay on female and male BrS patients and healthy controls. **A.** Male BrS patients vs male healthy controls. **B.** Statistical analysis of male BrS patients vs male healthy controls. **C.** Female BrS patients

vs female healthy controls. D. Statistical analysis of Female BrS patients vs female healthy controls. Number of samples: 20 for each condition. * $p < 0.05$

Since PBMCs contain all the different nucleated cell populations of blood, we performed the Ca^{2+} flux assay on T lymphocytes to determine if they were responsible for the differences previously detected. However, no significant differences were observed between BrS patients and controls (Figure 15A and 15B).

Calcium Channel Assay in T lymphocytes

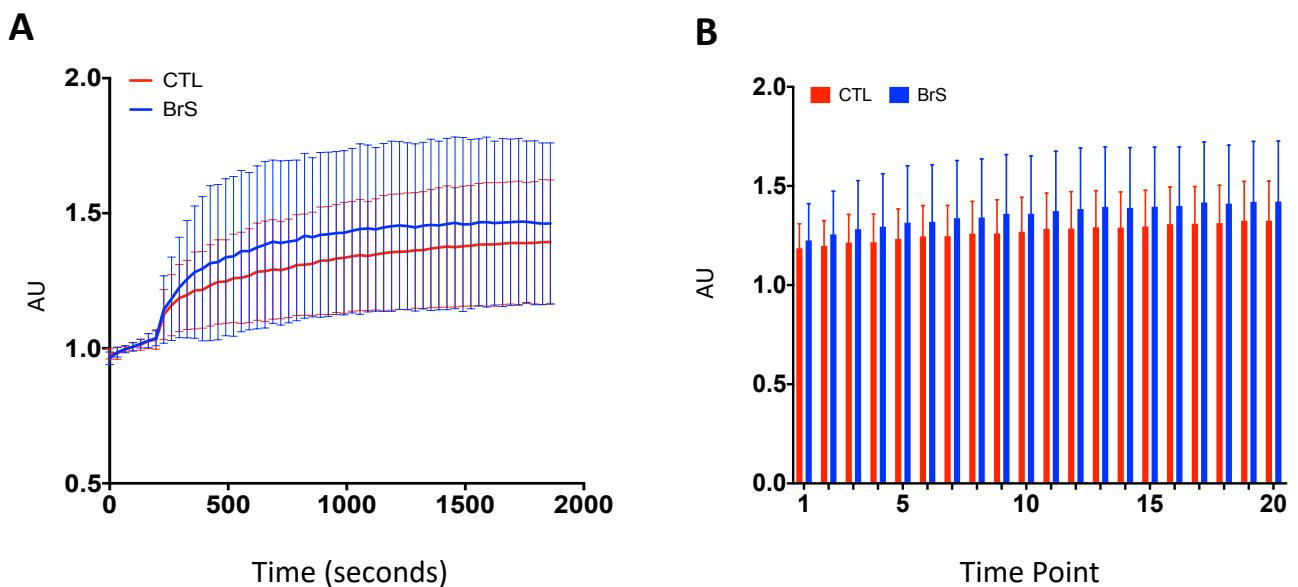


Figure 15. Fura-2 AM assay on T lymphocyte cells. **A.** BrS patients and healthy controls extracellular calcium storage. **B.** Statistical analysis of BrS patients and healthy controls extracellular calcium storage.

In addition, the gender-based analysis did not reveal any differences between male and female calcium flux in T lymphocytes in both study groups (Figure 16A, 16B, 16C, and 16D).

Calcium Channel Assay in T lymphocytes

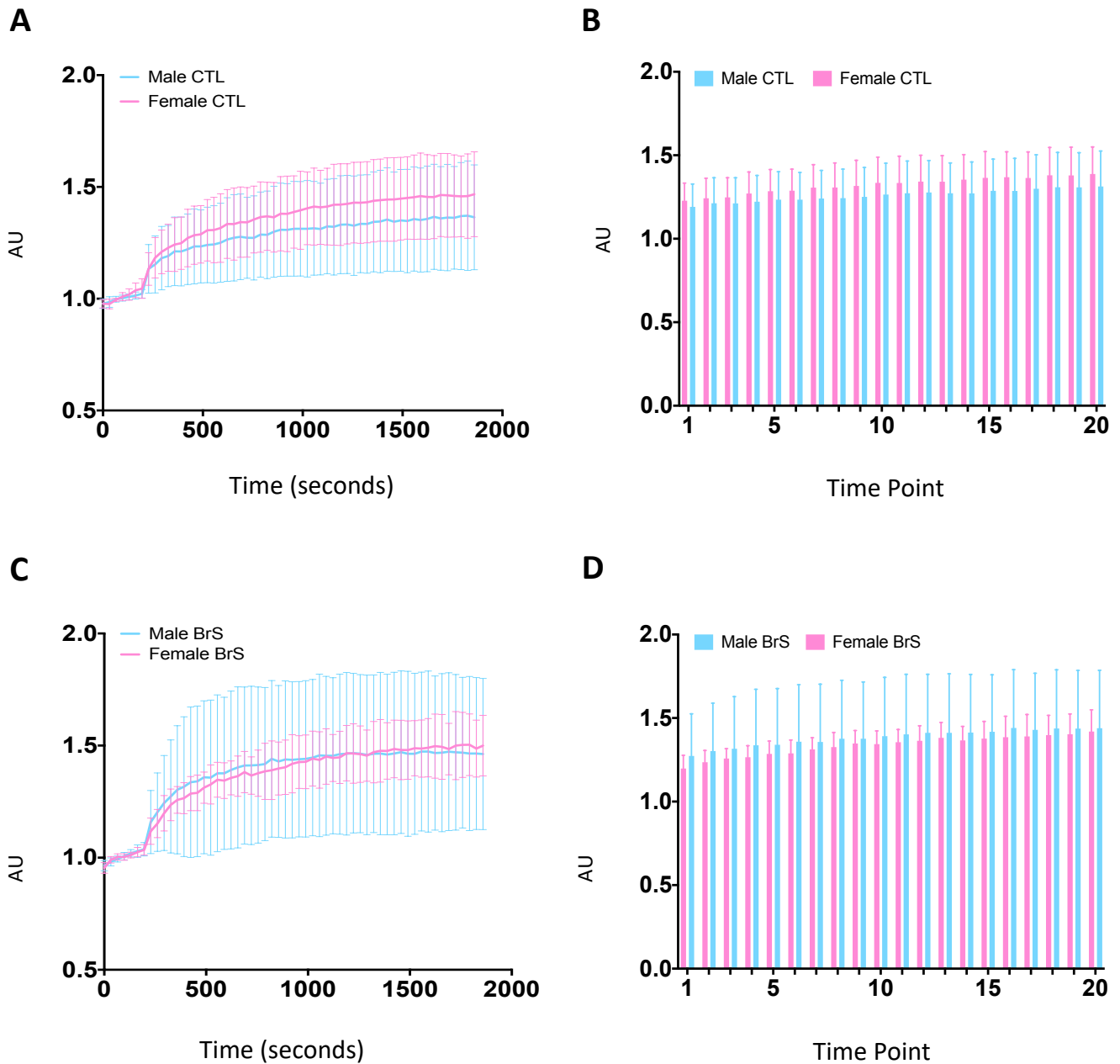


Figure 16. Fura-2 AM assay on T lymphocytes cells. **A.** Male healthy controls vs Female Male healthy controls **B.** Statistical analysis of male healthy controls vs female Male healthy controls. **C** Male BrS vs Female BrS. **D.** Statistical analysis of male healthy controls vs female healthy controls. Number of samples: 20 for each condition.

As expected, no gender-based differences between female BrS patients and their controls, and male BrS patients and their controls were detected (Figure 17A, 17B, 17C, and 17D).

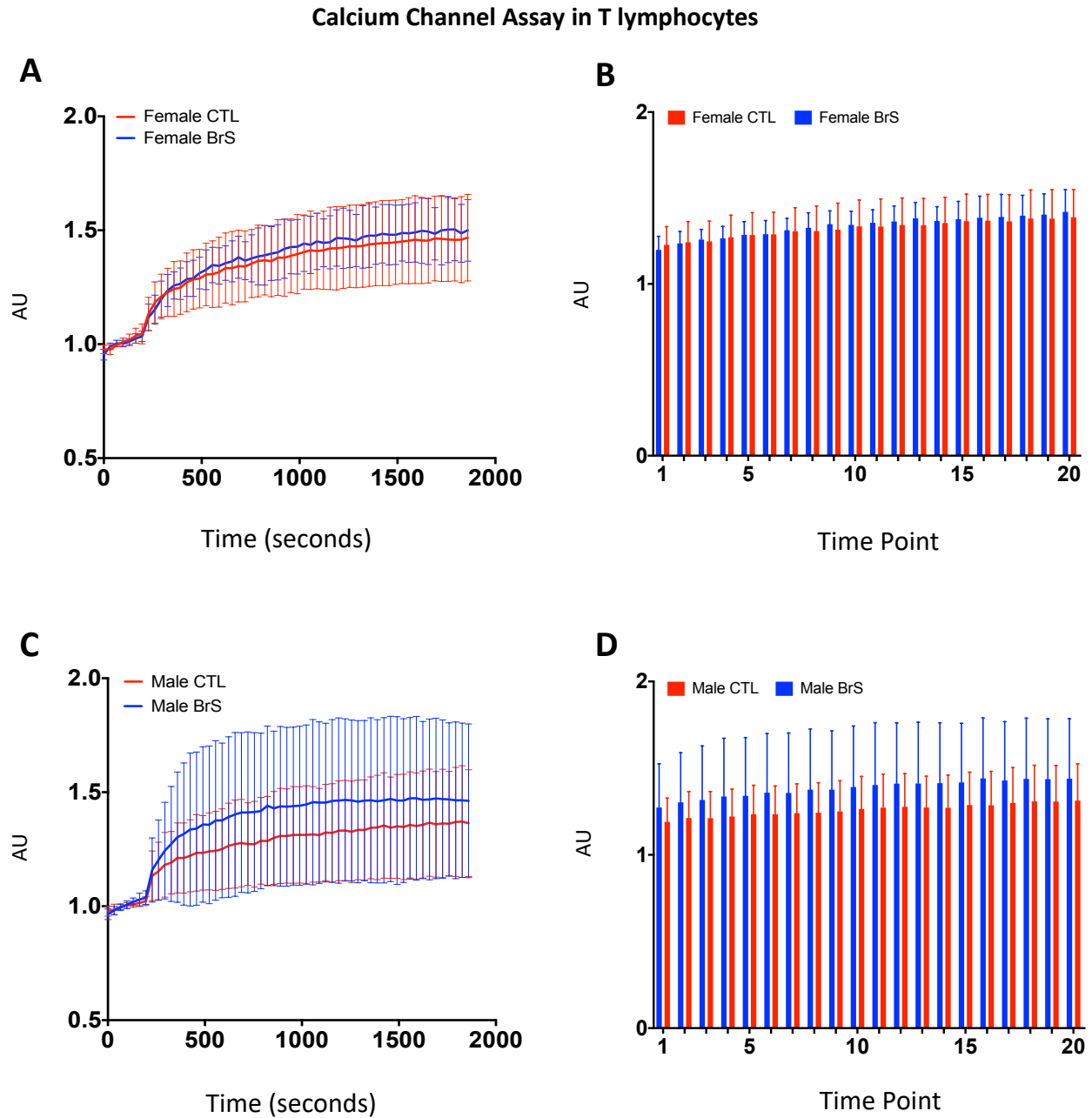


Figure 17. Gender dependence of calcium expression in BrS patients. **A.** Male BrS patients vs male healthy controls. **B.** Statistical analysis of male BrS patients vs male healthy controls. **C.** Female BrS patients vs female controls. **D.** Statistical analysis of female BrS patients vs female controls. Number of samples: 20 for each condition.

3.7 Fluxor™ II Green Potassium *in vitro* model

Potassium channel assay is based on the measurement of the intracellular thallium flux through the potassium channels, opened with a depolarizing stimulus.

After assay optimization, the potassium channel activity was analyzed between BrS patients and controls in PBMCs. No significant differences were detected between BrS patients and controls in all the conditions tested (Figure 18).

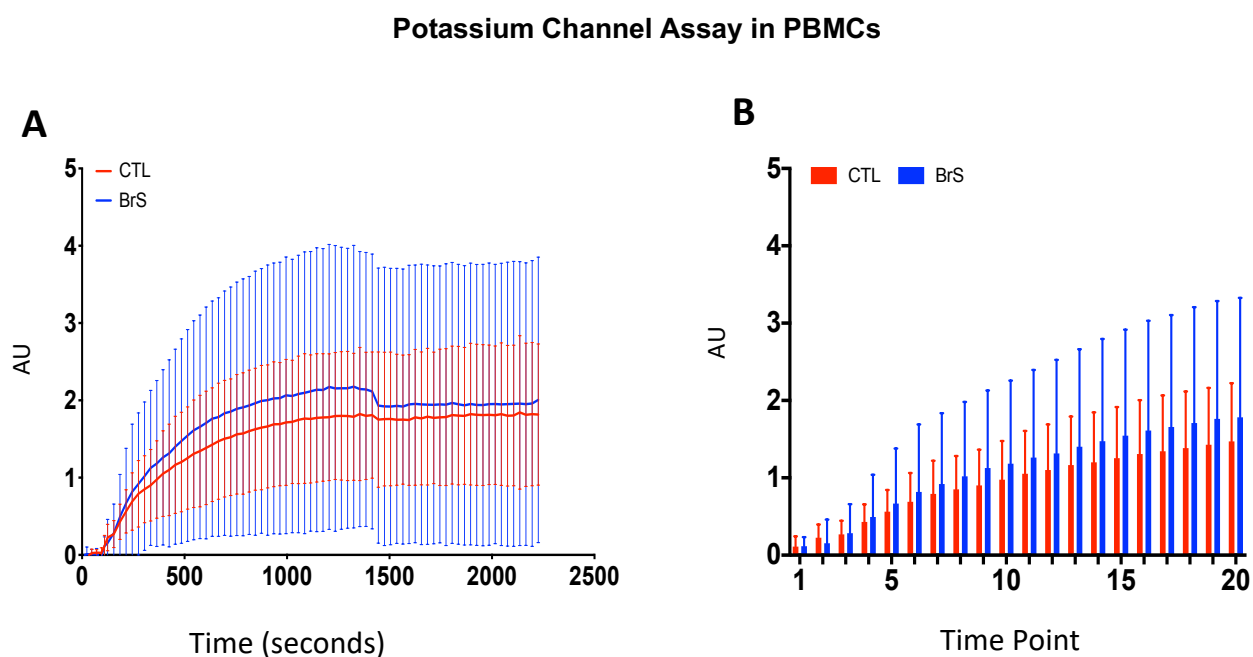


Figure 18. FluxOR™ assay on PBMCs. **A.** BrS patients vs healthy controls. **B.** Statistical analysis of BrS patients vs healthy controls. Number of samples: 20 for each condition.

In addition, no differences were detected in a gender-based analysis between male and female BrS patients, nor between male or female healthy controls in PBMCs (Figure 19).

Potassium Channel Assay in PBMCs

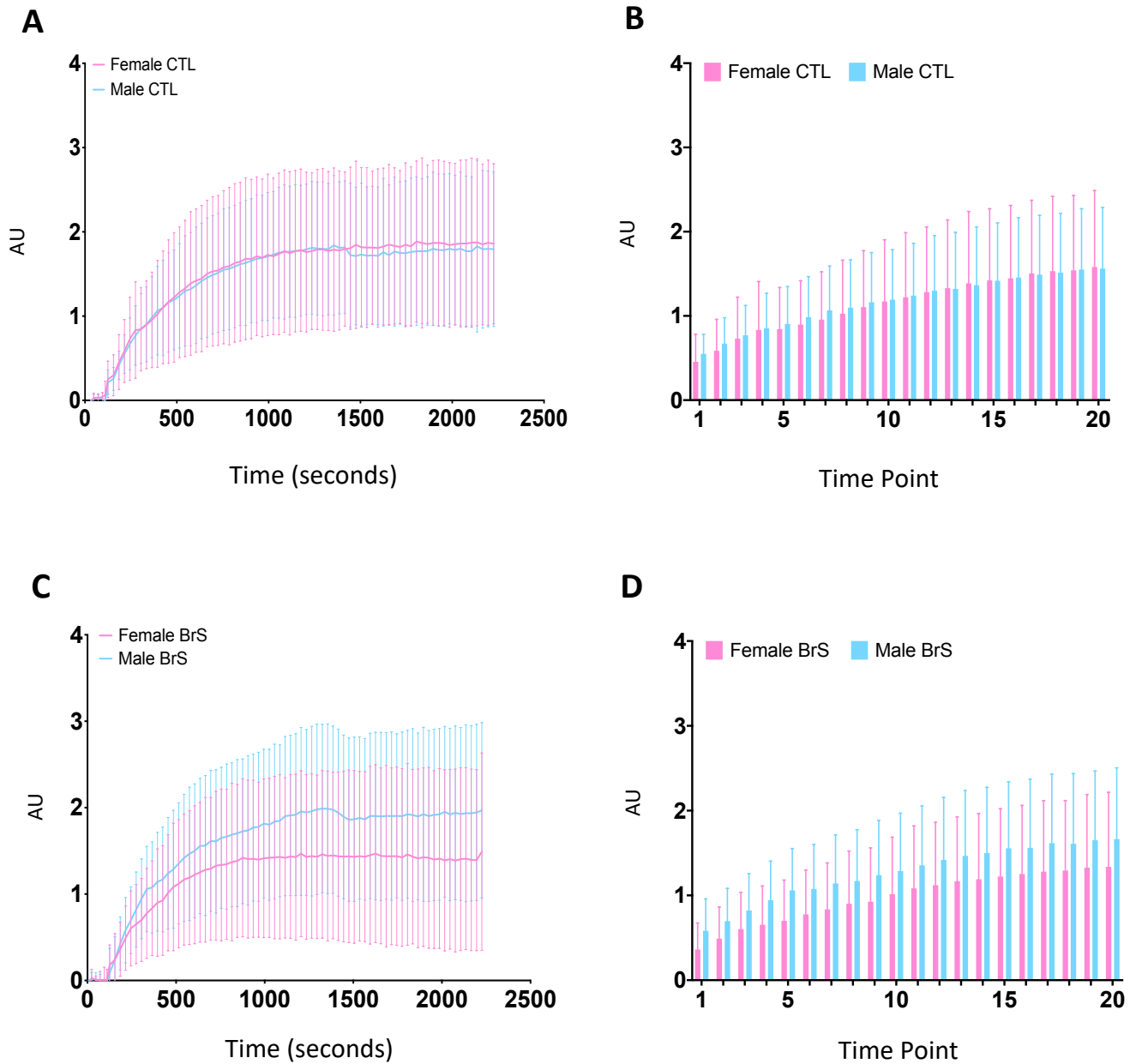


Figure 19. FluxOR assay. **A.** Male and female healthy controls. **B.** Statistical analysis of male and female healthy controls. **C.** Male and female BrS patients. **D.** Statistical analysis of male and female BrS patients. Number of samples: 20 for each condition.

Then, we performed the potassium channel functional assay on a specific PBMCs sub-population, in particular the T lymphocytes. As shown in Figure 20, we observed significant differences in potassium channel activity between BrS patients and controls after stimulus. Specifically, we observed a 2-fold-increase (Figure 20A) of the potassium flux in BrS patients from time point 15 to time point 20 after channel opening (Figure 20B).

Potassium Channel Assay in T lymphocytes

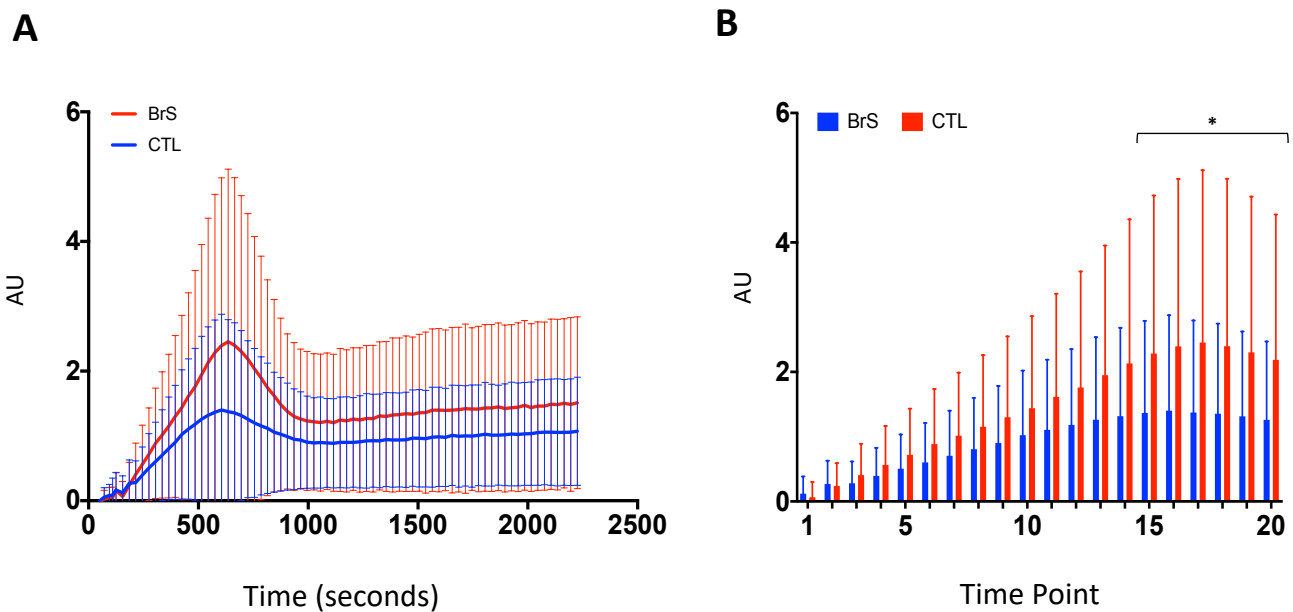


Figure 20. FluxOR™ assay on T lymphocytes. **A.** BrS patients vs healthy controls. **B.** Statistical analysis of BrS patients vs healthy controls. Number of samples: 20 for each condition. * $p < 0.05$

Then, we explored the gender dependence of potassium current in T lymphocytes cells. As shown in Figures 21A and 21B, we observed a 2.27-fold increase in the potassium flux of male healthy controls, as compared to female healthy controls, while in BrS we did not detect any significant difference in potassium flux between males and female (Figure 21C and 21D).

Potassium Channel Assay in T lymphocytes

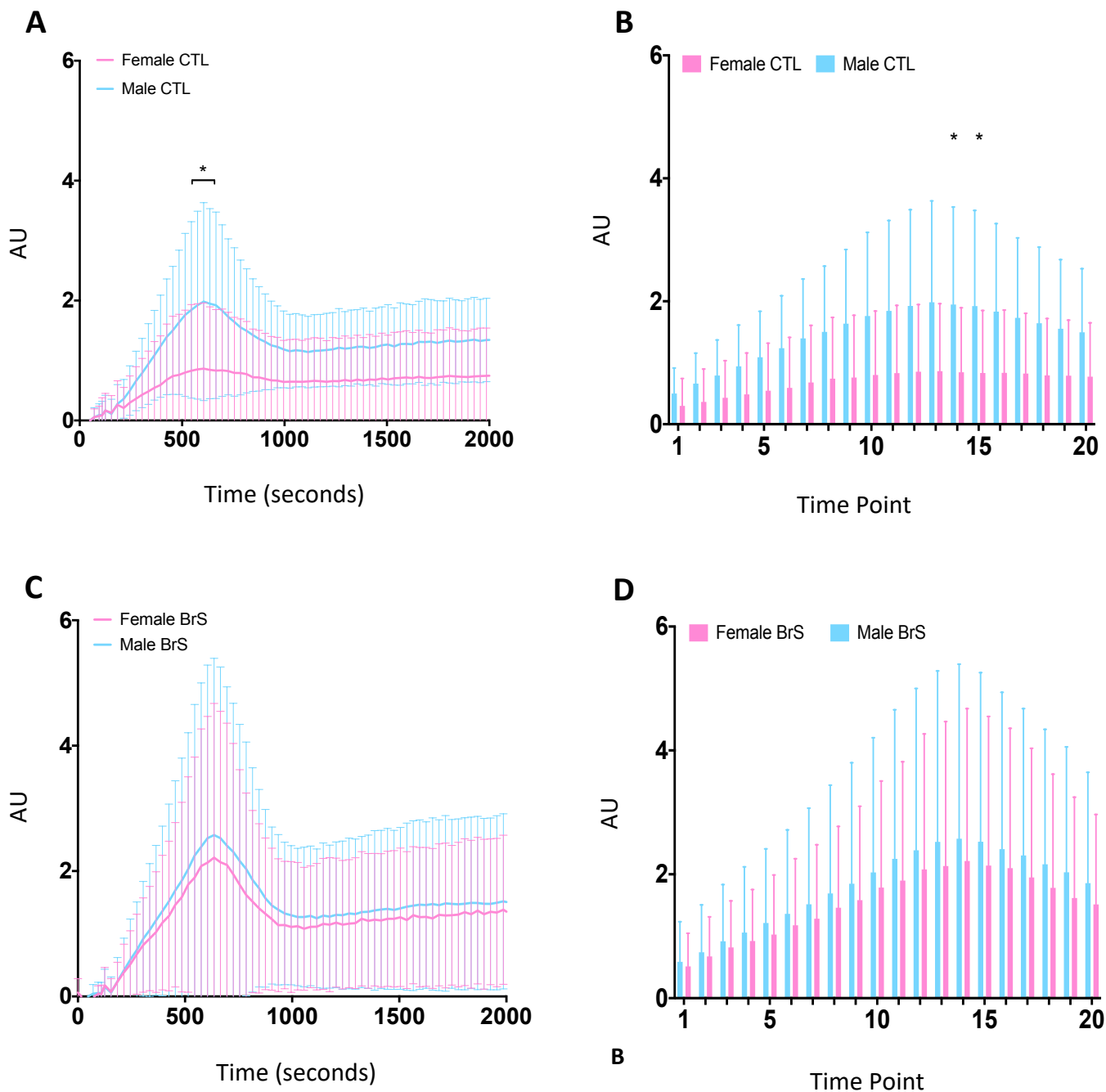


Figure 21. FluxOR™ assay on T lymphocytes. **A.** Male BrS healthy controls vs female BrS healthy controls. **B.** Statistical analysis of male BrS healthy controls vs female BrS healthy controls. **C.** Male BrS patients vs female BrS patients. **D.** Statistical analysis of male BrS patients vs female BrS patients. Number of samples: 20 for each condition. * $p < 0.05$

Finally, we explored the gender-dependence potassium channel activity in T lymphocytes cells. Figures 22A and 22B show no difference in potassium flux between male BrS patients, as compared to their controls. While, in Figures 22C and 22D, we observed a significant 2.52-fold increase in the potassium flux of female BrS patients, as compared to their controls.

Potassium Channel Potassium Channel Assay in T lymphocytes

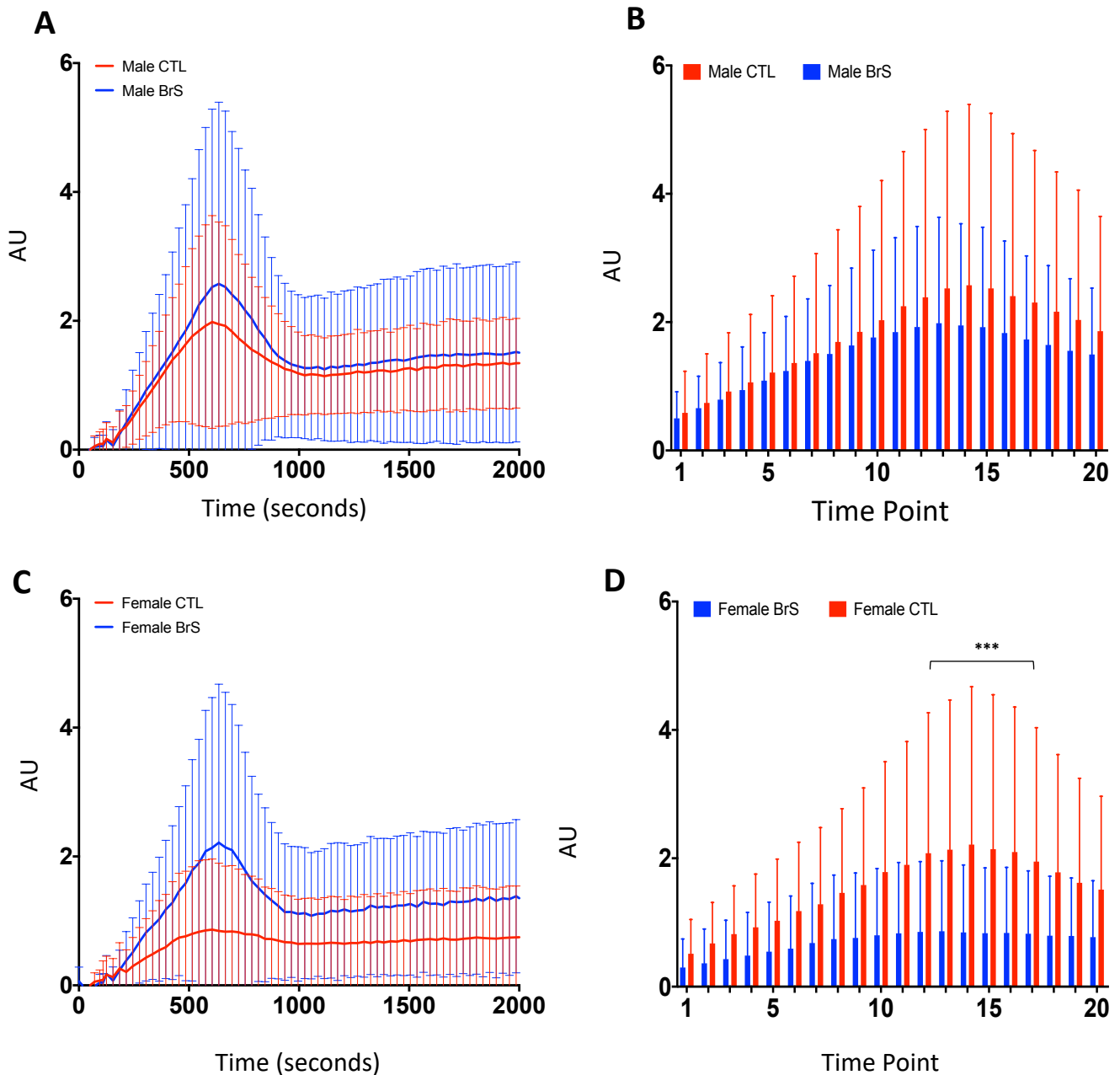


Figure 22. FluxOR™ assay on T lymphocytes. **A.** Male BrS patients vs healthy male controls. **B.** Female BrS patients vs healthy female controls. Number of samples: 20 for each condition. * $p < 0.05$, *** $p < 0.001$

Chapter 4

Discussion and Conclusions

BrS is a hereditary channelopathy related to an increased risk of SCD. Currently, from a clinical point of view, the disease is diagnosed through the identification of a peculiar ECG pattern and treated by an implantable cardioverter-defibrillator, followed by an experimental epicardial radiofrequency ablation of the arrhythmogenic pathological substrate, located in the RVOT. The ECG pattern could be either spontaneous or drug-induced by the administration of a sodium-channel blocker. To date, in the case of a non-spontaneous pattern, the drug-induced represents the only reliable diagnostic test. However, this procedure presents some critical limitations, as it is intrinsically dangerous as it could induce ventricular fibrillations, eventually leading to cardiac arrest. Thus, the test has to be performed only in highly specialized centers. Indeed, this represents a critical drawback, as it does not allow to a safe screening of the general population, thus probably causing an underestimation of BrS prevalence. Another critical point of BrS is represented by the risk stratification of BrS patients. Today, it only includes clinical features, such as the presence of spontaneous type I ECG pattern, of aborted sudden cardiac arrest events, as well as patients' age, gender, SCD familiarity, ECG variables, and SCN5A mutations. Thus, it would be instrumental to find biological parameters to improve the precision in BrS risk stratification. Moreover, from a biological point of view, the BrS pathogenic mechanisms are still under debate: different theories have been proposed, *i.e.* the repolarization or the depolarization model [154]. However, at the moment, there are no experimental evidences that clearly define the BrS etiology. All the theories imply a disfunction of the cardiac ion channels, especially the NaV1.5, responsible for the initiation and propagation of the action potential [150]. This aspect is supported by the fact that the SCN5A gene represents the most mutated gene in BrS [18], even if mutations in the gene encoding for the calcium and potassium channels have been detected in BrS patients. As a consequence, also calcium and potassium channels could have a role in the development of the pathology. Thus, ion channels probably represent the most important player of BrS onset, and the correct evaluation of their activity, both in mutated and unmutated BrS patients is a fundamental aspect to increase the knowledge and the comprehension of the disease.

For these reasons, in this thesis we focused our efforts on the optimization of fluorescent-based ion channels functional assays to develop rapid and easy tests to be applied with a HTS approach on BrS

patients, to gain new information that could be useful to improve the diagnosis, the patient management, and the biological comprehension of the disease.

Initially, we developed a fluorescence *in vitro* assay to evaluate the activity of NaV1.5 sodium channel. Our main goal, during this first part of this Ph.D. project, was to provide a rapid tool in order to screen the effects of SCN5A variants on channel functionality. Among the various cell systems reported in the literature [155], HEK293A cells were chosen for the *in vitro* experiments as they do not express the endogenous NaV1.5 channel. Moreover, these cells have no incoming currents because they minimally express native channels, therefore they are routinely used in electrophysiology studies [152]. The assay was based on the FRET technique which offers advantages in terms of sensitivity and speed of response. In particular, we employed Disbac₂(3), which is a dye sensitive to changes in the membrane potential. In literature, specific dyes for sodium are reported, such as Corona and SBFI [156], however, based on the literature and our experience, substantial transmembrane dye leakage and low sensitivity made these two sodium dyes less reliable compared to Disbac₂(3) dyes [157][156]. Thus, considering that HEK293A cells do not present incoming ion currents (nor endogenous sodium channel expression), and that we used the sodium channel agonist veratridine as the stimulus agent, we are confident that, in our experimental system, we measured the NaV1.5 channel activity, using a membrane potential indicator. The results of the assay gave an estimation of the general functionality of the channel, without specifically determining its biophysical characteristics, such as the rate of inactivation, time of inactivation, and gating properties. These aspects are usually studied with classic electrophysiology techniques such as patch-clamp [158], and a synergy between these two techniques could be an excellent approach for a future project aimed at the complete characterization of the effects of SCN5A mutations on the NaV1.5 channel.

During this Ph.D. project, we also have analyzed four de novo NaV1.5 mutations, three classified as VUS and one as Likely Pathogenic by VarSome. *In silico* analysis suggested a potential pathogenicity for all these mutations and in fact, by analyzing the degree of conservation of the mutated amino acids, both in the paralogues and in the orthologues, a high degree of conservation has been observed. This obviously suggested the importance of these amino acids within the protein and justified the almost total loss of function observed in the homozygous experiments, as compared to the wild type channel. Since, the four mutations are located in different areas of the protein, the mechanisms through which they can cause any dysfunction are probably different. Further studies are needed to better clarify this aspect. Furthermore, an interesting aspect concerns the functional test carried out under heterozygous conditions to replicate each patient's condition: in three out of four mutations there was an almost total recovery of sodium fluxes within the cell, as compared to wild type NaV1.5. This underlies that these

variants are detrimental for the channel, but it also indicates that in BrS patients with heterozygous mutations, the effects of these mutations may not be so detrimental, similarly to what we observed *in vitro*, as the co-expression with the wild type channel could often offset the effect of the mutations. Nonetheless, mut5089 even in the heterozygous model, the sodium flux was deeply reduced as compared to the wild type. This supports that the mutation is able to interfere with the wild type channel activity through a mechanism that need to be further investigated. It has been demonstrated that different channel mutations can lead to different BrS patients' phenotypes [34], possibly because the mutations can cause a different degree of channel dysfunction. However, to detect a possible correlation between channel function and patient phenotype, it will be necessary to increase the number of analyzed mutations. A speculative preliminary result was obtained from the analysis of the correlation between the predictive values of Panther software and some clinical data. Our SCN5A mutation database included 106 subjects; a probable pathogenicity index was calculated for all the mutations and then these values were correlated with different clinical parameters including age, sex, symptoms, ECG pattern type, pre- and post-ajmaline substrate areas. Indeed, a significant direct correlation was highlighted between the pathogenicity index and the post-ajmaline substrate area. Currently, this value is used as an index of the severity of the disease, as it indicates the area with altered membrane potentials and therefore more predisposed to the development of arrhythmic events. The positive correlation found in our predictive model, although still very preliminary, suggests to investigate in more depth the relationship between mutations and phenotype. A simple correlation between functional experimental data and phenotype is quite unrealistic, and other variables like age, sex, presence of other mutations, will have to be taken into account. However, this study could lead to the development of a predictive model based on the functionality of the channel which could be useful for the risk stratification process. Furthermore, given the importance of the SCN5A in the development of other cardiac pathologies, such as LQTS, AS, and CDM [87], [88][97], our *in vitro* assay could also be applied for other pathologies and not only for BrS.

The second part of this Ph.D. thesis was focused on the development of two functional assays for the analysis of calcium and potassium channels. Specifically, we wanted to screen the activity of these channels in blood cells, both in heterogeneous and specific cells population. We hypothesized that PBMCs from BrS patients could reflect the molecular alterations present in the heart. PBMCs were already characterized as a source of potential biomarkers and used for the study of molecular alterations in various diseases, including heart disease. For example, mitochondrial dysfunction, ROS production, and the mitochondrial DNA releases in patients with cardiac disease have been considered as

biomarkers for cardiovascular diseases [137]. Furthermore, we focused on calcium and potassium ion channels since dysfunctions in these channels have been found in BrS [122], [159]. Thus, our goal was to evaluate two aspects: firstly, if these alterations could be reflected in peripheral cells; secondly, if we could develop a diagnostic test based on the ion channel activity variations found between BrS patients and healthy controls. Therefore, as for the sodium assay, we developed a fluorescent assay for a useful clinical HTS, in particular, for the diagnosis of BrS.

Alteration of calcium channels was studied through the combination of ionomycin as an ionophore and two different assay buffers. As a matter of fact, ionomycin is able to increase intracellular calcium concentrations by the activation of the calcium influx through the native calcium channels, the phospholipase C-dependent mobilization of calcium directly correlated to the intracellular stores, and by a calcium store-regulated mechanism [153]. Indeed, this assay allows the investigation of the release of calcium from internal storage and the activity of the membrane channels responsible for the calcium influx. Therefore, the dynamic release of calcium from its storages was analyzed in the absence of extracellular calcium in the buffer. Our results showed no difference in either the speed or the amount of calcium released by the storage, suggesting that, in these cells, the calcium storage does not present alterations between BrS patients and healthy controls. However, when we focused our attention on the membrane calcium channel activity, BrS patients showed a higher calcium flux as compared to the healthy controls. Unfortunately, it is not possible to know if these alterations are due to different levels of expression of the membrane channels or by an alteration in the intrinsic activity of the calcium channels. Further experiments will be focused on the evaluation of the expression levels of the calcium channels and on the presence of other factors that could affect the channel functionality, as post-translational modifications that may affect their functionality. In addition, as PBMCs are a mixed population of lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells, we then assessed if T lymphocytes were responsible of the experimentally observed differences. Unfortunately, we did not detect significant differences in calcium fluxes between the T lymphocytes of BrS patients and controls. This indicated that the differences observed in PBMCs were probably given by a different cell population that needs to be investigated.

Regarding the potassium channel, the functional assay performed on PMBCs did not highlight any significant differences between BrS patients and healthy controls. However, by carrying out the assay on T lymphocytes, we highlighted a significant difference between BrS potassium flux and healthy controls; specifically, an increase of the potassium current was observed in BrS patients after the channel opening. Moreover, when we explored the gender-dependence potassium channel activity we observed

a significant difference between female BrS patients and healthy controls. Therefore, the heterogeneous population of PBMCs partially masked the difference found in T lymphocytes. Unlike what was detected in the calcium assay, where we observed a general alteration of calcium fluxes, the potassium assay showed significant differences in the initial stages of fluxes. Unfortunately, even in this case, we could not establish whether the difference was given by an altered expression or activity of the channels; further experiments will be required to elucidate this aspect.

Overall, these results helped us to identify calcium and potassium fluxes alterations in blood cells. Unfortunately, to date, the differences detected are not sufficient to clearly separate BrS patients from controls. However, this aspect is not totally surprising, as BrS patients do not present relevant clinical manifestations related to the immune system. Indeed, it has been shown that ion channels control fundamental functions of the immune response of blood cells [160], [161]: *e.g.* (1) K^+ ions indirectly control the influx of calcium, the immune cell signaling [162], the T lymphocytes activation [163], proliferation, and secretion of cytokines and chemokines [162]; (2) Ca^{2+} influx through CRAC channels are fundamental for the development of T and B lymphocytes [162]. Therefore, differences in ion channels activity would also have been accompanied by clinical manifestations. However, these data are very important as they confirmed that PBMCs could be used for the study of molecular alterations of the disease and they showed differences that made them suitable candidates for biomarker discovery. Further investigations are needed to understand if these alterations are responsible for the pathogenetic process or if they are due to other alterations, such as a chronic inflammatory state.

Another important aspect found in the analyses was the difference in ion fluxes between males and females. In particular, it was observed that males generally have a greater flux of both calcium ions and potassium ions, both in PBMCs and T lymphocytes, probably due to the effects of male hormones. Indeed, it has also been demonstrated that testosterone and other androgens are able to regulate the expression of calcium and potassium channels. Specifically, androgens are able to increase the expression of L-type Ca^{2+} channels and Na^+-Ca^{2+} exchanger [164][165], and it also enhances the expression of potassium channels [166]; while estrogens modulate the calcium transport pathways like Ca^{2+} -activated potassium channel subtype 3 [167], Na^+-Ca^{2+} exchanger [168], and regulates potassium (Kv10) channels expression [166]. Our analyses showed that there was a significant difference in the calcium fluxes in male PBMCs, while significant differences were found in the female potassium fluxes of T lymphocytes. Therefore, these data could provide the basis for the development of gender-based diagnostic tests.

In conclusion, during this Ph.D. work, we developed and tested fluorescent functional assays for the evaluation of the activity of NaV1.5 in an *in vitro* system and for the evaluation of calcium and potassium fluxes in peripheral blood cells. The application of these tests has provided the basis for future research aimed to improve the diagnosis, the management, and understanding of BrS. In particular, the systematic study of sodium channel activity of BrS patients' mutations will create a functional database of SCN5A mutations and could allow the development of a predictive model for risk stratification in BrS. This model could also allow a personalized clinical approach for each individual patient. The data obtained on blood cells, on the other hand, provided a new insight for both the identification of possible blood biomarkers useful for the diagnosis of BrS and for the definition of molecular pathways responsible for the onset of the disease.

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