SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of School of Medicine

PhD program in Neuroscience, Cycle XXXIII

Curriculum in Clinical Neuroscience, Executive

Role of Ryanodine Receptor type 3 (RyR3) in ischemic stroke

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ACADEMIC YEAR 2019/2020

Abstract

Stroke is a leading cause of mortality and acquired disability worldwide. The first genome-wide association study in Italian ischemic stroke patients found a significant association with the missense single nucleotide polymorphism (SNP) rs4780144 in the ryanodine receptor type 3 (RyR3) gene, which leads to a potential loss of function. Multiple evidences suggested that a reduced function of RyR3 could improve stroke outcome.

With this study we aimed at investigating the role of RyR3 in ischemic stroke at functional, genomic, and cellular level.

Adipose-derived mesenchymal stem cells (Ad-MSCs) express RyR3 but not the other ryanodine receptors (RyR1 and RyR2). We assessed the effect of the rs4780144 genotype on intracellular calcium homeostasis in Ad-MSC lines. Our results confirmed the reduction of the RyR3 function (reduced release of calcium ions into the cytoplasm) in Ad-MSCs with the mutated alleles, which was statistically significant in homozygous cells.

A second cohort of 319 Italian ischemic stroke patients with good clinical outcome was genotyped with Illumina Human-24 720. Genotypes of cases (both first and second cohorts) and controls were imputed using the TOPMed reference panel of human haplotypes. This second GWAS replicated the association with rs4780144 and other SNPs in the RyR3 gene.

Finally, ischemic injury induced by oxygen-glucose deprivation (OGD) was evaluated in organotypic brain slices from wild-type and RyR3-knockout mice. RyR3- knockout showed a decreased susceptibility to ischemic damage compared to wild-type slices as indicated by the reduced propidium iodide incorporation and LDH release at 48 and 72 h after OGD. RyR3-knockout slices showed also a reduced swelling after OGD, indicating reduction of cytotoxic edema. However, to date no significant differences were found in gene expression analysis performed on neuronal and oxidative stress related genes.

The results of this work confirm that RyR3 may play a role in ischemic stroke. In particular, the inhibition of RyR3 could positively modulate ischemic damage, resulting in a new and promising neuroprotective strategy in patients with acute ischemic stroke. However, further studies are needed to clarify the mechanisms that could possibly underlie the observed neuroprotection.

Acknowledgements

- Emilio Ciusani (Department of Diagnostics and Technology, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milano, Italy) extracted DNA from ischemic stroke patients;
- Maria Grazia Franzosi and Simona Barlera (Department of Cardiovascular Research, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy) provided control genotypes of healthy subjects;
- Anna Maria Di Blasio (Medical Genetics Laboratory, Istituto Auxologico Italiano, Milano, Italy) provided control genotypes of healthy subjects;
- Steven Kittner (University of Maryland School of Medicine, Baltimore, MD, US) imputed genotypes of cases and controls;
- Barbara Viviani (Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milano, Italy) performed the functional studies on adipose-derived mesenchymal stem cells;
- Silvia Bione (Computational Biology Unit, Istituto di Genetica Molecolare Luigi Luca Cavalli-Sforza, Consiglio Nazionale delle Ricerche, Pavia, Italy) performed the GWAS in the Italian cohort;
- Elisa Zanier (Laboratory of Acute Brain Injury and Therapeutic Strategies, Department of Neuroscience, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy) performed the cellular studies in the RyR3-knockout mouse model.

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List of Common Abbreviations

Ad-MSCs	Adipose-derived mesenchymal stem cells
CT	Computed tomography
CTA	Computed tomography angiography
EKG	Electrocardiogram
ER	Endoplasmic reticulum
GWAS	Genome-wide association study
HO-1	Heme oxygenase-1
KO	Gene knockout
LDH	L actate dehydrogenase
MAP2	Microtubule associated protein 2
MRI	Magnetic resonance imaging
MRA	Magnetic resonance angiography
NQO1	NAD(P)H:quinone oxidoreductase 1
OGD	Oxygen and glucose deprivation
PCA	Principal component analysis
PCR	Polymerase chain reaction
PI	Propidium iodide
RyR	Ryanodine receptor
RyR3	Ryanodine receptor type 3
SNP	Single-nucleotide polymorphism
SOD1	Superoxide dismutase 1
TIA	Transient ischemic attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
WT	Wild-type

INTRODUCTION AND BACKGROUND

Ischemic stroke and novel treatment paradigms

After ischemic heart disease, stroke is the leading cause of mortality worldwide. Stroke is also the primary cause of long-term acquired disability. Despite a pronounced decrease in age-standardized mortality rates in the last decades, stroke burden is likely to remain high. People living with stroke in the European Union is estimated to increase by one third between 2017 and 2047. This shift in stroke burden from mortality to morbidity is likely to be due to demographics change over time, the implementation of primary prevention strategies, and the availability of better care and treatment both in the acute and long-term stages after stroke [Wafa 2020]. Ischemic stroke has also substantial indirect costs related to complications, such as post-stroke dementia, depression, and falls, thus having a strong emotional and socioeconomic impact on patients and on the national health system [Hankey 2017].

About 85% of strokes are ischemic, caused by the interruption of blood flow in a brain-supplying artery; this lead to irreversible cell-damage in ischemic core, surrounded by a hypoperfused penumbra region. In the penumbra, the tissue is not directly damaged by the stroke but hypoperfusion and metabolic changes put it at risk of delayed cell death. The rescue of the ischemic penumbra influences the functional recovery and represent the target of the available therapies [Hankey 2017; Powers 2018]. In the last two decades, with the widespread adoption of organized stroke units, some reperfusion interventions given in the acute phase, such as intravenous recombinant tissue plasminogen activator and endovascular mechanical thrombectomy for large artery occlusion, have improved survival and residual disability [Powers 2018]. However, due to the selective criteria, only a minority of patients benefit from these therapies [Hankey 2017]. Therefore, once brain damage has occurred, little can be done to improve functional outcome, except for rehabilitation therapy and pharmacological management of co-morbidities, with very large healthcare and social costs. There is a strong demand for alternative therapeutic approaches.

While the primary aim of stroke therapy is to restore blood flow to the brain, without exacerbating the damage already caused by depriving the tissue of oxygen and glucose, the secondary aim is to modulate any factors that may exacerbate this damage. Therefore, in addition to recanalization and optimal management of different risk factors (blood pressure, glycemic profile, etc.), treatment with compounds designed to protect brain cells during ischemia seems a reasonable option. Several key players in ischemic cell death within the penumbra have been identified, including excitotoxicity, oxidative and nitrosative stress, and inflammation. Among these, excitotoxicity was the first molecular mechanism to be identified and the most intensively studied: it refers to the massive accumulation of glutamate as a result

of energy failure, which overactivates a plethora of downstream signaling pathways, many of which involve a surge in calcium influx, causing the intracellular calcium concentration to increase [Chamorro 2016]. However, over the past quarter century, quite literally hundreds of putative neuroprotectants have been evaluated in preclinical models, but not one has entered the clinical realm, despite promising preclinical data. Different specific problems were charged with prior translational failures, including poor relevance and validity of preclinical models (mostly young animals without comorbidities) and selection, assessment and attrition bias [Neuhaus 2017]. Yet, with the advent of endovascular thrombectomy and the ability to investigate patients in much greater detail through advanced imaging modalities, neuroprotective agents are being re-examined as adjunct therapies to recanalization. Looking back at prior clinical trial failures, there was a profound lack of attention to the recanalisation status of patients. One lesson from animal studies that likely applies to human patients with stroke is that brain protective therapies are much more likely to succeed when used in reversible occlusion models, that is, the animal version of endovascular thrombectomy [Chamorro 2021].

Finally, the emergence of 'omics approaches (genomics, next generation sequencing, transcriptomics, proteomics and metabolomics) provide us with new insights into the basic mechanisms of stroke-related damage and repair processes in humans and pinpoint new disease-specific targets. This has the potential to be the beginning of an exciting new field of target discovery in stroke research [Neuhaus 2017]. For example, the largest multiancestry genome-wide-association meta-analysis in 67.162 stroke cases and 454.450 controls, which included our Italian cases and controls, led to the discovery of 11 new susceptibility loci that indicate mechanisms not previously implicated in stroke pathophysiology; another interesting results was that the identified stroke risk loci were significantly enriched in drug targets for antithrombotic therapy [Malik 2018].

First Italian GWAS in ischemic stroke patients

At Besta Institute, from November 2001 to June 2009, 722 consecutive patients with cerebrovascular disease evaluated in the Cerebrovascular outpatient clinics, as well as in the inpatient Cerebrovascular Unit, were included in the Cerebrovascular Diseases Registry (CEDIR). All subjects were examined by a neurologist, provided informed consent and had DNA extracted. In 2009 genotyping was carried out using the Illumina Human610-Quad v1_B or Human660W-Quad v1_A® accordingly to the manufacturer's protocols.

Managing of genotype data and quality control procedures were performed with PLINK 1.0.7 [Purcell 2007]. Samples were excluded due to unexpected duplicates (n=1), sex mismatch

between CEDIR and genomic (n=4) or evidence of non-European ancestry based on principal components analysis (n=3). Participant-specific quality-control included filters for call rate (>95%, n=5), heterozygosity, and number of Mendelian errors per individual. SNP-specific quality control included filters for minor allele frequency (>0.05, n=39.991) and Hardy-Weinberg equilibrium (P-value >1×10-6, n=565). Before the following filters were applied we excluded SNPs with more than 10% missing rate.

Controls were Italian individuals without cardiovascular diseases that were genotyped with the Illumina HumanHap610-Quad chip within two different studies:

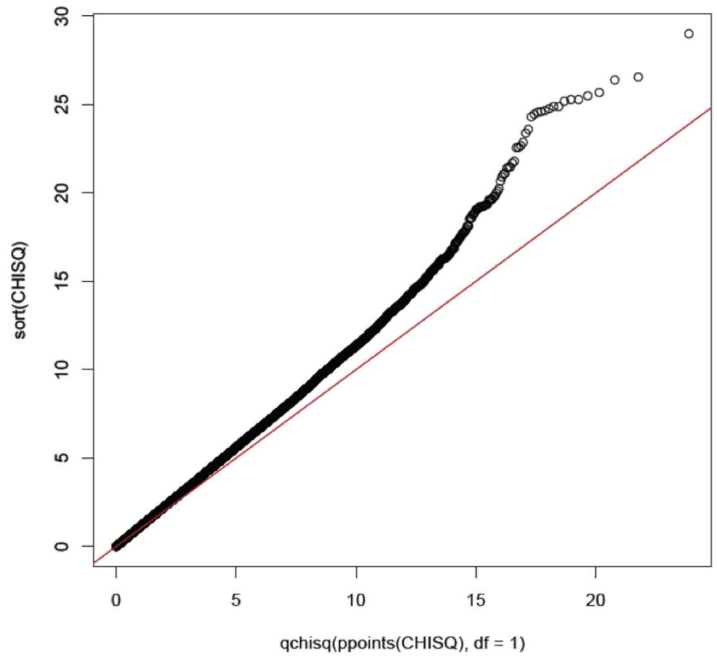
- 409 subjects were enrolled at Mario Negri Institute in the PROCARDIS (PRecOcious Coronary ARrtery DISease) Study, with no personal or sibling history of cardiovascular diseases before age 66 years [Clarke 2009];
- 547 subjects were enrolled at Auxologico Institute in a study on obesity [Berndt 2013].

Genotypes of control cases had already passed the quality control procedures.

Principal component analysis (PCA) with HapMap 3 [https://www.sanger.ac.uk/resources/downloads/human/hapmap3.html] on the Italian cases showed that Italian controls had similar ancestry to the cases.

The final cohort included 709 cases and 956 controls and analyzed 487.758 SNPs.

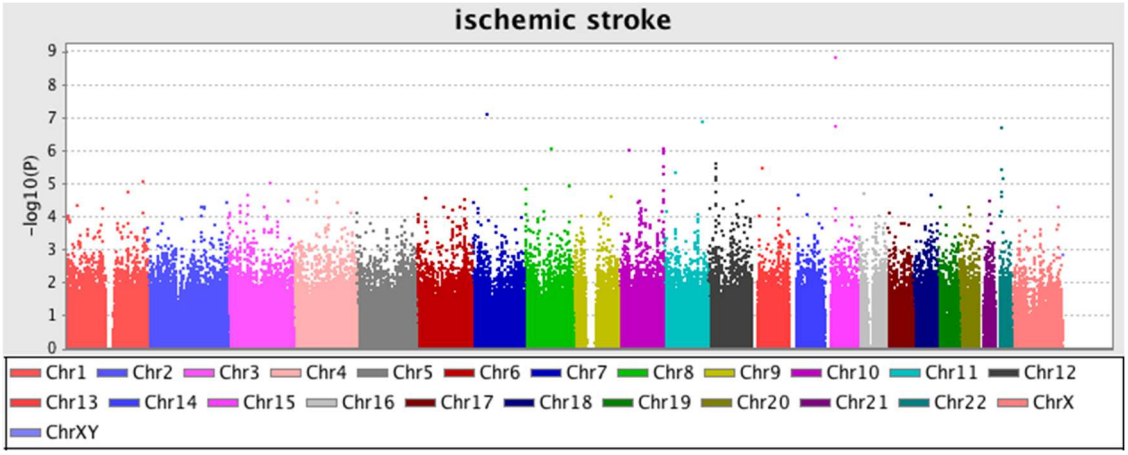
The following Quantile-Quantile Plot (QQ PLOT) for chi-squared tests ranked observed chi-squared test statistics against the corresponding expected order statistics and allows a qualitative evaluation. The resulting graph does not suggest population substructure and other sources of bias.



Within the 709 cases genotyped, 372 had an ischemic stroke, 39 had TIA, 263 had vascular cognitive impairment and 35 had other cerebrovascular diseases (cerebral venous thrombosis, cerebral haemorrhage, etc.).

The first Italian genome-wide association study (GWAS) in ischemic stroke patients was performed on the 372 subjects referred for ischemic stroke, subacute or chronic in most cases. Patients were aged > 18 years and previous ischemic stroke, first ever or recurrent, was confirmed on brain imaging. Ischemic stroke was defined as either (1) a radiographically proven (head CT or MRI) infarct associated with the appropriate clinical stroke syndrome, or (2) a fixed neurological deficit persisting more than 24 hours, consistent with a vascular pattern of involvement and without radiographic evidence of demyelinating disease, or other non-vascular structural disease. Clinical and laboratory data were collected for qualifying ischemic stroke event. Diagnostic work-up included: neuroimaging with head CT or brain MRI (100% - MRI was performed in 86.7%), EKG (100%), cervical and intracranial vessel imaging using CTA or MRA (68%) or carotid and/or transcranial ultrasound (32%), echocardiography (20%), and Holter-EKG monitoring (18%). All cases were phenotyped by an experienced stroke neurologist according to TOAST criteria [Adams 1993], based on relevant clinical imaging and available information on cardiovascular risk factors: 65 patients had cardioembolic ischemic stroke (CE), 74 had ischemic stroke due to large artery disease (LA), 25 had small vessel disease (SV), 56 had ischemic stroke due to other causes (arterial dissection, vasculitis, etc.) (OT), and 152 had ischemic stroke due to undetermined cause (UN). In the following years, this cohort was included in several international studies and contributed to move forward research in ischemic stroke genetics [Traylor 2012; NINDS Stroke Genetics Network 2016; Malik 2018].

The results of the first Italian GWAS in ischemic stroke patients are shown here in the Manhattan plot.



Genomic coordinates are displayed along the X-axis, with the negative logarithm of the association p-value for each SNP displayed on the Y-axis, meaning that each dot on the Manhattan plot signifies a SNP. Because the strongest associations have the smallest p-values (e.g., 10−9), their negative logarithms will be the greatest (e.g., 9). The different colours of each block shows the extent of each chromosome.

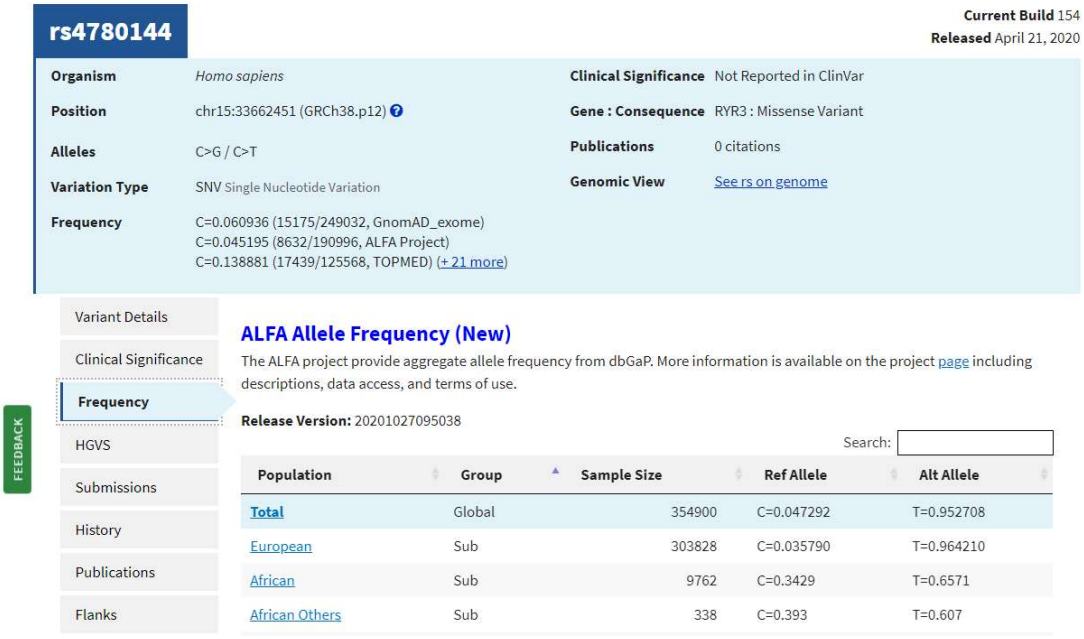
The following table reports the details on the 3 SNPs that reached the statistical significance thresholds (P-value below 5E−08) to differentiate true positives from false positives in GWAS [Fadista 2016].

Chr	SNP	A1	A2	F_cases	F_contr	CHISQ	P value	OR	L95	U95
15	rs4780144	C	T	0.1044	0.04289	36.46	1.56E-09	2.603	1.889	3.585
7	rs17172491	G	A	0.0953	0.04188	28.94	2.47E-08	2.41	1.734	3.349
7	rs11761143	A	G	0.08877	0.0377	28.77	3.16E-08	2.487	1.765	3.503

Chr: chromosome; SNP: SNP ID number according to dbSNP [https://www.ncbi.nlm.nih.gov/snp/]; A1 and A2: allele 1 and 2; F_cases and F_ contr: frequency of allele 1 in cases and controls; CHISQ: chi-squared test; P value: p value of the chi-squared test; OR: odds ratio; L95 and U95: lower and upper values of the 95% confidence interval of the odds ratio.

While the 2 SNPs located on the chromosome 7 mapped on intronic regions, rs4780144, which was the SNP with the strongest association in our ischemic stroke patients (p=1,56E-09, OR=2.6, 95% CI 1.89-3.58), seemed particularly interesting because the substitution of a

cysteine for a thymine (CGC > TGC) in position chr15:33662451 maps on the 35th exon of the Ryanodine Receptor type 3 (RyR3) gene and results in a missense variant of the protein with substitution of arginine in 1641 position for the amino acid cysteine (R1641C). This missense variant has a low prevalence in Caucasians and the frequency of the reference allele C observed in our controls (0.04289) was similar to what is reported in Europeans (0.035790, https://www.ncbi.nlm.nih.gov/snp/rs4780144).



rs4780144

Current Build 154
Released April 21, 2020

Organism	*Homo sapiens*	**Clinical Significance**	Not Reported in ClinVar
Position	chr15:33662451 (GRCh38.p12)	**Gene : Consequence**	RYR3 : Missense Variant
Alleles	C>G / C>T	**Publications**	0 citations
Variation Type	SNV Single Nucleotide Variation	**Genomic View**	See rs on genome
Frequency	C=0.060936 (15175/249032, GnomAD_exome) C=0.045195 (8632/190996, ALFA Project) C=0.138881 (17439/125568, TOPMED) (+ 21 more)		

Variant Details
Clinical Significance
Frequency
HGVS
Submissions
History
Publications
Flanks

FEEDBACK

ALFA Allele Frequency (New)

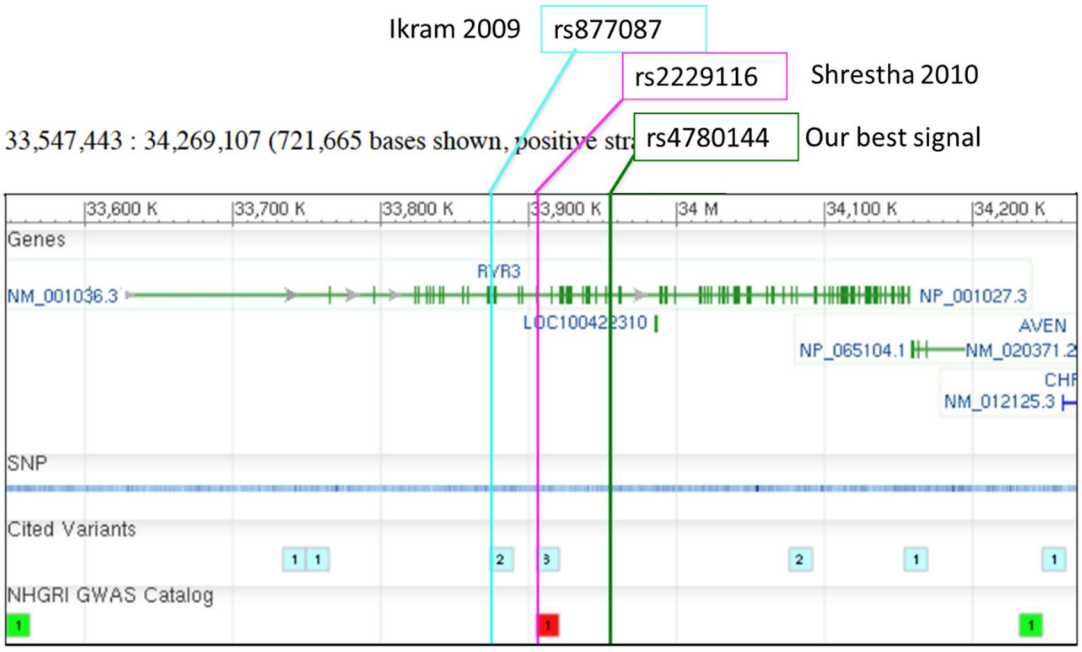
The ALFA project provide aggregate allele frequency from dbGaP. More information is available on the project page including descriptions, data access, and terms of use.

Release Version: 20201027095038

Search:

Population	Group	Sample Size	Ref Allele	Alt Allele
Total	Global	354900	C=0.047292	T=0.952708
European	Sub	303828	C=0.035790	T=0.964210
African	Sub	9762	C=0.3429	T=0.6571
African Others	Sub	338	C=0.393	T=0.607

The same gene was already associated with ischemic stroke [Ikram 2009] and intima media thickness, an intermediate phenotype of atherosclerotic stroke, in HIV infected individuals [Shrestha 2010].



Moreover, commonly used software tools for protein structure prediction [Yazar 2021] like SIFT suggest a significant loss of function in the mutated RYR3.

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PROVEAN

PROVEAN Genome Variants Result - Full version (Download)

Database: human37_66

Note: For each variant **all** protein isoforms are shown.

VARIATION		PROTEIN SEQUENCE CHANGE								PROVEAN PREDICTION				SIFT PREDICTION				ANNOTATION
ROW_NO.	INPUT	PROTEIN_ID	LENGTH	STRAND	CODON_CHANGE	POS	RESIDUE_REF	RESIDUE_ALT	TYPE	SCORE	PREDICTION (cutoff=-2.5)	#SEQ	#CLUSTER	SCORE	PREDICTION (cutoff=0.05)	MEDIAN_INFO	#SEQ	dbSNP_ID
1	15,33954652,C,T	ENSP00000354735	4866	1	ATC [C/T]GC CTC	1641	R	C	Single AA Change	-1.30	Neutral	151	30	0.050	Damaging	2.85	160	rs4780144
		ENSP00000373884	4870	1	ATC [C/T]GC CTC	1641	R	C	Single AA Change	-1.30	Neutral	151	30	0.051	Tolerated	2.85	160	rs4780144
		ENSP00000399610	4865	1	ATC [C/T]GC CTC	1641	R	C	Single AA Change	-1.30	Neutral	151	30	0.050	Damaging	2.85	160	rs4780144

To investigate the association of rs4780144 in our stroke patients (this SNP was available in 359 cases), as a first step, we studied the association between rs4780144 genotype and

possible confounding factors that were recorded in the CEDIR database, such as age at stroke onset, sex, positive family history for cerebrovascular diseases, smoke, previous cardiovascular ischemic event, arterial hypertension, hypercholesterolemia, non-insulin dependent diabetes mellitus, atrial fibrillation, and stroke subtype according to TOAST classification [Adams 1993]. The analysis, performed with chi-squared test, t-test or Fisher's test as appropriate, are reported in the following table. None of the possible confounding variables resulted significantly associated to the rs4780144 genotype.

	TT (n=286)	**CT (n=70) and CC (n=3)**	**p-value[1]**
Age at onset, years	55.40 ± 16.01	57.26 ± 14.0	0.36 [2]
Males, N (%)	178 (62%)	47 (64%)	0.73
Family history, N (%)	97 (34%)	25 (34%)	0.95
Smoke, N (%)	109 (38%)	32 (44%)	0.37
Atherotrombotic disease, N (%)	65 (23%)	22 (30%)	0.19
Arterial hypertension, N (%)	156 (54%)	40 (58%)	0.97
Hypercholesterolemia, N (%)	167 (58%)	47 (64%)	0.35
NIDDM, N (%)	40 (14%)	12 (16%)	0.60
AF, N (%)	17 (6%)	6 (8%)	0.60 [3]
TOAST subtype			
LA, N (%)	54 (19%)	15 (20%)	0.10
CE, N (%)	46 (16%)	16 (22%)	0.24
SV, N (%)	21 (7%)	3 (4%)	0.43 [3]
OT, N (%)	47 (16%)	7 (10%)	0.14
UN, N (%)	118 (41%)	32 (44%)	0.69

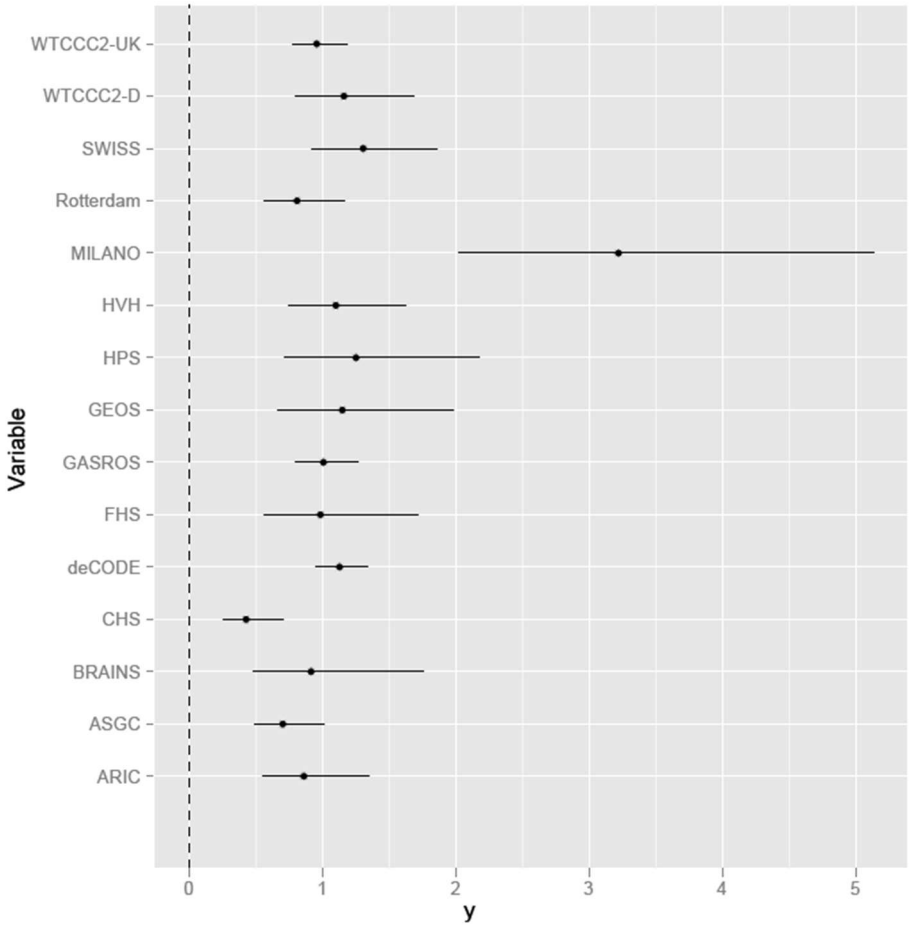
(1) chi-squared test, unless otherwise specified; (2) t-test; (3) Fisher's Test.

Replication in Metastroke and SiGN

As a second step to investigate the association of rs4780144 in our stroke patients, we tried to replicate our results in the Metastroke dataset, which is a collection of 15 ischemic stroke cohorts with a total of 12.389 cases and 62.004 controls, all of European ancestry [Traylor 2012]. In 2011 we requested genotypic data for a total of 15 SNPs in the three loci selected as the ones showing association p-value<5x10-3 and among which are the three with genome-wide significance. As the minor allele frequencies of the 3 associated SNPs are low/moderate, we avoided genetic heterogeneity bias by testing all the associated variant in the candidate

loci. For each SNP, allelic association tests was performed in each cohort separately or in the combined dataset controlling for cohort of origin (i.e. Cochran-Mantel-Haenszel test). Homogeneity of odds ratio was tested by Breslow-Day test. Logistic regression analysis was performed to take into account the impact of suitable covariate (i.e. age, sex). Association with stroke TOAST subtypes was also be evaluated.

For none of the 15 SNPs, we obtained evidence of replication of the results obtained in the Italian cohort. In particular, rs4780144 failed to show any correlation with ischemic stroke in any of the studies considered (see OR in the next figure – note that, in this figure, in the Italian cohort we included patients with ischemic stroke, TIA and vascular cognitive impairment). In some cases we observed sign of association but for the opposite allele (in the Italian cohort C is the risk allele, while in the CHS cohort C appear as the protective allele). Results did not change significantly when the analysis took into account the impact of suitable covariate (i.e. age, sex, TOAST subtypes).



Nevertheless, almost all our Italian cohort of stroke patients was enrolled in the outpatient clinic and, unlike the others, it was is composed of non-acute patients that had a good recovery after the cerebrovascular accident (first of all they survived and then, they were able to go to an outpatient visit, in most cases independently – the median modified Rankin Scale

was 1). For this reason, we hypothesize that the Metastroke dataset, which was composed mainly by stoke at onset, was probably not suitable for the replication.

A subgroup of the SiGN dataset [NINDS Stroke Genetics Network 2016], composed by 904 ischemic stroke cases, was used to test if rs4780144 was associated with stroke severity or stroke recovery. The rs4780144 SNP was not associated with stroke severity or stroke recovery, but 3 other SNPs in the RYR3 locus were significantly associated with stroke outcome at 3 months (covariables: C1, C2, sex, age, NIHSS initial, TOAST) [Sammali 2017].

Chr	rsID	position	alleleA	alleleB	pvalue	beta	SE
15	rs1878300	33688604	A	G	**0,799867**	-0,01649	0,064988
15	rs4780144	33954652	C	T	**0,386227**	0,083255	0,095964
15	rs11637619	33956155	C	T	**0,155208**	0,094265	0,066177
15	rs1390158	33957357	A	G	**0,606963**	0,049356	0,09586
15	rs6495228	34016274	G	A	**0,563999**	0,035928	0,062217
15	rs489832	34277907	T	C	**0,017314**	0,124093	0,051893
15	rs511422	34282982	G	A	**0,017685**	0,124432	0,052208
15	rs603152	34294637	T	G	**0,024096**	0,118598	0,052351

Ryanodine Receptor type 3

Ryanodine receptors (RyRs), together with inositol 1,4,5-triphosphate receptors, are the major intracellular calcium ion (Ca^{2+}) channels, located in the sarcoplasmic/endoplasmic reticulum membrane [Lanner 2010; Karagas 2019]. RyRs are homotetrameric proteins and form the largest known ion channels (>2MDa, each subunit, composed of > 4.800 amino acids, is more than 550 kDa) [Lai 1988]. The massive size and the dynamic nature of RyRs make their structural analysis a challenge. Advances in single-particle electron cryomicroscopy are beginning to elucidate many important structural features [Yan 2015]. RyRs are responsible for the release of Ca^{2+} from intracellular stores during excitation-contraction coupling in both cardiac and skeletal muscle and various neuronal activities such as activity-dependent neuronal gene expression (which includes cell death and synaptic plasticity), mitochondrial bioenergetics, and vesicle releasing in synapses. Within the diseases of the central nervous

system, most of the studies have associated RyRs to neurodegeneration [Chavis 1996; Zündorf 2011; Karagas 2019].

The plant alkaloid ryanodine, for which this receptor was named, has become an invaluable investigative tool: ryanodine binds to RyRs with high affinity and specificity and displays preferential interactions with the open state of the channel, allowing its usage to evaluate the functional state of the channel [Imagawa 1987; Lai 1988]. At nanomole concentrations, ryanodine locks the channel in an open subconductance state while at high concentrations (>100 mM) it inhibits the channel [McGrew 1989]. Most RyR channel modulators interact with the large cytoplasmic domain (four-fifths of the RyR protein) whereas the carboxy-terminal portion of the protein forms the ion-conducting pore. RyRs are modulated directly or indirectly by the dihydropyridine receptor and by various ions, small molecules and proteins, e.g., Ca^{2+} (calcium-induced calcium release - CICR), Mg^{2+}, protein kinase A, calmodulin, calsequestrin. RyRs are activated also by millimolar caffeine concentrations; caffeine concentrations above 5 mmol/L cause a pronounced increase (from micromolar to picomolar) in the sensitivity of RyRs to Ca^{2+}, such that basal Ca^{2+} concentrations become activatory [Lanner 2010].

In mammalians there are three major isoforms of the RyRs, 65% identical in sequence [Hakamata 1992], which are found in different tissues:

- RyR1 is primarily expressed in skeletal muscle [Takeshima 1989];
- RyR2 is primarily expressed in myocardium (heart muscle) [Nakai 1990];
- RyR3 is expressed more widely, but especially in the brain [Hakamata 1992].

RyR1 is the most thoroughly examined isoform because of its high expression levels and ease of purification from skeletal muscle. RYR1 is found also in cerebellar Purkinje cells. In humans, the gene encoding RyR1 is located on chromosome 19q13.2 and spans 104 exons. Mutations in the RYR1 gene underlie several debilitating and/or life-threatening muscle diseases including malignant hyperthermia [MacLennan 1990], heat/exercise induced exertional rhabdomyolysis [Capacchione 2010], central core disease [Zhang Y 1993], multiminicore disease [Ferreiro 2002], and atypical periodic paralyses [Zhou 2010].

The predominant form of RyR in cardiac muscle is RyR2. RyR2 is also expressed at high levels in Purkinje cells of cerebellum, in the cerebral cortex, the olfactory nerve layer, the motor trigeminal nucleus, and the facial nucleus. The gene encoding RyR2 is located on chromosome 1q43 and spans 102 exons [Lai 1992; Furuichi 1994].

The RyR3 gene has 107 exons and is located on chromosome 15q13.3-q14 [Sorrentino 1993; https://www.ncbi.nlm.nih.gov/gene?cmd=retrieve&dopt=default&list_uids=6263&rn=1]. RyR3

is expressed abundantly in restricted areas of the brain such as hippocampal neurons of the CA1 pyramidal layer, amygdala, thalamus, Purkinje cells, corpus striatum, caudate nucleus and at a much lower level in midbrain (superior and inferior colliculi), pons and medulla oblongata. No hybridization signal is observed with RNA preparations from cerebral cortex and cerebellum [Hakamata 1992; Lai 1992; Furuichi 1994; Nakashima 1997; Martin 1998]. Among non-neural tissues, RyR3 is expressed in skeletal muscles (highest expression in the diaphragm), the smooth muscle cells of the coronary vasculature, lung, kidney, ileum, spleen, uterus, ureter, urinary bladder, and esophagus [Giannini 1992; Giannini 1995]. Heterogeneity of Ryr3 splice variants may explain the unique tissue-specific pharmacologic and functional properties of Ryr3 [Jiang 2003].

It was estimated that RyR3 represents only 2% or less of total RyR in the brain [Murayama 1996] and, to date, it has been poorly studied. Mutations in the RYR3 gene were associated with a myopathy with nemaline bodies [Nilipour 2018] but this finding was not confirmed. GWAS gave some evidences that RyR3 could be associated with cardiovascular phenotypes, such as carotid intima-media thicknesses [Shrestha 2010; Zhi 2015], atherosclerosis [Zhao 2014], stroke [Ikram 2009] and circulating phospholipid trans fatty acids [Mozaffarian 2015].

Different laboratories independently generated Ryr3-deficient mice [Takeshima 1996; Bertocchini 1997; Futatsugi 1999]. In all cases, the homozygous mutant mice were fertile and displayed no gross defects. However, different abnormalities were detected in Ryr3 knockout mice such as:

- increased locomotor activity [Takeshima 1996; Matsuo 2009];
- impaired contraction of skeletal muscle during the first weeks after birth [Bertocchini 1997];
- impaired inhibition of hippocampal long-term potentiation and spatial learning, suggesting impaired synaptic plasticity [Futatsugi 1999; Balschun 1999; Matsuo 2009].

AIMS

The results of the first GWAS in Italian ischemic stroke patients in the chronic phase revealed a possible association with the RyR3 gene. In humans, this gene is particularly expressed in the brain and the SNP with the strongest association (rs4780144) is a missense mutation (R1641C) with a possible loss of function of the protein. RyR3 is a Ca^{2+} channel of the endoplasmic reticulum and its function regulates processes such as cell death, synaptic plasticity and mitochondrial bioenergetics. RyR3 is therefore an excellent candidate gene for a possible association with ischemic stroke. However, the replication of the association in other cohorts of acute ischemic stroke patients failed. This probably means that RyR3 does not modify the risk of incidence of ischemic stroke. Given the functions of RyR3 and the peculiarities of our cases, we hypothesized that RyR3 could be associated with stroke recovery and that its reduced functioning could lead to a more favorable outcome. Hence the increased prevalence in our cohort of the "variant" gene, since most of the cases enrolled in the CEDIR study were chronic stroke patients from the outpatient clinic (which means that they were stroke survivors with a moderate recovery, such as to allow them to go to the outpatient clinic). We therefore had evidence that some polymorphisms within the RyR3 gene were associated with stroke outcome at 3 months.

To date, RyR3 was poorly studied but there are some evidences that associate this gene to cardiovascular diseases.

The aim of this work is to collect further evidence on the possible role of RyR3 in influencing recovery after ischemic stroke. For this we have planned to investigate the role of RyR3 in ischemic stroke at 3 different levels:

- Functional: we assessed the effect of rs4780144 genotype on intracellular calcium homeostasis in adipose-derived mesenchymal stromal cells;
- Genomic: we performed a second GWAS in Italian ischemic stroke patients to replicate the association of rs4780144;
- Cellular: we studied the effect of in vitro oxygen-glucose deprivation in the RyR3-knockout mouse model.

METHODS

Functional study

We assumed that the rs4780144 SNP and the missense mutation (R1641C) in the RyR3 gene could led to a possible loss of function of the Ca^{2+} channel on the endoplasmic reticulum. In order to gain information on the impact of the rs4780144 genotype on RyR3 functions, we planned to monitor the intracellular calcium homeostasis under the control of RyRs in carriers homozygous for the wild-type alleles (TT, controls) and homozygous (CC) or heterozygous (CT) for the variant allele.

To identify an adequate cellular model, we determined RrR1, RyR2 and RyR3 expression as described elsewhere [Martin 1998] in two widely available cell lines obtained from healthy donors: peripheral blood lymphocytes and adipose-derived mesenchymal stem cells (Ad-MSCs) isolated from periumbilical fat tissue. We found that Ad-MSCs expressed RyR3 only, while peripheral blood lymphocytes expressed also RyR1 (data not shown). Consequently, we genotyped different Ad-MSC lines and isolated 3 wild-type lines, 1 heterozygous and 1 homozygous.

Cytosolic free Ca^{2+} concentration [Ca2+]i was monitored as previously described [Viviani 2001]. Briefly, cells were loaded with 10 uM Fura 2-AM, fluorescence ratio signal was measured in a Perkin-Elmer LS 50 B double-wavelength fluorimeter and calibrated in terms of [Ca2+]i [Grynkiewicz 1985]. Loaded cells were initially stimulated with RyR agonists Ryanodine (Ry) and Caffeine (Cf). Based on literature data [Meissner 1986; Zheng 2005], to obtain a dose-response curve we applied different concentrations of Ry (1 and 5 uM) and Cf (10, 20, 40 mM). Although Ry showed a slight [Ca2+]i increase with increasing dose, total amount of released Ca^{2+} (Δ = Ca^{2+} peak - basal Ca^{2+}) was similar for both Ry concentrations. Caffeine effect increases up to Cf 20 mM to reach a plateau at 40 mM.

Based on these results, Ry 1µM was chosen as stimulus to challenge homozygous and heterozygous for rs4780144 since able to induce the strongest Ca2+ release.

Ryanodine	**INTRACELLULAR CALCIUM CONCENTRATION $[Ca^{2+}]_i$**		
Stimuli	Ca $^{2+}$ Basal $[Ca^{2+}]_i$	Ca $^{2+}$ Response	Ca $^{2+}$ Δ (Response - Basal)
Ry 1µM (n=17)	47,7 ± 5,7	63,4 ± 6,5	15,6 ± 2,2
Ry 5µM (n=10)	63,8 ± 7,5	78,3 ± 8,2	14,5 ± 2,7

Caffeine	INTRACELLULAR CALCIUM CONCENTRATION $[Ca^{2+}]_i$		
Stimuli	Ca $^{2+}$ Basal $[Ca^{2+}]_i$	Ca $^{2+}$ Response	Ca $^{2+}$ Δ (Response – Basal)
Cf 10mM (n=6)	31,3 ± 5,64	33,9 ± 5,2	2,61 ± 0,65
Cf 20mM (n=23)	48,69 ± 5,7	56,7 ± 5,8	8 ± 0,9
Cf 40mM (n=9)	37,9 ± 5,8	43 ± 6,2	5,13 ± 0,57

Data are the means ± standard error of the means; cells were obtained from two different donors.

Genomic study

At Besta Institute, from June 2012 to October 2017, 403 consecutive patients with ischemic cerebrovascular disease evaluated in the Cerebrovascular outpatient clinics, as well as on the inpatient Cerebrovascular Unit, were enrolled in the spontaneous prospective observational study "Whole-genome study, metabolomics, phenomics and study of genotype-phenotype correlations in ischemic cerebrovascular disease" and their data were included in the Cerebrovascular Diseases Registry (CEDIR). All subjects were examined by a neurologist, provided informed consent and had DNA extracted.

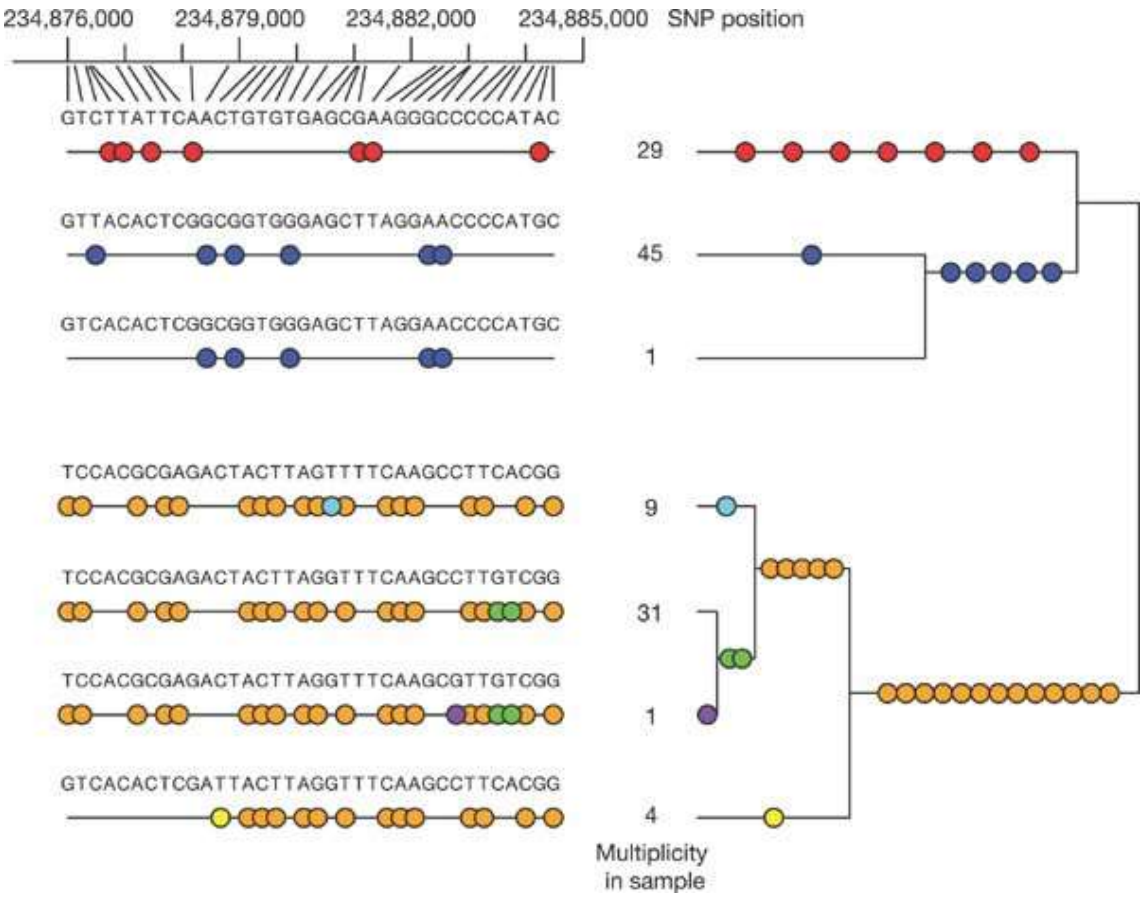
Patients were aged > 18 years and previous ischemic stroke, first ever or recurrent, was confirmed on brain imaging. Ischemic stroke was defined as either (1) a radiographically proven (head CT or MRI) infarct associated with the appropriate clinical stroke syndrome, or (2) a fixed neurological deficit persisting more than 24 hours, consistent with a vascular pattern of involvement and without radiographic evidence of demyelinating disease, or other non-vascular structural disease. Clinical and laboratory data were collected for qualifying ischemic stroke event. Diagnostic work-up included: neuroimaging with head CT or brain MRI (100%, MRI in 93%), EKG (100%), cervical and intracranial vessel imaging using CTA or MRA (72%) or carotid and/or transcranial ultrasound (52%), echocardiography (18%), and Holter EKG monitoring (21%). All cases were phenotyped by an experienced stroke neurologist according to TOAST criteria [Adams 1993], based on relevant clinical imaging and available information on cardiovascular risk factors.

In 2017 genotyping was carried out using the Illumina HumanOmniExpress - 24 BEAD - Chip® accordingly to the manufacturer's protocols.

As controls, we had the same 956 Italian individuals without cardiovascular diseases that were used for the first cohort of ischemic stroke patients (409 subjects from Mario Negri Institute

and 547 subjects from Auxologico Institute), that were genotyped with Illumina HumanHap610-Quad chip.

The new microarray, compared with older versions, included a higher number of SNPs; however, the genotyped SNPs were very different and direct comparisons were only rarely possible. Therefore, it was necessary to move to a common reference and impute our genotypes. In genetics, imputation refers to the statistical inference of unobserved genotypes. It is achieved by using known haplotypes in a population, usually from large whole genome sequencing projects like HapMap or the 1000 Genomes Project in humans, thereby allowing to test for association between a trait of interest (e.g. a disease) and experimentally untyped genetic variants, but whose genotypes have been statistically inferred ("imputed").



An example of how 7 tag SNPs, corresponding to the seven different colours, can capture all the SNPs of the seven different haplotypes observed over this region [International HapMap Consortium 2005].

Managing of genotype data and quality control procedures were performed with PLINK 1.0.7 [Purcell 2007]. Samples were excluded due to unexpected duplicates (n=2), sex mismatch between CEDIR and genomic (n=3) or evidence of non-European ancestry based on principal

components analysis (n=1). Participant-specific quality-control included filters for call rate (>95%, n=2), heterozygosity, and number of Mendelian errors per individual. This second cohort of patients with ischemic cerebrovascular disease included 395 new cases.

Principal component analysis (PCA) with HapMap 3 [https://www.sanger.ac.uk/resources/downloads/human/hapmap3.html] on the Italian cases showed that Italian controls had similar ancestry to the cases.

Genotypes of patients enrolled with cohort 1 (n=709, ischemic stroke=372), cohort 2 (n=395) and controls (n=956) were then imputed using TOPMed reference panel [Taliun 2021].

For the preparation and checking for the quality control process, we run the Imputation Preparation and Checking script found in this link: https://www.well.ox.ac.uk/~wrayner/tools/.

Usually, this is for HRC or 1000G only, but works with TOPMED reference panel too. There were 2060 samples with 271.849 SNPs. Sites with alternative allele frequency > 0.5 were 187.343. Reference overlap was 100%. SNPs call rate < 90.0% were 0. The imputation included 270.420 SNP, since 1.429 were excluded at this step because of allele mismatch.

We used the Michigan imputation server for imputation: https://imputationserver.sph.umich.edu/index.html#!

After imputation, each subject had > 10.000.000 SNPs.

The association analysis was performed using plink (v9.0). Since we did not have additional controls for the replication of the GWAS, we have decided to split the controls (n=956) into two independent groups, with a random algorithm, of 476 and 480 subjects, for comparison with cohort 1 and cohort 2 respectively.

Cellular study

Finally, as a proof of concept to assess the possible role of RyR3 in ischemic stroke, we planned to use the organotypic brain slice model from wild-type mice (C57BL/6) and RyR3 knockout mice (Envigo RMS srl, Udine, Italy and EMMA, Roma, Italy) undergoing oxygen and glucose deprivation (OGD).

Procedures involving mice and their care were conducted at Istituto di Ricerche Farmacologiche Mario Negri in conformity with institutional guidelines that are in compliance with national and international laws and policies. C57BL/6 and RyR3 KO mice were housed in a specific pathogen free vivarium at a constant temperature (21±1°C) with a 12h light–dark cycle and ad libitum access to food and water. The IRCCS-Istituto di Ricerche Farmacologiche Mario Negri adheres to the principles set out in the following laws, regulations, and policies

governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. N° 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). All efforts were made to minimize animal suffering and to reduce the number of animals used.

While primary dissociated cultures permit a single homogeneous cell population to be studied, organotypic brain slice cultures allow to explore the function of brain cells in a three-dimensional system where the main architecture of the cells is preserved [Humpel 2015]. Organotypic brain slice cultures are prepared from 2-4 day old mouse pups (P2-4) [Dossi 2013]. The mice are beheaded, the mouse pup brains were removed from the skull under sterile conditions and were immersed into a 3% agar solution. Tissue blocks containing mesencephalic and forebrain levels were dissected out, fixed onto a specimen stage of a vibratome (Leica, VT 1000S) with Super Attack glue, and placed in ice-cold (4°C) artificial cerebral spinal fluid (ACSF) solution (NaCl=87mM, NaHCO3=25mM, NaH2PO4=1.25mM, MgCl2=7mM, CaCl2=0.5mM, KCl=2.5mM, D-glucose=25mM, sucrose=75mM, Penicillin=50U/ml, Streptomicin=50µg/ml, equilibrated with 95% O2 and 5% CO2, pH=7.4). Prefrontal cortex coronal sections of 200-300 µm thickness were cut through the use of a vibratome. Cortical slices were transferred into petri dishes filled with ice-cold ACSF. Only intact cortical slices were placed on membranes of tissue culture inserts (Millicell Culture insert, 0.4µm pore size, Merk-Millipore) with two slices per insert and placed in 6-well plates, each filled with 1ml of culture medium (MEM-Glutamax 25%, basal medium eagle 25% (Invitrogen), horse serum 25% (Euroclone), glucose 0.6%, Penicillin 100U/ml, Streptomicin 100µg/mL (Euroclone); pH=7.2). All the cultures were maintained at 37°C in 5% CO2. After 2d, the incubation medium was changed with NB/B27 and replaced there after every 2d. On the basis of our experimental data, from each mouse we are able to obtain about 6 organotypic slices, of which on average 5 are usable experimentally (the organotypic slices that are damaged or degraded after a week in culture are eliminated) [Pischiutta 2016].

After one week in culture, cortical slices were subjected to OGD, an in vitro model of brain ischemia [Gesuete 2011]. The culture medium was removed, cortical slices were washed twice with PBS and transferred into a temperature-controlled (37°±1°C) hypoxic chamber (InvivO2 400, Baker Ruskinn) at [O2]=0.1%, [CO2]=5%, [N2]=95%. Once in the hypoxic chamber, the PBS was replaced with deoxygenatedglucose-free medium. After a 2h OGD period, cortical slices were returned to a normoxic incubator and medium was replaced with NB/B27. Control

cortical slices (CTRL, not exposed to ischemic injury) were maintained in normoxic incubator with NB/B27.

To evaluate the susceptibility to ischemic damage, organotypic slices from KO and WT mice were subjected to OGD. The susceptibility to damage was evaluated through two different assays: incorporation of propidium iodide (PI) and evaluation of the release of lactate dehydrogenase (LDH) in the culture medium. These outcomes were evaluated at 3 different times: 24, 48 and 72 hours after the damage. For each time point we had 4 WT groups and 4 KO groups of 8 replicates each. For each genotype we needed 4 X 8 = 32 organotypic slices, corresponding to 7 animals (considering 5 experimentally usable slices for each animal). Each experiment was replicated twice to have the results confirmed by independent experiments.

Total animals: WT: 7 (x2) x 3 time points = 42; KO: 7 (x2) x 3 time points = 42.

For the propidium iodide incorporation assay, at each time point, the inserts with cortical slices were moved to new plates and fresh NB/B27 containing propidium iodide (PI) 2 µM (Sigma-Aldrich) was added. After 30 minute incubation, images were captured at ×4 magnification using an Olympus IX71 microscope (Olympus, Tokyo, Japan) with TRITC filter. Images were analyzed with Fiji software [Schindelin 2012] for quantification.

Subsequently we evaluated the mechanisms of the effects of the lack of the RyR3 on different brain cell populations, at the protein level through qualitative histological analyzes (immunohistochemistry and immunofluorescence) or quantitative (western blot), and at the gene level (gene expression by RT-PCR) gene level. These outcomes were evaluated at 2 times (in the interval 24-72h) defined on the basis of the results obtained in the damage susceptibility experiment. For each time point had 6 WT groups and 6 KO groups of 8 replicates each. For each genotype we will need 6 X 8 = 48 organotypic slices, corresponding to 10 animals (considering 5 experimentally usable slices for each animal). Each experiment will be replicated twice to have the results confirmed by independent experiments.

Total animals: WT: 10 (x2) x 2 time points = 40; KO: 10 (x2) x 2 time points = 40.

For histologic analysis, 48 hours after injury, cortical slices were fixed in 4% paraformaldehyde in PBS and then rapidly frozen and stored at –70°C. To determine the total cell count, 20 µm cut cortical slices were stained with cresyl violet [Perego 2011;8:174] and acquired by Olympus BX61 microscope (Olympus) with motorized stage. For each slice, 16–20 fields at ×40 were analyzed over the cortical slice, using Fiji software.

For gene expression analysis, 48 hours after injury, slices were collected and total RNA was extracted by miRNeasy mini kit (Qiagen), as previously described [Zanier 2014]. Samples were treated with DNase (Life Technologies) and reverse-transcribed with random hexamer primers

using Multi-Scribe Reverse Transcriptase (Life Technologies). Real-time reverse transcription PCR was performed and relative gene expression determined by ΔΔCt method. Data are expressed as log2 of the fold difference from the control group. We determined the expression of microtubule-associated protein 2 (MAP2), a marker of dendritic injury [Lee 2020], and different markers of neuronal oxidative stress such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO-1) and superoxide dismutase 1 (SOD1) [Alhazzani 2018; He 2020;].

The number of experimental groups was calculated using the following formula:
$n=2\sigma^2 f(\alpha,\beta)/\Delta^2$

Where:

- σ represents the standard deviation of the groups;
- α represents the type I error;
- β represents type II error;
- Δ represents the minimum difference in % to be identified.

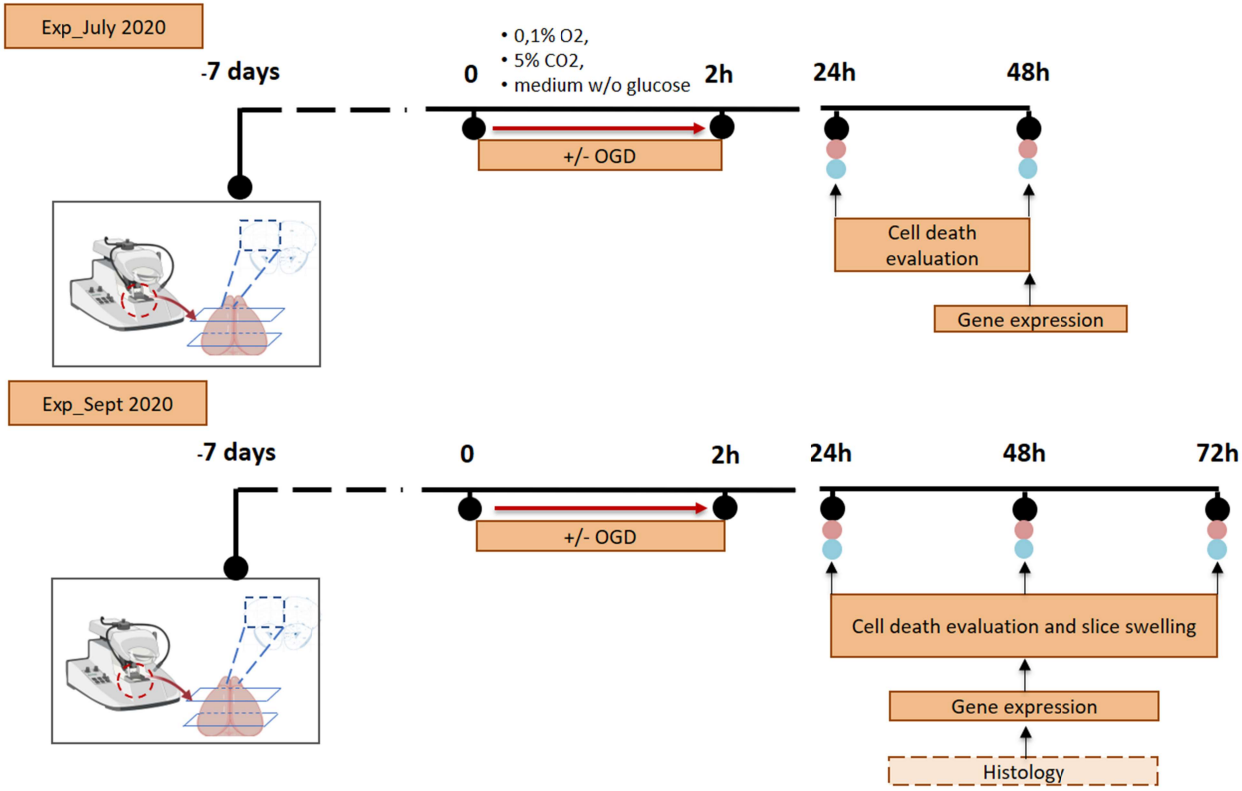
We aimed at minimizing the use of animals while maintaining good power in detecting significant effects. We therefore imposed α = 0.05; β = 0.2; Δ = 20 (an effect of less than 20% would in fact be of little biological significance).

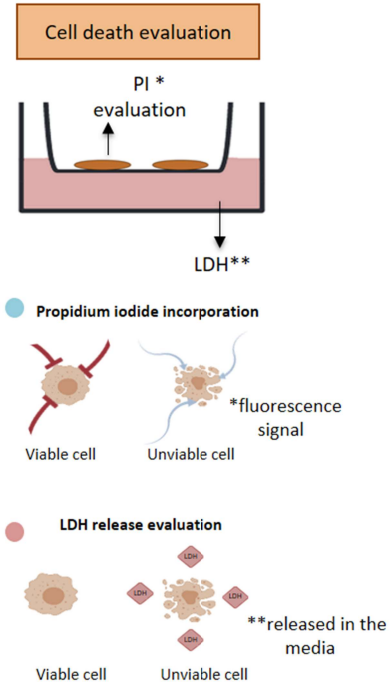
On the basis of previous experiments, for the evaluation of the effects of ischemic damage due to oxygen and glucose deprivation the value of σ is equal to 14.3, the sample size calculated per experimental group is therefore n = 8. Considering that we planned 20 experiments, the total number needed is 80 WT and 80 KO.

Data are presented as mean ± sd. For continuous variables, groups were compared by two-way analysis of variance (ANOVA) for repeated measurements followed by Tukey post-hoc test. All the other data were analyzed with one-way ANOVA, followed by Tukey post-hoc test (GraphPad Prism 6.0, La Jolla, CA).

The experimental design for in vitro experiments is summarized in the following figure.

$RyR3^{KO}$ brain slice susceptibility to in vitro ischemic injury



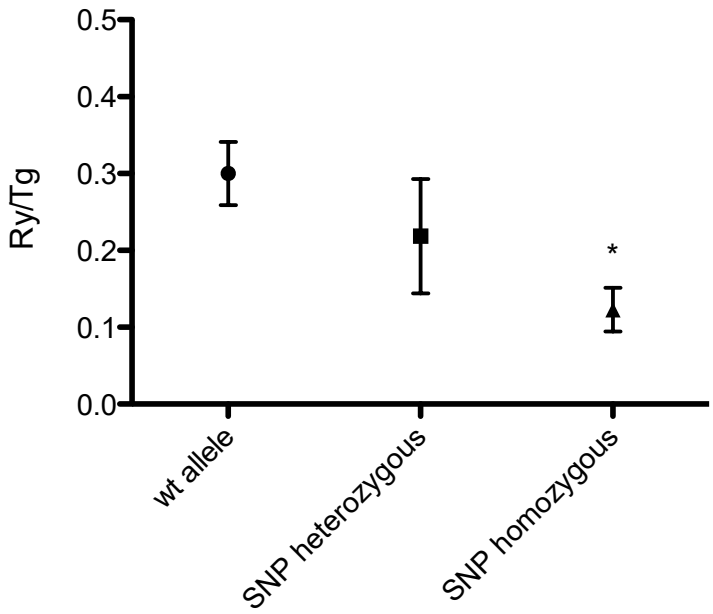


RESULTS

Functional study

Five Ad-MSC lines with different rs4780144 genotypes (3 wild-type TT, 1 heterozygous CT and 1 homozygous CC) were analysed for basal, Ry-induced [Ca2+]i, and total endoplasmic reticulum (ER) Ca^{2+}. Total ER Ca^{2+} was measured to take into account any difference among samples which may impact on RyR3 response, being this receptor located at the ER. Total ER Ca^{2+} was measured by means of thapsigargin (5µM), a potent inhibitor of sarco-endoplasmic reticulum Ca2+-ATPase [Davidson 1995; Andersen 2015] that empties the ER by releasing calcium in the cytoplasmic compartment [Rogers 1995]. Basal calcium levels did not significantly change among the different donors (controls [Ca2+]i: 51 ± 3,5; heterozygous [Ca2+]i: 40 ± 4,5 and homozygous [Ca2+]i: 55 ± 4,4). Significant differences in total ER Ca2+ among the different donor groups were detected (controls Δ Ca2+: 50 ± 6,7; heterozygous Δ Ca2+: 25,1 ± 4,4* vs Control and homozygous Δ Ca2+: 37,3 ± 4,03). For this reason Ry-induced [Ca2+]i was normalized on total ER Ca^{2+}.

Our data show that the calcium release induced by Ry 1uM and normalized on ER total content in Ad-MSCs loaded with Fura-2AM 10uM is reduced in CC homozygous cell line. This suggest that RyR3 function is reduced in carriers of the rs4780144 variant, significantly in homozygous subjects.



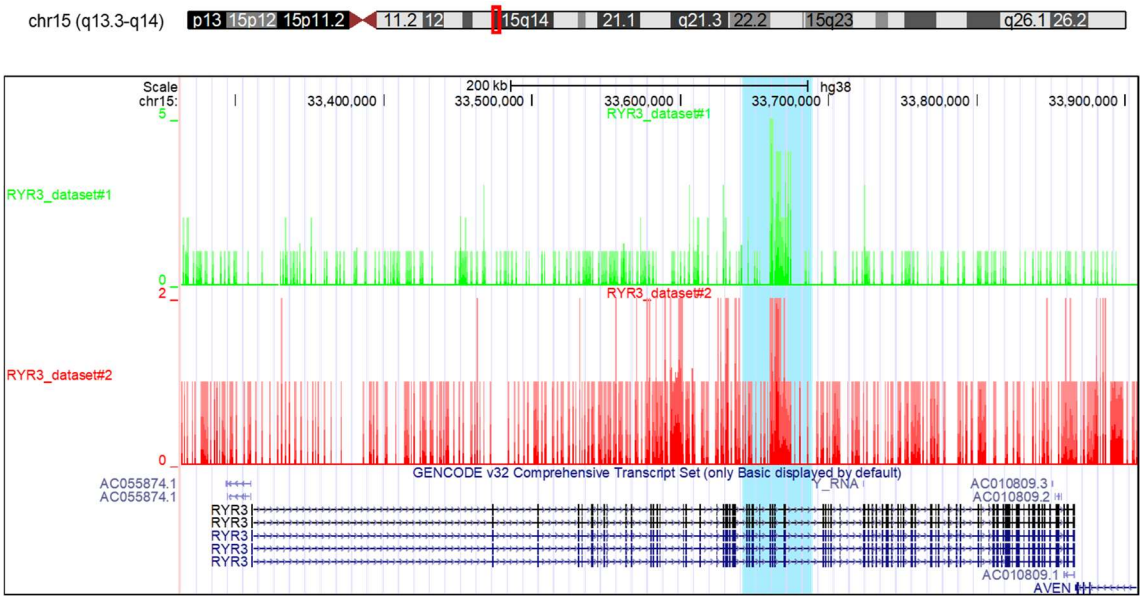
*Number of samples analyzed for each condition are as follow: wt allele n=32 from three different donors, SNP homozygous n=20 from one donor, SNP heterozygous n=16 from one donor. The statistical significance of differences was determined by one-way ANOVA followed by a multiple comparison test (Tukey's test), *p<0.05.*

Genomic study

Within the 395 new cases genotyped, 319 had an ischemic stroke, 18 had TIA, 7 had vascular cognitive impairment and 51 had other cerebrovascular diseases (cerebral venous thrombosis, cerebral haemorrhage, etc.).

Considering the 319 patients with ischemic stroke, 188 (59%) were males, with mean age at onset of 55 ± 15 years; 32 patients had cardioembolic ischemic stroke (CE), 62 had ischemic stroke due to large artery disease (LA), 45 had small vessel disease (SV), 52 had ischemic stroke due to other causes (arterial dissection, vasculitis, etc.) (OC), and 128 had ischemic stroke due to undetermined cause (UN).

The results of the second Italian GWAS in ischemic stroke patients confirmed the association with the RrR3 locus on chromosome 15, and are shown here in details.



Dataset#1 (green): cohort 1, 372 cases vs 476 controls. Dataset#2 (red): cohort 2, 319 cases vs 480 controls. The statistical value of the association is represented on the Y axis as -log10 (p-value). The region highlighted in blue represents the region with the highest concentration of significantly associated SNPs.

Here the details of the associations of the top SNPs in the two datasets, including rs4780144

			Dataset#1			Dataset#2		
rsID	**position**	**TYPE**	**CHISQ**	**P**	**OR**	**CHISQ**	**P**	**OR**
rs4780142	33660931	Intronic	23,89	1,02E-06	2,555	8,72	0,003147	1,921
rs4780141	33660889	Intronic	23,89	1,02E-06	2,555	6,694	0,009676	1,789
rs7181696	33661707	Intronic	23,04	1,59E-06	2,518	6,694	0,009676	1,789
rs7174989	33661740	Intronic	22,9	1,71E-06	2,492	6,694	0,009676	1,789
rs4780144	33662451	Missense	21,95	2,81E-06	2,433	6,694	0,009676	1,789
rs4780143	33661221	Intronic	23,04	1,59E-06	2,518	6,065	0,01379	1,746
rs4779627	33661222	Intronic	23,04	1,59E-06	2,518	6,065	0,01379	1,746
rs72713249	33661418	Intronic	20,92	4,80E-06	2,49	5,109	0,02381	1,717
rs2339296	33670754	Intronic	17,23	3,31E-05	2,088	5,805	0,01598	1,644
rs4780149	33674739	Intronic	17,14	3,47E-05	2,063	4,225	0,03984	1,512

To investigate the association of rs4780144 in our stroke patients (this SNP was available in 359 cases), we studied the association between rs4780144 genotype and possible confounding factors that were recorded in the CEDIR database, such as age at stroke onset, sex, positive family history for cerebrovascular diseases, smoke, previous cardiovascular ischemic event, arterial hypertension, hypercholesterolemia, non-insulin dependent diabetes mellitus, atrial fibrillation, and stroke subtype according to TOAST classification [Adams 1993]. The results of the analysis, performed with chi-squared test, t-test or Fisher's test as appropriate, are reported in the following table. As in the first cohort, none of the possible confounding variables resulted significantly associated to the rs4780144 genotype.

	TT (n=276)	CT (n=41) and CC (n=2)	*p-value*[1]
Age at onset, years	54.63 ± 15.58	55.70 ± 15.21	0.67 [1]
Males, N (%)	160 (60%)	28 (65%)	0.38
Family history, N (%)	53 (19%)	11 (26%)	0.33
Smoke, N (%)	119 (43%)	14 (32%)	0.19
Atherotrombotic disease, N (%)	43 (16%)	7 (16%)	0.91
Arterial hypertension, N (%)	148 (54%)	19 (44%)	0.25
Hypercholesterolemia, N (%)	94 (34%)	17 (39%)	0.48
NIDDM, N (%)	44 (16%)	7 (16%)	0.95
AF, N (%)	14 (5%)	4 (9%)	0.26 [2]
TOAST subtype			
LA, N (%)	55 (20%)	7 (16%)	0.57
CE, N (%)	26 (9%)	6 (14%)	0.41[2]
SV, N (%)	38 (14%)	7 (16%)	0.66
OT, N (%)	46 (17%)	6 (14%)	0.65
UN, N (%)	111 (40%)	17 (40%)	0.93

(1) chi-squared test, unless otherwise specified; (2) t-test; (3) Fisher's Test.

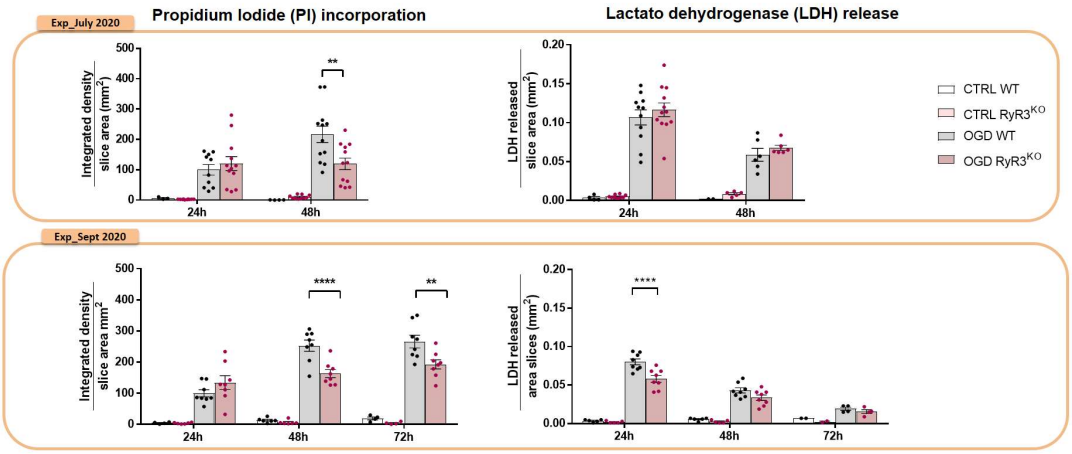
Cellular study

RyR3KO slices showed a reduced cell death assessed by PI incorporation and LDH release indicating a decreased susceptibility to ischemic damage.

In particular, in the PI assay, the results of the first experiments that were done in July 2020 were replicated in the second set completed in September 2020, confirming the reduced neuronal death at 48h in RyR3KO slices (172±8 vs 253±11, p = 0.00008), which continues also at 72h (191±7 vs 261±9, p = 0.007).

On the LDH assay, while the results of the first experiments that were done in July 2020 did not show any statistically significant difference in the two groups (RyR3KO slices showed a slightly increased damage, not statistically significant), the second set of experiments completed in September 2020 showed a significant reduction in cell damage at 24h (0.061±0.005 vs 0.078±0.004, p = 0.0004).

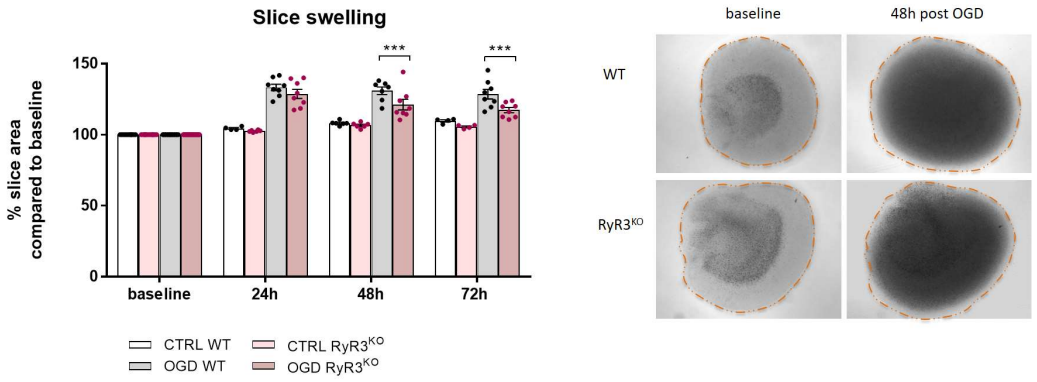
$RyR3^{KO}$ brain slice susceptibility to in vitro ischemic injury



2way ANOVA followed by Tukey post hoc test, ** p<0.01, *** p<0.001

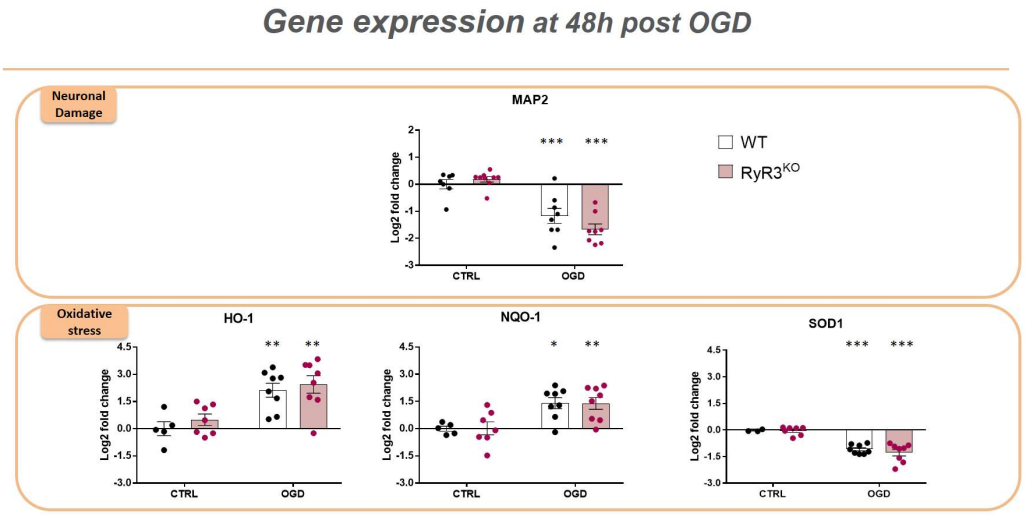
RyR3KO slices showed also a reduced swelling after OGD at 48h (117%±4 vs 128±3, p = 0.0005) and 72 h (121%±3 vs 132±4, p = 0.0009), indicating reduction of cytotoxic edema.

$RyR3^{KO}$ brain slice susceptibility to in vitro ischemic injury



2way ANOVA followed by Tukey post hoc test, *** p<0.001

Finally, in the gene expression analysis that was completed in December 2020, no significant differences were found in neuronal (MAP2) and oxidative stress (HO-1, NQO-1 AND SOD1) related genes.



2way ANOVA followed by Sidak post hoc test: *vs genotype matched control
*p<0,05, ** p<0.01, *** p<0.001*

DISCUSSION

Role of RyR3 in ischemic stroke

The fist GWAS in Italian ischemic stroke patients, most of which were in the chronic phase, found a significant association with the SNP rs4780144, a missense variation in the RyR3 gene. RyR3 is a Ca^{2+} channel of the endoplasmic reticulum and it is expressed mainly in the brain. RyR3 plays a key role in the regulation of neuronal calcium homeostasis, thus controlling processes such as cell death, synaptic plasticity and mitochondrial bioenergetics [Hakamata 1992; Furuichi 1994; Nakashima 1997; Karagas 2019]. Commonly used software tools for protein structure prediction [Yazar 2021] like SIFT suggest a significant loss of function in the mutated RyR3. Therefore, rs4780144 was an excellent candidate SNP for a GWAS in ischemic stroke patients [Manolio 2013]. However, this association was not replicated in the Metastroke dataset, which is a collection of 15 ischemic stroke cohorts with a total of 12.389 cases, most of which were genotyped at stroke onset, and 62.004 controls, all of European ancestry [Sammali 2017].

Given the functions of RyR3 and the peculiar characteristics of the stroke patients included in our cohort (most of them had a good clinical outcome after stroke), we hypothesized that RyR3 does not affect the risk of incidence of ischemic stroke but it may be associated with stroke recovery and its reduced functioning could lead to a more favorable outcome. We had a fist confirmation of this hypothesis in a preliminary study in a cohort of stroke patients with outcome data [Sammali 2017].

With this work, we obtained further multiple evidences that confirm the possible role of RyR3 in conditioning the clinical outcome after an ischemic stroke.

The rs4780144 variant, responsible for a missense mutation in the RyR3 gene, causes a reduced release of calcium from the ER into the cytoplasm after stimulation. This is a confirmation of the functional relevance of this SNP.

In the second GWAS in Italian patients with ischemic stroke we confirmed the statistically significant associations with various SNPs located in the RyR3 gene and rs4780144 was within the top ones. The levels of statistical significance achieved in this second GWAS, although adequate for a replication study, were lower than those of the first GWAS. This could be explained considering that, in order to be able to perform the association study in two independent cohorts, the number of controls was halved (therefore the statistical evidence is a little lower). As for the first GWAS, we did not find any significant association with the other possible confounding variables included in the CEDIR dataset, most of which are associated with the incidence of ischemic stroke. As we have already noticed, other GWAS found that

RyR3 was associated with cardiovascular phenotypes, such as carotid intima-media thicknesses [Shrestha 2010; Zhi 2015], atherosclerosis [Zhao 2014], stroke [Ikram 2009] and circulating phospholipid trans fatty acids [Mozaffarian 2015]. However, none of these mentioned a possible association with stroke recovery.

Finally, in the most important experiments, we demonstrated that, after OGD, RyR3KO slices showed a decreased susceptibility to ischemic damage, a reduction of cytotoxic edema and a reduced cell death.

To date, most of the studies on the role of ER Ca^{2+} pool in neurological diseases, have been focused on chronic conditions such as autism spectrum disorder, lysosomal storage diseases, neuropsychiatric diseases, peripheral neuropathies, and age-related neurodegenerative diseases [Marambaud 2009; Okubo 2018; Del Prete 2014; Karagas 2019; Sun 2020]. Actually, in cerebral ischemia, considering that high extracellular glutamate causes excitotoxicity, which results in N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation, allowing a massive influx of calcium from extracellular space into the cytoplasm, only few studies have investigated the possible role of the limited ER Ca^{2+} pool. Moreover, since the RyR2 mRNA is rather uniformly distributed and the RyR3 is only 2% or less of total brain RyRs [Murayama 1996], most of the studies have focused on the former [Bull 2008]. However, RyR3 is expressed abundantly in the specific regions such as corpus striatum, thalamus, cerebellum, and hippocampus. This restricted distribution may indicate that, in these regions, Ca^{2+} regulation is different from that in other part of the brain. Furthermore, since these regions roughly correspond to the areas where 'delayed neuronal death' first occur after hypoxia in the brain [Kirino 1982], RyR3 may be important in cerebral ischemia [Hakamata 1992; Nozaki 1999]. It has been demonstrated that, in neurons exposed to OGD, it is the ryanodine-sensitive ER Ca^{2+} pool which is affected [Pisani 2000]. This pool is thought to be critical for survival of cells following metabolic stress, as indicated by the observation that the RyR antagonist dantrolene protect cells from damage in various pathological states [Wei 1996; Zhang L 1993; Paschen 2001].

Limitations

Strengths of the present study include the multiple evidences, obtained at different levels, that the reduced functioning of RyR3 may influence positively the outcome after ischemic stroke. However, there are also different limitations that need to be mentioned.

The experiments with the major limitations are those carried out for the study of RyR3 functionality, in which the greatest limit is represented by the model adopted for the experiments: lines of Ad-MCS from different subjects that differed in the rs4780144 genotype.

In fact, having hypothesized an altered functionality of RyR3 at the neuronal level, we had to use cells from a different stem (Ad-MSC), which could have different mechanisms for controlling calcium concentrations in the cytoplasm. Furthermore, despite having directly stimulated the RyR3 channel with an agonist, the final effect, namely the release of calcium into the cytoplasm, could have been affected by other variables that in our model could not be controlled: first of all, the presence of other possible variants in the RyR3 gene, which could have influenced its functionality; then the presence of other factors that could have altered the complex control of cytoplasmic calcium concentration [Karagas 2019].

As for the genetic study, the major limitation was the lack of a second independent control group. To remedy this, we had to use a "statistical trick": we randomly divided the controls into two groups which were then compared "independently" with the two cohorts of stroke cases. Although the division into two groups was performed with a completely randomized automated procedure, if in our original cohort of controls we had an increased prevalence of the more common genotype (TT) of the SNP rs4780144, this would have reproduced, albeit to a different extent, in the two subgroups that we got out of it. However, it should be recognized that the association with RyR3 did not concern rs4780144 only, but also many other adjacent SNPs. Finally, it should be mentioned that, due to the evolution of the kits used for genotyping, in the second cohort of cases the rs4780144 genotype was not determined directly, but imputed. While maintaining a high level of reliability, imputation may obviously introduce another possible source of variability.

The cellular study, while giving the strongest evidence in favor of a role of RyR3 in influencing the outcome of cerebral ischemic, also has some limitations. In fact, the experiments have not yet clarified how a reduced functioning of RyR3 could improve the outcome after a cerebral ischemia. We have several hypotheses, but only experimental verification will be able to clarify this point before we can confirm what have observed and before we can propose possible studies in humans.

Future Directions

Before proposing a treatment that can reduce the functionality of RyR3 in patients with ischemic stroke, further confirmations of what we have already observed would be recommended.

First of all, we will try to have access to genotypes obtained with Illumina technology from a second control group of 500-1.000 Italian subjects without cardiovascular diseases, in order to carry out a truly independent replication of our GWAS results. Even if it were not possible to

directly replicate rs4780144, because it may be absent in the panel of SNPs of the new gene-chips, it could be interesting to verify other SNPs in the RyR3 gene locus.

Secondly, we should clarify the molecular mechanism through which RyR3 inhibition can provide neuroprotection after stroke. For this purpose, expression studies of genes related to apoptosis, the main mechanism on which RyR3 could affect, and *in vivo* studies could be useful. In fact, the variations of ischemic damage assessed from the histological point of view in the *in vivo* model could provide further information to clarify the possible mechanisms involved in neuroprotection (i.e. variation of the ischemic core could suggest an effect on the excitoxic cascade, while a variation of the penumbra would suggest more an effect on programmed cell death).

Dantrolene, a drug that is currently used in clinical practice as skeletal muscle relaxant, has already been considered as a promising neuroprotective agent that could increase the rescue of the ischemic penumbra caused by the interruption of blood flow in a brain-supplying artery. Dantrolene is a hydantoin derivative and it is a peripherally acting skeletal muscle relaxant currently used intravenously (1-2.5 mg/kg) in the treatment of malignant hyperthermia, a life-threatening disorder triggered by general anesthesia. More rarely, it is used also for neuroleptic malignant syndrome, spasticity, shivering, heat stroke and serotonin syndrome [Lanner 2012]. Dantrolene acts directly on RYR1 and RYR3, but not on RYR2. Its unique mechanism of blocking intracellular Ca2+ release made it an attractive drug in treating or preventing cell death resulting from a multitude of neuronal injuries, including ischemia, epileptic seizures, and spinal cord injury, with promising results [Muehlschlegel 2009; Boys 2010; Xu 2015]. In particular, dantrolene significantly decreased infarct volume and provided neuroprotective effect on rat brain after transient middle cerebral artery occlusion [Li 2005]. It was proposed as a possible new neuroprotective therapy [Inan 2010]. Intravenous administration of dantrolene in healthy volunteers may result in skeletal muscle weakness and dyspnea; within CNS effects, dizziness, lightheadedness and drowsiness are the most commonly reported. The most serious reported adverse effect, hepatotoxicity, refers to high doses in chronic oral administration. While dantrolene itself has no myocardial effects, marked myocardial depression was reported in animals after coadministration of verapamil, but not with other calcium-channel blocking agents [Muehlschlegel 2009]. Recently, intra-arterial dantrolene was effective for refractory cerebral vasospasm after aneurysmal subarachnoid hemorrhage [Majidi 2012; Ortiz Torres 2019] and high doses of intravenous dantrolene (1.25 mg/kg every 6 h for 7 days) were feasible, safe and well tolerated in critically ill patients with aneurysmal subarachnoid hemorrhage [Muehlschlegel 2015].

Considering all these premises, the current research in new neuroprotective agents as an adjunct therapy to recanalization, and the results of our work, dantrolene could be a good candidate.

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