

Cardiovascular Risk Profile of Patients Hospitalized for Myocardial Infarction is Undestimated by Traditional Risk Factors and is Better Estimated by a Genetic Analysis Based Upon Single Nucleotide Polymorphisms: A Retrospective Study

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ABSTRACT

Aims: Acute myocardial infarction (AMI) may occur in patients without traditional cardiovascular risk factors (TRFs). The aim of our study was to compare, in patients with AMI, the cardiovascular risk estimate obtained from the analysis of TRF with the integrated genetic risk (IGR), calculated in the same population by the addition to TRF of the genetic analysis of 11 single-nucleotide polymorphisms (SNPs), which have been previously associated with cardiovascular disease (CVD) in genome-wide association studies (GWAS). **Methods:** We evaluated 118 patients hospitalized for AMI in our institution from June 2016 to June 2017. In these patients, a retrospective analysis of the risk of CV events at 5 years was done using a dedicated software. In this population, the IGR was compared to the TCR. **Results:** In the study population, the retrospective estimate of the CV risk according to TRFs yielded a high-risk profile (5-year CV events risk >20%) in only 16 patients (14%). When CV risk was estimated by the genetic analysis, the prevalence of high-risk patients increased significantly (41 patients, 35%, $P < 0.0001$ vs. TRF-based analysis). However, we observed no association between any single SNP and a family history of CVD. **Conclusions:** In a population of AMI patients, IGR analysis based on 11 SNP associated with CVD in GWAS recognized a high cardiovascular risk status in a significantly higher proportion of patients otherwise classified at low risk by TRF. We speculate that the prospective application of the IGR analysis to primary prevention might improve CV risk stratification.

Key words: Cardiovascular disease, genome-wide association (GWA) studies, risk assessment, software, race, myocardial infarction

INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of death around the world, as about 30% of all deaths worldwide were caused by CVD, twice the number of deaths caused by cancers.^[1,2]

It has been estimated that heritable factors account for 30–60% of the interindividual variation in the risk of coronary artery disease (CAD).^[3] Particularly, in “premature” forms of CAD, a positive family history is often the only cardiovascular risk factor identifiable, suggesting a crucial role for genetic factors in the genesis of the disease.^[4]

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Recently, numerous studies aimed at identifying genes involved in this complex pathology have been conducted. In particular, interesting results have emerged from genome-wide association (GWA) studies, based on the screening of human genome to identify common DNA variants associated with phenotype of interest.^[5] Indeed, GWA studies have identified several common variants associated with risk of CAD.^[6-9]

However, these polymorphisms, when considered individually, explain only a modest proportion of the overall cardiovascular risk. Therefore, it has been suggested that a combination of several genetic polymorphisms in a single risk score could help to identify a cardiovascular haplotype for the redefinition of clinical risk scores.

To date, many sophisticated algorithms considering interactions between lifestyle and genetic polymorphisms have been designed,^[10-13] and recently, a new algorithm has been developed to calculate, using a dedicated software (Ω race, <https://www.fondazioneinuit.it/race>), a new integrated genetic risk (IGR) score taking into account the well-known traditional CVD risk plus the analysis of 11 single-nucleotide polymorphisms (SNPs) associated with coronary heart disease in GWA studies ($P < 5 \times 10^{-8}$; Cardio Kit AC097, Nuclear Laser Medicine, Milan).^[9,14-16]

The purpose of this study was, therefore, to investigate retrospectively the potential contribution of the “ Ω risk score” in defining a whole cardiovascular risk in a cohort of patients hospitalized for acute myocardial infarction (AMI).

MATERIALS AND METHODS

Patient samples and sample processing

This is a retrospective study based on blood samples of 118 patients hospitalized with AMI at the Department of Cardiology, University Hospital “Maggiore Della Carità” (Novara, Italy), collected for routine examinations. All patients were recruited from June 1, 2016, to June 31, 2017.

Inclusion criteria were as follows: (i) The presence of biochemical data from 2011 (5 years before the cardiovascular event), in particular, reporting the well-known CVD risk factors such total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol and (ii) the presence of clinical data from 2011, in particular, systolic blood pressure and information about smoking, type 2 diabetes, hypocholesterolemic medications, hypertension-lowering medications, and a family history of ischemic heart disease. No exclusion criteria were applied.

Clinical data were obtained from the laboratory information system (LMX-Siemens Healthcare Diagnostics) and the TDR software (Siemens Healthcare Diagnostics). Baseline

demographic and clinical data, as well as comorbidities such as TD2 and current therapies, were gathered from medical records and the software PC Care 2.0 (F.C.S. Solutions SRL).

All data were entered into a database, and the identifying information of each patient (name, surname, and date of birth) was replaced by a numerical code.

After the routine tests were performed, the biological material scrap, in particular, the whole blood collected in EDTA vacutainers (Becton, Dickinson and Company), was stored at -32°C in test tubes marked only by a numerical code in a controlled access fridge, at Clinical Biochemistry Laboratory, Maggiore della Carità Hospital, Novara, Italy.

These residual whole blood samples were subsequently used for the analysis of the 11 aforementioned SNPs (Cardio Kit AC097 NLM, Nuclear Laser Medicine S.R.L., Milan, Italy).

As approved by the institutional review board for human subjects,^[17] the residual blood used in this study was considered discarded and no informed consent was necessary. We obtained the informed consent from all the participants to perform the genetic tests and to publish the results in anonymous form.

Genetic analysis

The Cardio Kit AC097 (Nuclear Laser Medicine S.R.L., Milan, Italy), based on reverse-hybridization principle, was employed for the identification of the following 11 SNPs found to be associated with CAD: 9p21 (rs1333049); 1p13 (rs599839); 1q41 (rs17465637); 10q11 (rs1746048); 21q22 (rs9982601); 6p24 (rs125264531); 2q33 (rs6725887); 19p13 (rs1122608); 3q22 (rs2306374); 10q24 (rs12413409); and 12p13 (rs3736235).

After the target sequence was amplified using biotinylated primers on MasterCycler (Eppendorf S.R.L., Milan, Italy), the resulting polymerase chain reaction product was hybridized with a specific oligonucleotide probe immobilized on membrane-based strips. The exact match between probes and amplified product generates a signal exploiting the bond between biotin and streptavidin conjugated with alkaline phosphatase and a subsequent color developer.

Automated genomic DNA isolation

Maxwell[®] 16 DNA Purification Kits (Promega Italia S.R.L., Milan, Italy) that utilized paramagnetic particle technology on Maxwell[®] 16 Instrument (Promega Italia S.R.L., Milan, Italy) were used to extract genomic DNA from 350 μL venous whole blood collected in buffered EDTA.

CVD risk score

The biochemical, clinical, and genetic data of all patients were used for evaluating the individual CVD risk score.

In particular, the risk of CV events was evaluated using a dedicated software (Ω Race, <https://www.fondazioneinuit.it/race>) that calculates the risk of CV events at 5, 10, 15, and 20 years, combining well-known traditional CVD risk factors (age, sex, obesity, smoking, diabetes, hypertension, and hypercholesterolemia; CUORE software, www.cuore.iss.it) (14) with genetic factors: 9p21 (rs1333049); 1p13 (rs599839); 1q41 (rs17465637); 10q11 (rs1746048); 21q22 (rs9982601); 6p24 (rs125264531); 2q33 (rs6725887); 19p13 (rs1122608); 3q22 (rs2306374); 10q24 (rs12413409); and 12p13 (rs3736235).^[16]

For all the subsequent analyses, we employed the 5-year risk score.

Statistical analysis

Statistical analyses were performed using MedCalc® Version 9.2.1.0 (MedCalc Software, Belgium). The differences of the 5-year CV events risk calculated using CUORE and the Ω -Race were estimated by Fisher’s exact test. $P < 0.05$ was considered statistically significant. The differences of genetic polymorphisms frequencies between patients with ST-segment elevation myocardial infarction (STEMI) and patients with non-ST-segment elevation myocardial infarction (NSTEMI) were estimated by Fisher’s exact test. Finally, the association between the polymorphisms investigated and traditional risk factors was estimated in NSTEMI patients using Fisher’s exact test.

RESULTS

Overall study population

The clinical and biochemical data of the study population are shown in Table 1. A CUORE predictive equation that takes into account only the traditional risk factors, and a new mathematical model that combines the traditional risk factors with genetic risk factors^[14,16] [Table 2] was applied to assess the 5-year CV events risk in 118 patients hospitalized for AMI.

The retrospective application of the risk prediction model based on TRFs predicted a high 5-year CV risk (events risk >20% in 5 years) in 16 patients (14%) of the study population. The implementation of the CV risk estimation with the IGR analysis significantly increased the number of patients retrospectively estimated at high risk for CV events to 41 (35% of the study population, $P < 0.0001$ vs. traditional cardiovascular risk factors [TRF] prediction) [Figure 1].

It is known that genetic variants that do not affect TRFs have a greater clinical impact, if used in the calculation of a risk score. Therefore, we examined whether there was a correlation between the 11 SNPs analyzed and the TRFs, in particular, TD2: 3q22 polymorphism (rs2306374) was

Table 1: Clinical and biochemical data of 118 patients with acute myocardial infarction

	Number	Mean±SD
Age (years)	118	56±13
Body weight (Kg)	118	76.5±16
Height (cm)	118	168.4±9.9
	Number	%
Male	87	73.7
Family history of cardiovascular diseases	39	33
Hypertension	69	58.5
Hypercholesterolemia	52	44.1
Cigarette smoking	51	43.2
Diabetes mellitus	23	19.5
Myocardial infarction with ST elevation (STEMI)	67	56.8

The data are from 2011, utilized to calculate the 5-year CV events risk, using a dedicated software (Ω race, <https://www.fondazioneinuit.it/race>). STEMI: ST-segment elevation myocardial infarction, CV: Cardiovascular, SD: Standard deviation

Table 2. The 11 SNPs analyzed in the present study, associated by GWA studies with coronary heart disease (Schunkert *et al.*, 2011; Donfrancesco *et al.*, 2010; Romeo *et al.*, 2016)

LOCUS	SNP	Nearest genes
9p21	rs1333049	<i>CDKN2A CDKN2B</i>
1p13	rs599839	<i>CELSR2 PSRC1 SORT1</i>
1q41	rs17465637	<i>MIA3</i>
10q11	rs1746048	<i>CXCL12</i>
21q22	rs9982601	<i>SLC5A3 MRPS6 KCNE2</i>
6p24	rs12526453	<i>PHACTR1</i>
2q33	rs6725887	<i>WRD12</i>
19p13	rs1122608	<i>LDLR</i>
3q22	rs2306374	<i>MRAS</i>
10q24	rs12413409	<i>CYP17A1 CNNM2 NT5C2</i>
12p13	rs3736235	<i>OLR1</i>

SNPs: Single-nucleotide polymorphisms

significantly associated with diabetes ($P < 0.01$, Fisher’s exact test).

Moreover, since 39 patients had family history of ischemic heart disease, we examined whether there was a correlation

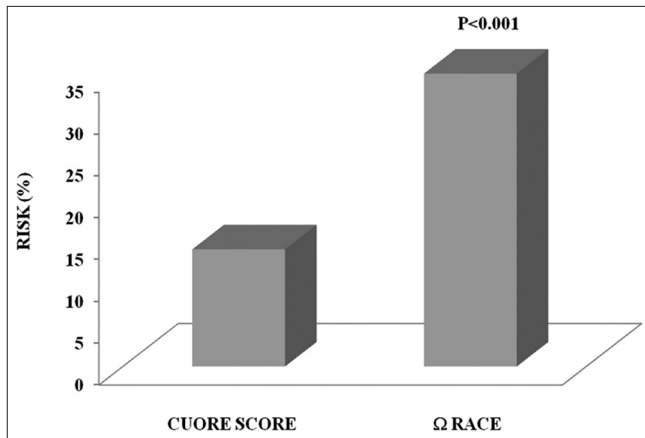


Figure 1: Proportion of patients with myocardial infarction recognized at high cardiovascular risk by traditional risk factors analysis (CUORE score) and by single-nucleotide polymorphisms enhanced analysis (Ω race program)

between the familiar anamnesis to ischemic heart disease and the analyzed SNPs. No significant association was found (data not shown).

STEMI versus NSTEMI patients

Of the 118 patients enrolled in our study, 67 have been hospitalized with STEMI and 51 with NSTEMI. The clinical and biochemical data of STEMI and NSTEMI patients in 2011 are shown in Table 3. Despite the widespread use of electrocardiographic changes to characterize patients with AMI, little is known about the possible genetic differences between STEMI and NSTEMI. Therefore, we analyzed the 5-year CV events risk, calculated using CUORE and Ω Race software in the STEMI and NSTEMI patient groups.

Five of the 67 (7%) STEMI patients had 5-year CV events risk profile $> 20\%$ calculated with CUORE; however, this percentage was significantly higher (25%: 17/67; $P < 0.001$ Fisher's exact test) when the risk score was calculated with Ω Race.

Eleven of 51 (22%) NSTEMI patients had 5-year CV events risk profile $> 20\%$ using TRFs, but this percentage increased significantly (47%: 24/51; $P < 0.001$ Fisher's exact test) when the IGR score was applied [Figure 2]. Moreover, our preliminary studies show that the new algorithm is able to predict the risk of heart attacks in a significantly higher percentage of NSTEMI cases compared with STEMI cases [Figure 2].

We then assessed whether there was an association between the 11 SNPs investigated and STEMI or NSTEMI infarction, and indeed, we found that the 9p21 polymorphism (rs1333049) was significantly associated with NSTEMI ($P < 0.05$; Fisher's exact test).

Finally, we assessed whether traditional risk factors, in particular, hypertension, hypercholesterolemia, and TD2 were associated with STEMI or NSTEMI infarction; the presence of hypertension, hypercholesterolemia, and TD2 was significantly associated with the NSTEMI ($P < 0.01$; Fisher's exact test).

DISCUSSION

In a population of patients hospitalized for AMI, the retrospective CV risk evaluation with an IGR analysis based on 11 SNP associated with CVD in GWA studies recognized high-risk patients in a significantly higher proportion, compared to risk evaluation with TRF-based models. According to this striking difference, we speculate that IGR analysis might improve CV risk stratification when prospectively applied to primary prevention patients.

It is well known that CVD is a multifactorial disease caused by genetic, social, physiological, and environmental factors that interact with each other to determine the level of coronary risk and over the last decades, numerous epidemiological studies have focused their attention on the global absolute risk for identifying high-risk individuals.^[18-21] Moreover, all current guidelines stress the need to consider the impact of all risk factors before making clinical management decisions and, in most cases, to recommend a system of evaluating combined risk factor effects.^[15]

Recently, the attention has focused on trying to improve risk estimation through the incorporation of new common variants associated with the risk of CVD by GWA studies. In particular, GWA studies have identified 56 genetic loci associated with CVD at genome-wide significance.^[6-9,22] Therefore, many sophisticated algorithms considering interactions between lifestyle and SNPs were designed.^[10-13] However, the addition of new risk factors often shows small improvements in CVD risk prediction.^[10,13,23-29]

In this context, a new algorithm has been developed to calculate, using a dedicated software (Ω race, <https://www.fondazioneinuit.it/race>), a global absolute risk that takes into account both traditional and genetic risk factors.^[9,14,16]

In our population of AMI patients, the Ω Race software allowed a significantly higher number of patients to be classified as having 5-year CV events risk $> 20\%$. In particular, the new algorithm would have been able to predict the risk of heart attacks in 47% of NSTEMI patients if used in primary prevention. Therefore, the addition of new genetic risk factors could be useful in correctly reclassifying those at intermediate risk as above or below a chosen intervention threshold.

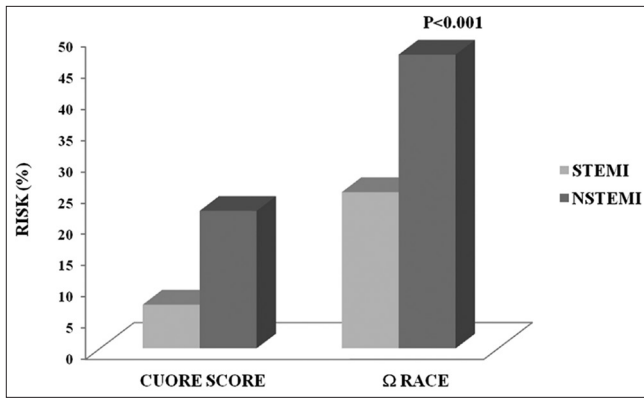


Figure 2: Proportion of patients with myocardial infarction recognized at high cardiovascular risk by traditional risk factors analysis (CUORE score) and by single-nucleotide polymorphisms enhanced analysis (Ω race program), according to the clinical presentation with ST-segment elevation myocardial infarction and non-ST-segment elevation myocardial infarction.

Table 3: Clinical and biochemical data of 118 patients with acute myocardial infarction divided into two groups: Patients with STEMI and patients with NSTEMI

	STEMI (67)	NSTEMI (51)
Male (n)	53	34
Age (years)	56±13	63±12
Body weight (Kg)	77.7±16.6	75±15.3
Height (cm)	166.8±10.2	169.6±9.6
Family history of cardiovascular diseases	36	29
Hypertension	48	73
Hypercholesterolemia	33	59
Cigarette smoking	52	32
Diabetes mellitus	9	33

The data are from 2011, utilized to calculate the 5-year CV events risk, using a dedicated software (Ω Race, <https://www.fondazioneinuit.it/race>). STEMI: ST-segment elevation myocardial infarction, NSTEMI: Non-ST-segment elevation myocardial infarction

It is known that genetic variants not associated with traditional risk factors have a greater clinical impact, if used when calculating the risk score. Hence, we dedicated a specific analysis to the correlation between the 11 SNPs employed by the Ω Race model and traditional risk factors (in particular, diabetes, hypertension, and hypercholesterolemia). Our data suggest that the 3q22 polymorphism (rs2306374) previously associated with the risk of developing CAD in GWA studies^[30-32] may also be associated with diabetes. These data are of particular interest as they never been reported in the literature before. This polymorphism has been associated with the *MRAS* gene, encoding the M-ras protein belonging to

the *ras* superfamily of GTP-binding proteins. It is expressed in the heart and aorta,^[33] and it may be involved in adhesion signaling, which is important in the atherosclerotic process.^[34]

The association between the SNP and TD2 should be investigated in a larger cohort, but if confirmed, the 3q22 polymorphism analysis should not be included in the new mathematical model for the calculation of CVD risk score (Ω race), as already associated with another risk factor (i.e., TD2).

Our data also suggest that the 9p21 polymorphism (rs1333049) strongly associated with the risk of developing CAD,^[30] myocardial infarction, and coronary artery calcification in GWA studies^[32] may also be associated with NSTEMI, data never reported before in literature.

Despite compelling genetic evidence for the association of 9p21 with CAD,^[35,36] the mechanism by which 9p21 confers an increased CAD risk is unknown. The variant is not associated with established CAD risk factors such as plasma lipoprotein levels, hypertension, or diabetes, suggesting that CAD pathogenesis is modulated through a previously unappreciated pathway.^[37,38] The polymorphism is located within a 58-kb linkage disequilibrium block on chromosome 9p21.3 that contains a newly annotated gene designated *ANRIL*. This locus encodes a long antisense non-coding RNA that regulates the expression of other genes.^[39] *ANRIL* is expressed in atheromatous human vessels, vascular endothelial cells, monocyte-derived macrophages, and coronary smooth muscle cells, all of which are involved in atherosclerosis.^[39]

Visel *et al.* suggested that this polymorphism affects CAD progression by altering the dynamics of vascular cell proliferation.^[40] Therefore, it could be hypothesized that the 9p21 polymorphism (rs1333049) influences susceptibility to CAD by altering *ANRIL* at the transcriptional levels, therefore, modulating cell proliferation.^[41,42]

It is known that NSTEMI involves less than full-thickness (partial thickness) damage of heart muscle, while STEMI involves full-thickness damage of heart muscle. Therefore, NSTEMI is considered a less severe type of heart attack compared to STEMI. However, the general clinical setting of NSTEMI patients is usually more severe than STEMI patients (e.g. age, previous history of cardiac and cerebral ischemia, and history of revascularization). These data are confirmed by our study since traditional risk factors such as hypertension, TD2, and hypercholesterolemia were more closely associated with NSTEMI than with STEMI.

It is also known that in the follow-up NSTEMI patient presented a higher risk of reinfarction and death than STEMI patients.^[4,43] Therefore, the prevalence of 9p21 polymorphism in the NSTEMI population could explain, from a genetic

point of view, their most serious general clinical setting compared to STEMI patients. However, it will be necessary to analyze a larger population to confirm the new association found between the 9p21 polymorphism (rs1333049) and NSTEMI patients.

The preliminary results presented in this study suggest that analysis of the abovementioned 11 SNPs (Cardio Kit NLM AC097), and the calculation of the IGR using a dedicated software Ω Race (<https://www.fondazioneinuit.it/race>) could be helpful to predict the occurrence of a greater number of adverse cardiovascular events, in comparison with the analyses based on traditional risk factors.

That aspect is of great importance since the traditional cardiovascular risk can be implemented with the genetic approach, in particular, in healthy persons without a previous CVD event at the moment of CVD risk assessment.

However, although none of the patients included in the present study experienced other coronary or cerebrovascular events during the 5-year period investigated, it cannot be excluded that such events could occur well before. Therefore, the retrospective risk assessment for 5 years of follow-up could have underestimated the occurrence of cardiovascular events.

As concerning clinical application and usefulness of the integrated approach described in our study, it can be speculated that it could mainly modulate the clinical intervention through specific and targeted treatments able to reduce the overall risk of CAD.

In particular, on the basis of genetic evidence, the finding of different degrees of cardiovascular risk calculated with Ω Race should lead to pursue different therapeutic targets for primary prevention, such as LDL cholesterol levels <130 or 100 mg/dL. To date, this has not yet been validated by scientific studies.

Therefore, prospective studies conducted in populations in which the weight of the genetic component is greater (e.g. young AMI patients) will be necessary to understand if Ω Race can really improve the prediction and stratification of CV risk. Only in this way, the knowledge of the genetic basis of AMI may lead to the ambitious goal of personalized medicine, modulated on the specific biological characteristics of the individual.

Finally, it should be stressed that in the analyzed population, we did not find an association between familiarity medical history for coronary heart disease and the analyzed genetic risk factors. This is an important observation, raising the issue of which weight should be given to the concept of familiarity, clinically evaluated on the basis of medical history. The term “familiarity” is generally automatically associated with

genetics. However, probably, familiarity for the disease clinically evaluated, is not necessarily associated with the inheritable genetic component, but it most affected by (i) the social context in which the person lives and (ii) all its habits that are usually inherited as dominant traits, bypassing, or even overcoming the Mendel’s genetic laws.

In conclusion, modern genetics reveal a new view on the biology of CAD. In particular, it appears that its genetic pathogenesis is largely independent from the traditional risk factors. Nevertheless, it is likely that genetic factors act in a network that also includes modifiable cofactors.^[44]

A better knowledge of these interactions will be vital to gain the greatest benefit from this emerging information on the genetic predisposition to CAD.

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