Gene-Gene and Gene-Clinical Factors Interaction in Acute Myocardial Infarction: A New Detailed Risk Chart

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Abstract: *Aims*: The complex pathogenesis of acute myocardial infarction (AMI) implicates phenotypic and genetic heterogeneity. In this pilot case-control study single nucleotide polymorphism (SNP) in several inflammatory genes, such as interleukin (IL)-1β, IL-6, IL-10, α-1-antichymotrypsin (ACT), tumor necrosis factor alpha (TNF)-α and interferon gamma (IFN)-γ genes along with SNPs of genes regulating vascular functions (vascular endothelial growth factor; VEGF) and cholesterol synthesis (hydroxy-methyl-glutaryl CoA reductase; HMGCR) were investigated.

Methods: Patients were genotyped with RT-PCR technique and data were analyzed with a new mathematical algorithm named Auto Contractive Map.

Results: The Auto Contractive Map (AutoCM), was applied in AMI patients with the aim to detect and evaluate the relationships among genetic factors, clinical variables and classical risk factors. Genes were selected because their strong regulatory effect on inflammation and SNP in these gene were located in the promoter region. In the connectivity map generated by AutoCM a group of variables was directly linked with the AMI status; these were: gender (male), early age at onset (50-65 years), HMGCR gene (CC wild type genotype), IL-1βCT, IL-6 GG and VEGF CC genotypes. This direct link suggested a possible pathogenetic association with AMI. Other genetic, clinical and phenotypic variables were associated to the disease under a statistically defined hierarchy showed in the new connectivity map generated by AutoCM.

Conclusion: These analyses suggested that genotypes of few inflammatory genes, a SNP in HMGCR gene, middle age, gender, low HDL and diabetes were very informative variables to predict the risk of AMI.

Keywords: Acute myocardial infarction, gene-risk factor interaction, connectivity map.

INTRODUCTION

Coronary heart disease is one of the major cause of morbidity and mortality worldwide [1,2]. A major complication of coronary artery disease is the acute myocardial infarction (AMI). AMI is multi-factorial disease and lifestyle factors, individual genetic background and environmental risk factors influence the clinical manifestation of the disease. Therefore, the pathogenesis of AMI is complex and not yet fully defined.

Hypercholesterolemia, hypertension, smoking, diabetes, obesity, and sedentary lifestyle are considered "classical" risk factor of AMI [3]. Abnormal blood lipids and lipoproteins [4] are also associated with increased risk of cardiovascular diseases. Additional peripheral plasma biomarkers influencing AMI risk such as blood homocysteine [5], fibrinogen [6] and lipoprotein(a) [3] have been proposed as indicators of an increased predisposition to the disease. More recently, markers of altered immune activation such as elevated blood levels of C-reactive protein and cytokines have been suggested to promote atherogenesis and lead to AMI [7-11].

Although recognition and treatment of established risk factors for AMI have considerably reduced the disease burden, phenotypic markers vary over time and their predictive potential is affected by age, gender, diet, co-morbidity, drug treatment and other environental variables. Moreover, the relevance of these variables on the atherosclerosis progression and clinical manifestation of AMI might be also differentially influenced by the individual genetic background.

Assessment of genetic risk factors might help in better defining individual risk or predisposition to AMI. In fact, inherited gene variants are less influenced by environmental factors and might provide a better indication for individual susceptibility to AMI. Moreover, efforts for unraveling the genetic basis of AMI are pivotal for the development of new diagnostic tools and innovative therapeutic approaches. Multiple pathogenetic pathways leading to AMI implicate genetic heterogeneity, or in other words, the association of multiple genetic traits with the disease. For instance, low plasma HDL may be influenced by several genes regulating different metabolic pathways. Transition from stable to unstable atherosclerotic plaque may be affected by several gene polymorphism in different genes with immune regulatory functions. Moreover, a single gene polymorphism (SNP) can account for a limited contribution (low odds ratio values) to the total genetic load for the disease and both common and rare gene polymorphisms may differentially influence the susceptibility to the disease. On the other hand, the presence of one or more established risk factors for AMI might differentially influence gene expression and in turn the clinical relevance of one or more genes associated with the disease. The above notions may partially explain contradictory results of genetic association studies from AMI and coronary heart disease [12,13]. Therefore, investigations designed to evaluate gene-gene and gene-environment interactions for identification of a multi variable network associated with increased risk of developing AMI might be highly informative.

In this pilot case-control study SNPs of inflammatory genes interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), alpha-1-antichymotrypsin (ACT), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) along with SNPs of genes regulating vascular functions (vascular endothelial growth factor; VEGF) and cholesterol synthesis (hydroxy-methyl-glutaryl CoA reductase; HMGCR) were investigated. Selected genes were chosen because their relevant role in regulating inflammatory responses. SNPs were chosen in the promoter region of the gene and they

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affected the rate of cognate mRNA and protein synthesis. To evaluate the relationship among genetic, clinical traits and classical risk factors in AMI a new statistical analysis based upon the Auto Contractive Map algorithm (AutoCM) has been applied. This novel data mining algorithm [14] was aimed to explore the concomitant association of different variables with AMI and the potential relationships among variables in a multi factor network relevant for the disease. The ultimate goal of this data mining model was to discover hidden trends and associations among variables, since this algorithm was able to create a semantic connectivity map in which non linear association were preserved and explicit connection schemes were described [15]. This analysis showed an age and gender dependent hierarchy of genetic and biological factors with the disease.

MATERIALS AND METHODS

Subjects

283 patients with clinical diagnosis AMI (mean age= 67.5± 2.1; 72.8% male and 27.2% female) from the Cardiology Unit of Ferrara University Hospital were enrolled. Each patient met diagnostic criteria for AMI based on electrocardiography changes and standard laboratory findings confirmed by echocardiography and coronary angiography. Controls consisted of 318 healthy subjects (mean age= 72.0±5.1; 49.4% male and 50.6% female) belonging to a longitudinal population study, i.e. the "Conselice Study of brain aging" [16]. All controls did not show cardiovascular or inflammatory diseases at the beginning of the follow up (1999-2000) and were still free of these pathological conditions at the end of the follow up (2004-2005).

The research protocol was approved by relevant institutional review boards, all participants gave a written and informed consent and the investigation conforms with the principles outlined in the Declaration of Helsinki.

SNP Detection

Genomic DNA from peripheral blood leukocytes of AMI and healthy subjects was obtained by a method described elsewhere [17]. Genetic determination of polymorphisms in promoter regions of IL-1β-511C/T, IL-10 -1082G/A, TNFα -308G/A, ACT -51G/T, VEGF -2578C/A and HMGCR -911C/A genes was performed by Real Time-PCR method. SNP-specific primers and probes were designed according to the TaqMan genotyping assay by ABI (Foster City, Ca, USA) and assays were performed in 25 μl total volume on Stratagene MX3000P following manufacturer's instructions.

IFNγ (rs2430561) and IL-6 (rs1800795) genotypes were assayed by Real-Time using allele specific modified LNA primers [18].

IFNγ(+874)rs2430561,(TTTATTCTTACAACACAAAATCAAAT C+T,TTTTATTCTTACAACACAAAATCAAATC+A, TGTGCCTTCCTGTAGGGTATTATTA)

IL6 (-174) rs1800795 (TCCCCCTAGTTGTGTCTTGC+C, TCCCCCTAGTTGTGTCTTGC+G, AATCCCACATTTGATAAATCTTTGT). The polymorphic SNP was located at the 3'-position of the forward primers, and a single-LNA base was incorporated at this position (+) (Proligo, Italy).

Real-time PCR was performed in 96-well plates using a Stratagene MX3000P platform. Reaction volume (25 μl), included a SYBR Green PCR Master Mix with the enzyme, Mg^{2+} and dNTPs (ABI, Foster City, CA, USA; 200 nmol/L) PCR primers and genomic DNA (0.5 ng/μl). A start of 10 min at 95°C was followed by 40 cycles at 95°C for 15 s and 60°C for 60 s.

Plasma Analyte Detection

Plasma levels of total cholesterol, triglycerides, and HDL were measured by commercial clinical lab assays.

Statistical Analysis

Interactions among variables influencing the AMI pathogenesis was investigated by an innovative mathematical approach that was able to assess in a limited number of case/controls the relevance of each variable in representing a major biological hub or point of aggregation for other variables in a connectivity map. Therefore, the relatively small number of AMI cases and controls was sufficient to evaluate multiple variable interactions with the disease by AutoCM. This method is based on an artificial adaptive system and defines the association strength of each variable with all the others factors in any dataset; the algorithm is named the Auto Contractive Map (AutoCM). The mathematic of AutoCM was invented and tested, as described elsewhere [14]. An extended description of the AutoCM learning equations, the specific mathematics linked to the "contractive factor", the association to minimum spanning tree (MST) algorithm and its application to clinical cases was described elsewhere [15].

Briefly, this approach describes a context typical of living systems where a continuous time dependent complex change in the variable value is present. After the training phase, the matrix of the AutoCM represents the warped landscape of the dataset. A simple filter (minimum spanning tree by Kruskal) to the matrix of AutoCM system was introduced; this approach shows the map of relevant connections between and among variables and the principal hubs of the system. Hubs can be defined as variables with the maximum amount of connectivity in the map.

RESULTS

A panel of 8 SNPs in IL-β, IL-8, IL-10, IFN-γ, TNF-α, ACT, VEGF and HMGCR genes was studied. Gene were selected because their strong regulatory effect on inflammation and SNP in these gene were selected in the promoter region since their regulatory function upon the synthesis of the cognate protein. Genes, phenotypic and clinical variables used in the statistical analysis are listed in Table **1**.

Table 1. Gene Polymorphism and AMI Classical Risk Factors Used in the Auto CM Statistical Analysis

Gene polymorphism
IL-1β= SNP at -511, allele mutation = T
IL-6= SNP at -674, allele mutation= C
IL-10 = SNP at -1082, allele mutation= A
TNF-α= SNP at -308, allele mutation= A
IFN-γ= SNP at +874, allele mutation= A
ACT = SNP at -51, allele mutation= T
VEGF = SNP at -2578, allele mutation= A
HMGCR= SNP at -911, allele mutation= A
Classical AMI risk factors
Gender = male or female
Age ranges = <50 age, 50-65 age, >65 age
High-BMI = Body Mass Index >25
Diabetes = presence of diabetes
Low HDL = serum HDL < 40mg/dl,
Total high cholesterol = serum cholesterol >200mg/dl
High-triglycerides = serum triglycerides >100mg/dl,

To investigate gene-gene interactions (8 SNPs) with AMI and the relationship of these genetic variants with gender and different

ages at onset of the disease (<50yrs, 50-65yrs, >65yrs), the AutoCM analysis was performed.

A first connectivity map with a limited number of variables (28 factors) was generated and reported in Fig. (**1**).

This map showed most relevant associations among variables and the number between two variable referred to the statistical strength of association of variables. In this map a group of variables was directly linked with the AMI status. These were: gender (male), early age at onset (50-65 years), HMGCR gene (CC wild type genotype), IL-1β CT, IL-6 GG and VEGF CC genotypes; this direct link suggesting a possible pathogenetic association with AMI. Male gender was a secondary point of aggregation for other variables such as, earlier age at onset of AMI (>50 years), TNF-α AA and IFN-γ TA genotypes.

The second hub in the map, the HMGCR CC genotype, was either connected to AMI and a secondary point of connectivity for several other variables. These were: advanced age at onset, ACT TT, IL-6 CT, IFN-γ AA and TNF-α GA and GG genotypes. Female gender was connected to AMI via late age at onset. Finally, the TNF-α GG genotype was a tertiary point of convergence for IL-1β CC, ACT GG and GT genotypes and appear to play a minor role in the pathogenesis of the diseases.

To further investigate interactions or epistasis of these genetic variants with other classical risk factors relevant for AMI such as, different age at onset (<50yrs, 50-65yrs, >65yrs), the presence of diabetes, high BMI, low plasma HDL, high total cholesterol, high triglycerides, a second AutoCM analysis was performed. A second connectivity map with 36 variables was generated and presented in Fig. (**2**).

In this map VEGF CC, IL-10 GA, ACT GT, IL-1β CT genotypes were still directly linked to AMI, as well as low plasma HDL, presence of diabetes and male gender and two statistical hubs, e.g. HMGCR CC genotype and age range over 65 years, were observed. Once again gender (male) was a secondary point of aggregation for other variables.

HMGCR CC genotype was the second hub directly linked with AMI and a point of aggregation for several genetic variables (IL-6 GG, IFN-γ AA, TNF-α GG). Other variables converged upon HMGCR CC genotype in an advanced (> 65 years) age-dependent manner. Among these, female gender, high BMI scores, high total plasma cholesterol levels, ACT GG and IL-10 AA appeared of relevance for AMI in this age group.

DISCUSSION

Several genetic and environmental factors may differentially contribute to biological mechanisms leading to the clinical manifestation of AMI. Investigations focused on a single SNP or several SNP in a single gene may result in a limited and segmental information that is difficult to replicate in case control studies across different populations with partially different genetic background. Moreover, gene-gene and gene-environment interactions, as well as the influence of other biological factors, such as gender, age and classical risk factor for AMI are usually not included or poorly

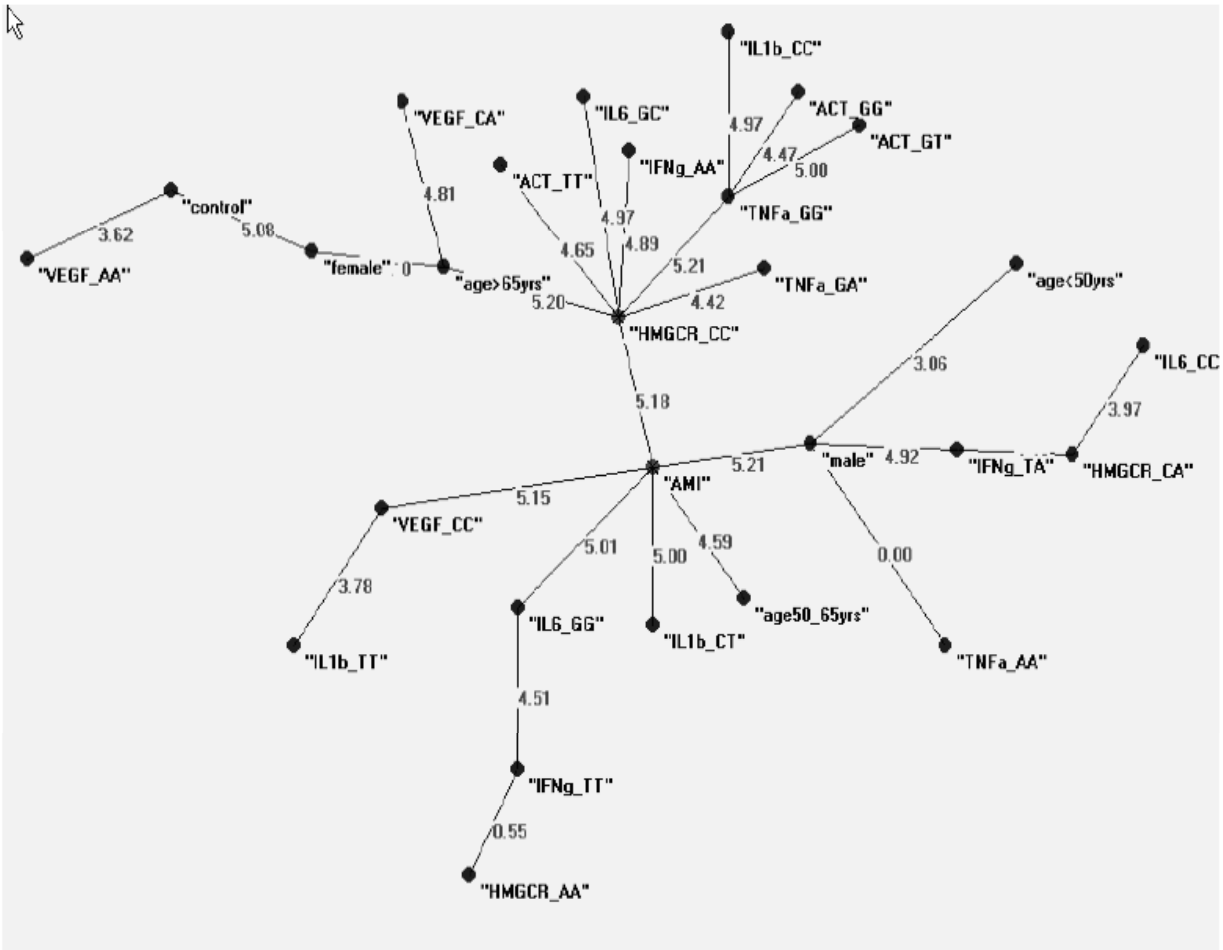

Fig. (1). Connectivity map of 28 variables from AMI/control database.

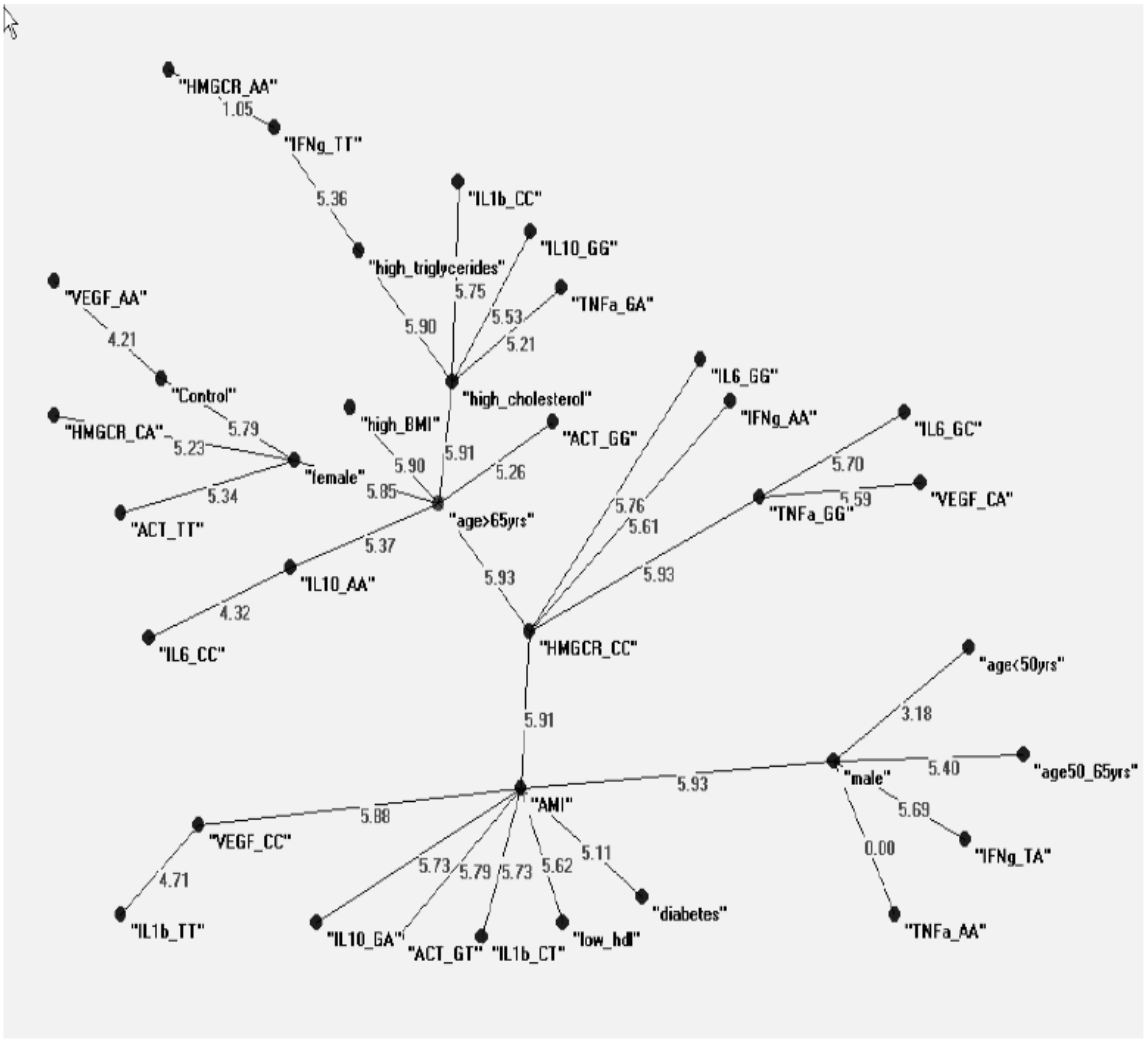

Fig. (2). Connectivity map of 36 variables from AMI/control database including classical risk factors.

evaluated in genetic association studies. These reasons might partially explain conflicting results from gene association studies in AMI [8,9,11,19-24].

In this pilot case-control study SNPs in the promoter region of IL-1β, IL-6, IL-10, ACT, TNF-α and IFN-γ inflammatory genes, as well as SNPs of genes regulating vascular functions (VEGF) and cholesterol synthesis (HMGCR) were investigated. Most SNPs selected in the present report have been shown to have functional relevance, since the presence of a given genotype or allele affected gene expression or plasma concentrations of the cognate protein [25-29].

A complex statistical analysis of genetic, clinical and epidemiological data was then applied. The statistical method derived from the minimum spanning tree (MST) algorithm that was originally described by Kruskal [30]. The algorithm has been modified, based on an artificial adaptive system and named AutoCM. This algorithm was developed to assess the association strength of each variable with all the others factors in any dataset [14], was able to investigate links among variables and has been recently used in biology and medicine [14,15].

This new statistical analysis showed a connectivity map among variables overall describing an age and gender dependent interaction of different gene variants with AMI Fig. (**1**). Moreover, additional interactions among these genetic variants and clinical-biological factors in the second connectivity map were shown Fig. (**2**).

In this context variables directly converging upon the clinical status of AMI appeared to play a major influence on mechanisms leading to the disease. These variables were: 1) SNPs in genes with inflammatory regulatory actions (VEGF CC, IL-10 GA, ACT GT and IL-1 β CT genotypes); 2) low HDL plasma levels; 3) diabetes; 4) gender (male) and 5) the HMGCR CC genotype. These mixed genetic, clinical and epidemiological factors could be considered as primary players in a partially new risk chart describing factors associated with AMI.

It is of interest that the HMGCR CC genotype was also a secondary point of connectivity for many other genetic, clinical and biological variables. Few other genetic factors were directly linked to HMGCR gene, such as IL-6 GG, IFN-β AA and TNF-α GG genotypes. These findings indirectly confirm the hypothesis that some inflammatory regulatory genes might affect AMI risk independently by cholesterol metabolism. Finally, many other genetic or non genetic factors converged on the HMGCR gene, but in older patients (age> 65 years). Therefore, other inflammatory genes by affecting cholesterol synthesis might be associated with a later clinical manifestation of AMI. Other immune regulating gene appeared to play role in AMI history by influencing cholesterol blood transportation (HDL and LDL blood levels) and influencing

caloric metabolism (high BMI). Finally, our data suggested age and gender differentially affected the interactions of genetic and classical risk factors with AMI. Further studies will better discriminate gender-related factors associated with the disease.

It is important to note that the main difference between Figures 1 and 2 was related to the introduction of additional clinical-epidemiological factors in the second analysis Fig. (**2**). Major variables described in Fig. (**1**) maintained their direct link with the AMI status also in the second map Fig. (**2**). Therefore, their relevance in AMI were not altered by introduction of other well established risk conditions for the disease such as diabetes, cholesterol, HDL or BMI.

Our observations suggest that statistical models describing multiple interactions among genes and between genes and other biological factors could increase the level of information relevant to the disease. In fact, according to these findings, several conclusions could be suggested: 1) a group of gene variants (VEGF CC, IL-10 GA, ACT GT) with regulatory effects on inflammation directly affected immune mechanisms related to AMI. A modulation of their cognate molecule by specific inhibitor might results in secondary prevention of AMI by an early intervention therapeutic effects. 2) Other immune regulating genes (IL-6, IFN-γ and TNF-α) operated via HMGCR influence. This observation indirectly supported the notion that statins, drugs targeting HMGCR gene and lowering cholesterol levels, also showed anti-inflammatory effects [31]. 3) Low plasma HDL and diabetes were confirmed to be of primary relevance for the clinical manifestation of the disease by AutoCM analysis. 5) In this case-control investigation, high BMI, high total plasma cholesterol and triglycerides appeared to influence AMI risk in women at older age (> 65 years) and *via* the HMGCR gene.

Our findings have shown relevant genetic, phenotypic and clinical risk factors in a AMI population ranging between 50-70 years of age. We can not exclude that others, partially different risk factors, might play a pivotal role in older (> 80 years) AMI patients. Recent investigations indeed showed that in very old people with no history of cardiovascular diseases classical risk factors did not predict cardiovascular mortality [32].

Genotypes in several pro-inflammatory genes such as IL-6 GG, IL-1β CT and VEGF CC were directly associated with AMI in the connectivity map. This data confirm previous observations showing in a different case/control investigation an association of IL-6 and IL-1β. SNPs with AMI [9,11]. Other Authors showed that VEGF polymorphism influenced the severity of atherosclerosis [24]. Therefore, some gene regulating inflammatory responses and angiogenesis appear to directly influence mechanisms involved in AMI pathogenesis possibly by affecting leukocyte activation and migration in the vessel intima.

SNPs in other pro-inflammatory genes such as TNF-α GG, IFN-γ AA and ACT TT were linked to AMI via HMGCR gene. These findings suggested that the above genes might influence AMI by affecting lipid metabolism. In fact TNF-α was recently shown to affect the cholesterol efflux in adipocytes by influencing ABCA1 expression and endothelial lipase activity [33]. Furthermore, TNF-α SNPs were found to independently influence HDL and apoA1 blood concentrations [34].

Finally, it is of important to note that AutoCM analysis was successfully applied in other human disease such as Alzheimer's disease and the algorithm was able to identify new risk factors for dementia [15]. Artificial neural network analysis was also used in stroke by other investigators to assess disability outcome [35] and gait classification scores [36] with useful clinical results.

Our findings suggest that the concomitant assessment of the association of multiple variables with complex diseases by powerful algorithm may results in a new data mining method to apply in the developing field of predictive diagnostics. Moreover, this method suggest a road leading to an innovative risk evaluation map that, by including genetic factors, integrates already established risk charts to evaluate individual predisposition to AMI.

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CONFLICT OF INTEREST

None to declare.

ABBREVIATIONS

ACT	=	Alpha-1-Antichymotrypsin
AMI	=	Acute Myocardial Infarction
AutoCM	=	Antocontraptive Map
BMI	=	Body Max Index
HDL	=	High Density Lipoprotein
HMGCR	=	Hydroxyl-Methyl-Glutaryl-CoA Reductase
IL-1β	=	Interleukin-1 beta
IL-6	=	Interlukin-6
IL-10	=	Interleukin-10
IFN-γ	=	Interferon-gamma
LDL	=	Low Density Lipoprotein
MST	=	Minimum Spanning Tree
RT-PCR	=	Real Time PCR
SNP	=	Single Nucleotide Polymorphism
TNF-α	=	Tumor Necrosis Factor-alpha
VEGF	=	Vascular Endothelial Growth Factor

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