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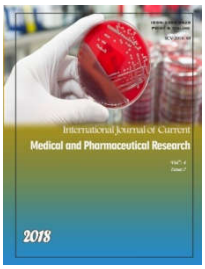

CYP2C19 GENE COPY NUMBER VARIATIONS AND CLOPIDOGREL PHARMACOGENETICS: ASSOCIATION OF GENE DOSAGE AND CARDIOVASCULAR EVENTS IN SARDINIAN AND ITALIAN CONTINENTAL PATIENTS WITH ACUTE CORONARY ARTERY SYNDROMES

Nicola Marziliano[1,2,3]., Damiana Fiscella[3,4]., Giovanni Lorenzoni[1]., Ivan Meloni[1]., Pierluigi Merella[1]., Simonetta Bonano[1]., Andrea Marras[1]., Sara Uras[1]., Antonio Fiscella[3], Maria Agata Longo[3]., Michele Massimo Gulizia[4]., Gavino Casu[1] and Mariano Intrieri[2]

[1]UOC di Cardiologia, ASSL3 NUORO-ATS Sardegna, Nuoro-IT
[2]Dipartimento di Medicina e di Scienze della Salute "Vincenzo Tiberio" -Università del Molise, Campobasso-IT
[3]Fondazione Floresta Longo, Catania-CT
[4]UOC di Cardiologia, PO Garibaldi, Catania-CT

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ABSTRACT

Variability in pharmacokinetics and drug response accounts for single-nucleotide variants/ polymorphisms (SNVs/SNPs) as well as copy-number variants (CNVs). While the role of SNVs/SNPs on drugs metabolism has been extensively studied, little is known about the CNVs. Cytochrome P450 2C19 (*CYP2C19*) gene variants and their overall effects on the clinical outcomes of patients with Acute Coronary Syndromes (ACS) treated with Clopidogrel in a dual antiplatelets therapy, remain still controversial although bed-side genetic-driven care has been shown to be feasible. We sought to evaluate the impact of *CYP2C19* CNVs on the clinical outcomes in Sardinian patients who underwent percutaneous coronary interventions (PCI) and received clopidogrel therapy having as control population Italian continental (Sicilian ancestry included). The prevalence of *CYP2C19* CNVs were assessed by means of three dedicated TaqMan assays (Hs05148033_cn, Hs02932336_cn and Hs05107177_cn) in 100 Sardinian patients who underwent PCI. The control population was made of 200 Italian continental patients (of whom 60 individuals of Sicilian ancestry). Clinical relevant outcomes (adverse cardiovascular events, stent thrombosis and bleeding) and *CYP2C19* CNVs were then associated in these two groups. The primary observation was the identification of *CYP2C19* gene CNVs in the Sardinian population at higher rate: 7.2% of deletion and 3.2% of duplication alleles respectively in Sardinian vs 1.2% and 0.7% in the control group. The second finding showed that the *CYP2C19* deletion allele is at increased risk of a composite of cardiovascular death, myocardial infarction, symptom-driven revascularisation compared with non-carriers (10.58% vs 6.07%, OR: 1.99, 95% CI, pz<0.001). Stent thrombosis (ST) is also more frequent in the deletion allele carriers (2.22% vs 0.44%, OR: 4.77, 95% CI, p<0.001). The risk of bleeding is higher in the duplication allele carriers. In conclusion, genetic testing including the search for CNVs, may be helpful to personalize patients' care being the dual antiplatelets therapy pivotal for patients undergoing PCI.

INTRODUCTION

Genetic variations overall contribute to the variability in response to drugs between 18-32% (1). Although the incorporation of pharmacogenomics (PGx) into clinical practice is here to come and the recent introduction of a portable USB compatible handheld DNA sequencers offers available bed-side genotyping of individuals without need of a laboratory (2), this stage has not been reached yet.

The Cyotchrome P450 (*CYP*; 10q23.33) enzymes are the major system that catalyse phase I drug metabolism of about 20% of the clinically used drugs (3). About 52 allelic variants of the *CYP2C19* gene due to Single Nucleotide Polymorphisms (SNPs)/Variations (SNVs) have been reported (https://www.pharmvar.org/gene/CYP2C19) with evidences of different genotype-phenotype associations according to the ethnic groups (4-6).Based on to the genotype and its effects, subjects are categorized as poor metabolizers (PMs) if carrying

Corresponding author:* **Nicola Marziliano
UOC di Cardiologia, ASSL3 NUORO-ATS Sardegna, Nuoro-IT

either of the most common defective *CYP2C19* allele namely *CYP2C19*3* (c.636G>A; rs4986893) or the *CYP2C19*2* (c.681G>A; rs4244285); extensive metabolizers (EM) if carrying no variant alleles; ultra-rapid metabolizers (UMs) if carrying the *CYP2C19*17* (c-806C>T; rs12248560; Table 1). In addition, discrepancies observed between the genotype-phenotype association studies in the *CYP2C19* led to the identification of Copy Number Variants of this gene (CNVs; 7).

Table 1 List of the recurrent *CYP2C19* (Ref Seq NM_00769) allelic variants and their clinical effects (if known): allele name, coding effect, change at the protein level, *rs* name (as from the dB SNP database) and clinical annotations are provided.

Gene	Allele name	Coding	Protein	Rsname	Effetc
CYP2C19 NM_00769	CYP2C19*1	-	-	-	wild type
	CYP2C19*17	c.-806C>T	5'UTR	rs12248560	higheractivity
	CYP2C19*4	c.1A>G	splice site	rs28399504	none
	CYP2C19*8	c.358T>C	p.Trp120Arg	rs41291556	poor
	CYP2C19*6	c.395G>A	p.Arg132Gln	rs72552267	poor
	CYP2C19*3	c.636G>A	p.Trp212X	rs4986893	none
	CYP2C19*2	c.681G>A	splice site	rs4244285	none
	CYP2C19*7	c.819+2T>A	splice site	rs72558186	none
	CYP2C19*5	c.1297T>C	p.Arg433Trp	rs56337013	reduced

CYP2C19 is important for the metabolism of a variety of drugs including clopidogrel, omeprazole and phenytoin (8-10). In the setting of acute coronary syndromes (ACS), among patients treated with clopidogrel, carriers of a reduced-function *CYP2C19* allele (namely the *CYP2C19*2*) have significantly lower levels of the active metabolite of clopidogrel, diminished platelet inhibition, and a higher rate of major adverse cardiovascular events, including stent thrombosis, with respect to noncarriers(11) and the *CYP2C19* genotyping for Clopidogrel resistance at the point of care, although debated, has been recently shown to be effective (12-13). However, the individuals' response to Clopidogrel is not fully explained based solely on the available genotypes. Therefore, in this paper we sought at evaluating the presence of *CYP2C19* gene CNVs within a genetically homogenous population of ACS patients of Sardinian origin compared to Italian continental subjects (30% of whom of Sicilian ancestry) with the aim of explaining such differences in drug response (14).

MATERIAL AND METHODS

Blood samples and informed consent. Genomic DNA of all the enrolled individuals (n=300) was extracted from whole blood using the Maxwell 16 and associated AS1010 chemistry (Promega). Quality and quantity of isolated DNA were measured using the Nanodrop 2000 (ThermoFisher). All of the patients were of Italian ancestry and were hospitalized because of ST-segment elevation or non-ST-segment elevation ACS (Figure 1).

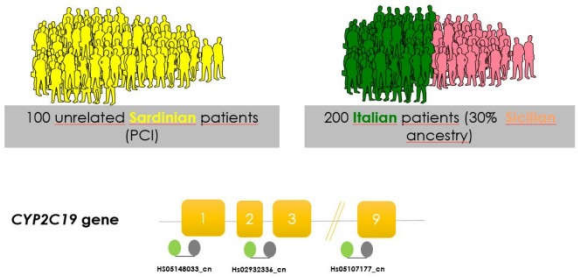

Figure 1 The study enrolled 300 ACS patients (100 Italian Sardinian and 200 Italian Continental; upper part of the figure); the *CYP2C19* gene was studied by means of three Taqman Copy Number Assays (Hs05148033_cn, Hs02932336_cn and Hs05107177_cn) spanning respectively the exon/intron boundaries of exons 1, 2 and 9 (lower part of the Figure).

Each enrolled individual gave his/her informed consent for DNA banking and DNA genotyping as per the research aims of this study. The patients'clinical characteristics are listed in Table 2.

Table 2 Baseline demographics and clinical characteristics of the two population present inthis study. Date are presented as percentage (± standard deviation when present).

	Allpatients (n=300)	Sardinians (n=100)	Italian Continental (n=200)
Demographics			
Age, yrs	71.5±13.7	74.2	68,8
>80 yrs	38.2±8.9	37,2	39,1
female	29±5.6	32,5	25,5
Cardiovascular Risk Factors			
Family History	24,2	25,8	22,6
Hypertension	65,2	63,9	66,5
Dyslipidemia	62,5	67,4	57,6
Acute CoronarySyndromes			
STEMI	32,4	33,9	30,9
NSTEMI	64,8	63,7	65,9
Unstable angina	2,1	3,2	1
Revascularisation			
PCI	61.3	60.2	62.4
CABG	9.35	9.8	8.9

Patients outcomes. The primary purpose of the study was the assessment of the presence of CNVs in the *CYP2C19* gene and their possible association in the setting of ACS. To this end we looked at the presence of composite cardiovascular death and the first occurrence of nonfatal myocardial infarction, nonfatal stroke, and major bleeding (defined according to the Bleeding Academic Research Consortium type 3 to 5) within 12 months after hospitalization. Subsequently we looked at the composite of the primary endpoint plus the occurrence of definite or probable stent thrombosis.

Copy number variation assessment. The variations in the allelic content of the *CYP2C19* gene were made by means of the comparative ΔΔCt method (or relative quantification) as elsewhere described (7,15). The primers and probes set used for the *CYP2C19* (target gene) and *RNAseP* (reference gene; *RPPH1*A30064) amplification are listed in Figure 1 (lower part). Total PCR reaction was of 25 ul containing the following: 12.5 ul of 2X TaqMan Gene Expression Master Mix, 1.25 ul of TaqMan assay and 10 ng of gDNA (all of the reagents were purchased by ThermoFisher) and run on a ABI PRISM 7500 platform under universal amplification conditions.

Statistical analysis.STATA4 (Stata software) was used for secondary statistical data analysis after performing primary analysis for CNVs by means of the SDS Software (ThermoFisher). Data were expressed as mean ±SD. The copy number range between 1.6-2.1 was considered as normal diploid copy number for the *CYP2C19* gene. Descriptive statistics were used to compare the baseline characteristics of the patients' groups. The cumulative incidence of the endpoints during the 12 months of follow-up were graphically represented by means of Aalen-Johansen curves, and the significance of the differences between the sub-distribution of the hazards was tested by using the Fine-Gray model. All of the tests were 2-sided at a significance level of 0.05.

RESULTS

A total of 300 patients were enrolled in this study: 100 of Sardinian ancestry and 200 Italian Continental (30% of whom of Sicilian ancestry; Figure 1). The mean age of the population was 71.5± 13.7 years (range between 30 to 98 years), with the 38.2% of the patients > 80 years. Taking into account the proportion of revascularization by means f PCI (61.3%) and CABG (9.35%), these patients can be considered a high-risk study population.

Genotyping results. In Table 3 are summarised the genotyping results of the enrolled patients.

Table 3 Number of deleted and duplicated CYP2C19 alleles in the two populations (Italian Sardinian and Italia continentals); MACE (major cardiovascular events in the follow-up); NS: not significant; NA: not applicable.

	CYP2C19 **Deletiontion**	***CYP2C19*** **Duplication**
Sardinians (n=100)	7 (7%)	3 (3%)
Italian Continental (n=200)	2 (1%)	1 (0.5%)
P	<0.001	<0.001
MACE Sardinians (n=100)	12 (12%)	-
MACE Italian Continental (n=200)	27 (13.5%)	-
P	NS	NA
BleedingSardinians (n=100)	-	5 (5%)
BleedingItalian Continental (n=200)	-	15 (7.5%)
P	NA	NS

By assessing the presence of large gene InDels, we found CNVs to be present within the *CYP2C19* gene at a higher rate in the Sardinian DNAs compared to the Italian continental DNAs. More in depth, by analysing the full *CYP2C19* gene length with three TaqMan copy number assays spanning from the 5'UTR to the 3' UTR (Hs05148033_cn, Hs02932336_cn and Hs05107177_cn; Figure 1), we found in the Sardinian population the presence of 7% (n=7) deleted and 3% (n=3) duplicated alleles respectively vs 1% (n=2) and 0.5% (n=1) in the control group. Figure 2 shows the raw data as from the SDS software when applying the ΔΔCt method in order to assess the copy number variation (s).

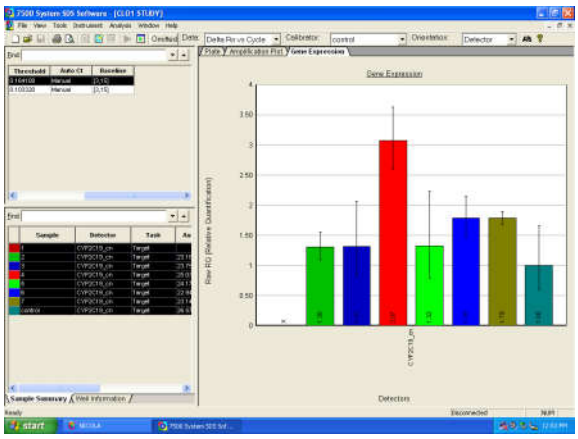

Figure 2 Raw quantitative data as from the SDS System 7500 software (run in Gene Expression mode): the red sample showed a duplicated allele compared to all of the other samples.

Patients outcomes. All of the 300 patients were followed up for at least one year (14±2 months). Major cardiovascular events (a composite of the following cardiovascular death, re-occurrence of nonfatal myocardial infarction, nonfatal stroke and stent thrombosis) occurred in 12(12%) Sardinian patients, 7 of them with deletion allele vs 27 Italian continental (13.5%) with the same genetic make-up (p<0.001). Major bleeding events occurred in 5 (5%) Sardinian patients, 3 of them carrying the *CYP2C19* duplicated allele with respect to 17(7.5%) Italian continental, 1 of them carrying the duplicated allele (p<0.011).

DISCUSSION

Biological mechanisms and genetic factors underlying the individual Clopidogrel response are heterogeneous and still remain elusive. Previous genetic and clinical trials have focused almost exclusively on the role of common, recurrent genetic variants, mostly involving single nucleotide change (Single Nucleotide Polymorphisms or SNPs) and have identified only a small fraction of the expected heridability (16, 12).

CYP2C19 is the main factor for metabolism of drugssuch as omeprazole, lansoprazole, imipramine, propranolol, diazepam and clopidogrel. Some *CYP2C19* genetic variants may produce non-functional alleles and therefore no enzyme activity is left to metabolize these drugs correctly. The frequencies of these genetic variants show regional variability. For instance, the *CYP2C19*2*allele has a frequency of 21.4%, 14.4%, 15%, 13.6%, 13.1%, 23%, 32%, 39% and >75% respectively in the Iranian, Swedish, German, Ethiopian, Zimbabwean, Chinese-Taiwanese, Filippino and Malenesian populations respectively (6). Among patients treated with Clopidogrel, carriers of the *CYP2C19*2* show markedly lower levels of the active metabolite of the drug, decreased platelet inhibition and higher rate of subsequent cardiovascular events compared with non-carriers. Conversely, *CYP2C19*17* allele is reported to be associated with higher risk of bleeding in carriers (17).Hence testing for *CYP2C19* variants in the patients' population may be useful since Clopidogrel still is a high-priority drug for clinical treatments of Acute Coronary Syndromes.

CYP2C19 copy number variations so far have little or not fully evaluated. Only recently, CNVs *of CYP2C19, CYP4F2* and *SLCO1B3* have been investigated in a large cohort of 2,504 whole genomes from the 1000 Genomes Project and 59,898 exomes from the Exome Aggregation Consortium identifying 208 genes' rearrangements (0.33%; 18). However, the present study was aimed at evaluate the presence of *CYP2C19*alleles' deletions/duplications in a cohort of 300 individuals (600 chromosomes) with Acute Coronary Syndromes of whom 100 of Sardinian ancestry. A total of 13 *CYP2C19* large Ind/Dels were found. Deleted alleles (n=9) were more frequent in patients with MACE events, while Insertion alleles (n=4)were more abundant in patients with bleeding events. Although the aim of this study was to evaluate the presence of gross Cytochrome P450 2C19 gene-rearrangements and not their association with clinical outcomes, these findings may prompt to further genetically characterise patients when guided genetic-therapies are in place.

Study limitations. There are several limitations to our study. First the sample size accounts only for 600 chromosomes but all of the 300 patients were completely followed-up for one year. In addition, the ethnicity is solely Italian and the genetic data arise from a single centre. Therefore, duplication studies with other European populations are warrantied as well as the involvement of other genotyping facilities.

CONCLUSION

Our study demonstrates once more the complexity of developing personalised therapies for treatment of ACS. Genotype data should include not only recurrent SNPs but

additional search for large gene-rearrangements although much less frequent but with a strong effect on the final phenotype.

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