



## Myocardial infarction marker levels are influenced by prothrombin and tumor necrosis factor- $\alpha$ gene polymorphisms in young patients

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### ABSTRACT

Polymorphisms of genes encoding key factors for the control and activation of inflammatory response and coagulation cascade regulation may play a role in genetic susceptibility to acute myocardial infarction (AMI). This study sought to analyze the effect of TNF  $-308G/A$  and pro-thrombin (FII) 20210G/A polymorphisms on the laboratory parameters of young patients affected by AMI. Results indicated that TNF  $-308A$  positive genotype frequencies were increased in these patients and that a genetically determined higher production of TNF- $\alpha$  is associated in young subjects to a more severe cardiac damage as depicted by higher levels of troponin, Creatine kinase-MB Isoenzyme (mCK-MB) and a significant increased plasma fibrinogen levels. Similar and probably additive effects on might have a genetically determined increased production of pro-thrombin even if no significant differences in genotype frequencies of pro-thrombin (FII) 20210G/A polymorphisms were observed in this study. All together these results, indicating the relationship among genetically determined TNF $\alpha$  and FII production and increased levels of tissue damage markers of AMI, suggest that a complex genetic background, might be involved in susceptibility to AMI in young men influencing the extension and severity of the disease.

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### 1. Introduction

Acute myocardial infarction (AMI) the leading cause of mortality and morbidity in industrialized countries is a common outcome of coronary atherosclerosis. As other multifactorial disease, AMI probably involves many different gene variants that might interact to result in an additive or a synergistic co-effect. Young adults are a relatively small proportion of patients who experience AMI [1]. They have some distinct characteristics compared to older patients as young people are more likely to have normal coronary arteries

*Abbreviations:* AMI, acute myocardial infarction; aPTT, time (in seconds) of activated partial thromboplastin; AST, aspartate aminotransferase; CHD, coronary heart disease; CTnl, cardiac troponin I isoenzyme; FII, factor II (pro-thrombin); FV, factor V Leiden; HLA, human leukocyte antigen; IL-6, interleukin-6; IL-10, interleukin-10; INR, international normalized ratio; mCK-MB, creatine kinase-MB; PLTs, platelets; PT, prothrombin activity; SNPs, single nucleotide polymorphisms; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; UTR, upstream untranslated region.

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[2]. Although the exact pathogenetic mechanism of AMI in young patients remains unknown it has been proposed that thrombus formation due to atherosclerotic plaque rupture or erosion is, always, the main mechanism [3].

The importance of genetic factors seems to be particularly relevant in younger subjects, where it is assumed that the genetic background influences the susceptibility of these subjects to environmental risk factors for AMI identified in the general population. In particular a prominent role of genetic factors in the onset of this disease has been documented in twins and family based studies [4].

Recently a number of candidate genes and chromosomal loci have been identified to be associated with the susceptibility to myocardial infarction and a majority of these genes have been implicated in the processes of inflammation [5,6]. The single nucleotide polymorphisms (SNPs) present in the genes CD14 ( $-159 C/T$ ), TNF $\alpha$  ( $-308 G/A$ ), IL-1a ( $-889 C/T$ ), IL-6 ( $-174 G/C$ ), PSMA6 ( $-8 C/G$ ), and PDE4D (SNP83 T/C, respectively), were found to be associated with increased risk of cardiovascular diseases in different populations [7–12]. In addition previous studies have shown that polymorphisms of genes encoding key factors for the control and

activation of inflammatory response may play a role in genetic susceptibility to AMI in young men [13,14].

In particular the pleiotropic pro-inflammatory cytokine tumor necrosis factor (TNF)-alpha, lying at the telomeric end of the class III region of the human leukocyte antigen (HLA) [15–18]. Play a central role in the inflammatory responses with multiple biologic activities.

Wilson and his colleagues [19] first reported the bi-allelic polymorphism within the 5' genomic region of the TNF- $\alpha$  gene promoter at position –308 (–308G  $\rightarrow$  A; rs1800629). Carrying the A allele enhances transcriptional activity and is reported to be associated with higher levels of circulating TNF- $\alpha$  [20,21]. Serum levels of TNF- $\alpha$  is elevated in coronary heart disease (CHD) patients and may modify the risk for developing coronary events since it affects endothelial cell hemostatic function [22] and in a recent metanalysis [23] it has been demonstrated that this polymorphism might be a risk factor for coronary heart disease.

Moreover congenital or acquired mutations located on genes coding for antithrombin III, protein C, protein S, factor V Leiden (FV) and prothrombin (FII), normally associated to the occurring of deep venous thrombosis, were also inconstantly found in patients with arterial thrombosis. Recent observations stressed the hypothesis that mutations of FV and FII may be risk factors for acute myocardial infarction [24–28].

The prothrombin gene is organized in 14 exons, separated by 13 introns with the 5' upstream untranslated region (UTR) and the 3'-UTR which may play regulatory roles in gene expression [29,30]. One genetic variation in the 3'-UTR region of the prothrombin gene is the G to A transition at nucleotide position 20210, at or near the cleavage site of the mRNA precursor to which poly A is added. This was termed as the factor II G 20210A mutation. The prevalence of carriers of factor II 20210G/A substitution (rs1799963) in healthy Northern Europeans is 1.7% whereas in Southern Europeans the prevalence is nearly double (3%) [31].

In 1996, Poort et al. [29] reported that the 20210G/A single nucleotide polymorphism (SNP) is associated with an increased risk of venous thrombosis. Several studies confirmed this initial observation both in venous thrombosis and arterial disease [30,32].

In the present study, we analyze the frequencies of polymorphisms 20210G/A of FII and –308G/A of the promoter region of TNF- $\alpha$  in a group of Sicilian patients aged <46 years affected by AMI and evaluate the effect of these polymorphisms on the blood levels of myocardial tissue damage and clotting markers, to evaluate the possibility to use typing of these polymorphisms in association with selected haematochemical parameters in prognostic evaluation of these patients.

## 2. Materials and methods

### 2.1. Subjects

In this study, we analyzed two cohorts of men affected by acute myocardial infarction (AMI) and unrelated controls matched for age and sex. The cohort comprised 60 young male patients (age range 23–46 years) affected by acute myocardial infarction, consecutively admitted to the Cardiac Unit of Palermo University Hospital in the last year, and 130 healthy age and gender matched controls, all living in western Sicily. The diagnosis of acute myocardial infarction was based on standard laboratory (troponin I greater than decision limit, 3.0 ng/mL) [33] findings, typical electrocardiographic changes and confirmed by echocardiography and coronary angiography. According to the Helsinki declaration and to local ethical committee recommendations for the observational studies, written informed consent for enrolling in the study and for personal data management was obtained from all the

subjects. Blood samples from patients were collected 48 h from symptoms, transported and processed promptly according to pre-analytical recommendations [34].

### 2.2. Biochemical and hematological analyses

Cardiac markers were examined by automatic analyzers in the fresh blood sample collected 48 h after myocardial infarction. The activity of aspartate aminotransferase (AST) and levels of CK-MB isoenzyme (CK-MB mass, mCK-MB) and Troponin I were quantified by routine chemical and immunochemical clinical laboratory methods (Biochemical automatic analyzer: Modular P and E, Roche, Basel, Switzerland); haemochromocytometric test was performed by analyzer Sismex 9500, coagulation tests: prothrombin activity (PT), time of activated partial thromboplastin (aPTT), fibrinogen Clauss by automatic analyzer Futura Advance.

### 2.3. DNA genotyping

Genomic DNA was isolated by a standard method using proteinase K digestion followed by a standard salting-out technique [16].

#### 2.3.1. Molecular analysis of alleles at the –308 nucleotide (–308G $\rightarrow$ A) of TNF- $\alpha$ gene

The –308G/A polymorphism (rs1800629) of TNF- $\alpha$  was identified using a modification of PCR-Restriction Fragment Length Polymorphism assay described by Galbraith and Pandey [35]. Briefly, 0.5  $\mu$ M of forward and reverse primers (5'AGG CAA TAG GTT TTG AGG GCC AT 3' and 5'GGC GGG GAT TTG GAA AGT T 3') were mixed to 5–10 ng of DNA template, with a final concentration of 0.2 U Taq DNA polymerase (Perkin Elmer BioSystem, Rome, Italy), 200  $\mu$ M of each deoxynucleotide and 1X reaction buffer. PCR was performed for 35 cycles at 94 °C, 58 °C and 72 °C for 35 s, respectively. Restriction enzyme digestion with NcoI (M-Medical, Milan, Italy) of the PCR amplified product (159 bp) and subsequent electrophoresis on a 2–5% agarose gel discriminated between the two alleles; –308A showed two fragments of 146 bp and 13 bp, while –308G was undigested and resulted in a single band of 159 bp. Heterozygous individuals were detected by the presence of all the three fragments.

#### 2.3.2. Molecular analysis of alleles at the 20210 nucleotide (20210G $\rightarrow$ A) of prothrombin (factor II) gene

The identification of the alleles in the polymorphic site of Factor II was obtained by Real Time PCR using TaqMan Pre-Developed Assay Reagents for Allelic Discrimination essays optimized by Applied Biosystems on a 7300 Real Time PCR System as previously described [36]. Briefly, 10 ng of DNA for each sample were used in a PCR reaction, containing 1X optimized master mix and 1X specific primers/probes mix assay, according to manufactory protocol in a final volume of 25  $\mu$ l. Two identical probes, except for the central nucleotide that specifically recognizes the single nucleotide polymorphism (SNP) were used, each one labeled at the 5' extremity with different dyes (for the wild-type allele the fluorochrome FAM, and the fluorochrome VIC for probe specific for minor allele) and at the 3' extremity with a quencher dye, that in this case was the Minor Groove Binder (MGB) dye. Then, the amplification was performed in 7300 Real-Time ABI Prism PCR System (Applied Biosystems, USA), using a standard amplification protocol (1 cycle of 2' at 50 °C; 1 cycle of 10' at 95 °C and 40 cycles of 15" at 95 °C plus 15" at 60 °C), and the results available in the report sheet of 7300 System SDS v1.3 Software. Finally, samples were graphically grouped in 3 genotypic clusters, easily recognizable in the Allelic Discrimination plot on the basis of the two probe's fluorescence intensity emissions, whereas the uncertain cases were also evalu-

ated for the grow up of the fluorescence emission curve of each dye on the component's sheet.

#### 2.4. Statistical analysis

The Hardy–Weinberg equilibrium was confirmed by Pearson's test (goodness of fit between the observed and expected genotype ( $3 \times 2$  tables) and allele ( $2 \times 2$  tables) frequencies). Fisher's exact tests were performed to calculate significant different genotype or allelic distributions between patients with acute myocardial infarction and healthy controls. Odds ratio and 95% of confidence intervals were calculated with Woolf's approximation to quantify the risk in carriers of minor allelic variants. Differences in quantitative and qualitative data were analyzed using formal statistical tests (ANOVA followed by *t*-Student test). Differences were considered significant when  $p < 0.05$ .

### 3. Results

In previous studies we have demonstrated that proinflammatory gene variants determine an increased individual's risk for myocardial infarction [14]. Table 1 shows the analysis of genotypic frequencies of the single nucleotide polymorphisms of TNF ( $-308G/A$ ) and of the prothrombin (factor II, FII 20210G/A) among the 60 male patients and 130 healthy controls. Both patient' and control' genetic frequencies fit the Hardy–Weinberg equilibrium for both the two SNPs. We observed a significant increase of frequency of genotypes positive for the minor allele (A) of  $-308G/A$  SNP ( $p = 0.0018$ ), that are represented with a percentage of 36.7% among patients, against 14.6% among the healthy controls ( $p = 0.0048$ , odds ratio 4.00; 95% confidence interval: 1.51–10.56). In addition a not significant increase of the percentage of subject bearing the 20210A allele of the factor II gene (8.3% vs 6.9%).

As well known AMI is diagnosed using some serum specific markers as Cardiac troponin I (CTnI) and Creatine kinase-MB Isoenzyme protein (mCK-MB). Table 2 reports data on the effect of TNF  $-308G/A$  and FII 20210G/A polymorphisms on the levels of these haematochemical markers, on the levels of Aspartate Transpeptidases (AST) measurement, nowadays considered obsolete for this purpose and leukocytes. Stratifying haematochemical data according the two SNP genotypes the higher levels of CTnI were observed in subject bearing the TNF- $\alpha$   $-308A$  or the FII 20210A alleles.

On the other hand subjects positive for  $-308A$  shows the higher levels of mCK-MB whereas the FII 20210A ones shows the higher levels of leukocytes. The analysis of the effect of  $-308G/A$  TNF $\alpha$  and 20210G/A factor II genotypes on coagulation parameters, showed a significant increasing of plasma fibrinogen levels and of circulating platelets concentration in young men with acute myocardial infarction homozygous or heterozygous for  $-308A$  allele TNF $\alpha$  (Table 3). A similar pattern of fibrinogen concentration was observed in subjects bearing Factor II GA or AA genotypes.

### 4. Discussion

Signs of a systemic inflammatory response such as fever, leucocytosis and elevated acute phase reactants are frequently observed in patients with AMI and CHD. In patients with extensive myocardial infarction a pronounced inflammatory response may further complicate the clinical course [17]. After acute myocardial infarction, systemic inflammatory response is associated with the increases in plasma cytokines, such as TNF- $\alpha$ , interleukin-6 (IL-6) and IL-10, in myocardium and blood. TNF- $\alpha$  as a pro-inflammatory cytokines and can cause severe damage to cardiomyocytes and suppress cardiac function [36].

In addition excessive thrombin generation has been described in individuals at high risk of fatal CHD [37]. It seems biologically plausible that the higher prothrombin levels related to the 20210A variant may also confer an increased risk of arterial disease.

Results reported in Table 1 confirm reports from previous researches [13,14] on the role of TNF  $-308A$  allele in AMI and suggest that this gene variant might be an AMI risk factor for young men but do not allow to confirm the association between FII 20210A allele and AMI. To date, actually, studies attempting to answer this question have yielded conflicting results. In some reports, being a carrier of the mutation was associated with an increased risk of acute myocardial infarction (AMI) [38,39]. Nevertheless, prospective studies failed to establish any association between the 20210A allele and AMI [40].

As reported in Table 1 the cumulative frequencies of genotypes positive for Prothrombin 20210A allele in our patients and control populations are almost doubled respect the published frequencies for the South Europe. These differences are probably due to the patient and control populations sampling (young men). So our subjects cannot be considered representative of the general population. On the other hand the North to South gradient in distribution of genotypes in Caucasians was established at 50°N latitude [8]. Sicily is at very South of this distribution so one could speculate that the frequency of Prothrombin 20210A allele might be further increased in our population. In all cases a different and larger population sample is necessary to determine Prothrombin 20210G/A genotype and allele frequencies in general Sicilian population.

Our data indicate that TNF  $-308G/A$  and FII 20210G/A polymorphisms impinge upon the levels of haematochemical markers associated to acute myocardial infarction. As well known the cardiac form of Cardiac troponin I (CTnI) levels are routinely measured for diagnosing acute myocardial infarction. Cardiac troponin measurements also provide information concerning prognosis and the effect of early intervention in patients with acute coronary syndromes. Similarly measurement of concentration of Creatine kinase-MB Isoenzyme protein (mCK-MB) represent an important marker of degree of myocardial damage immediately after AMI [41]. On the other hand Cardiac tissue necrosis induces an

**Table 1**  
–308G/A TNF and 20210G/A FII genotype frequencies in 60 young patients affected by acute myocardial infarction (AMI) and 130 health sex and age matched controls.

Genotypes		AMI	%	Healthy controls	%	O.R.	95%C.I.	<i>p</i>
TNF SNP –308G/A	GG	38	63.4	111	85.4	0.29	0.14–0.62	0.0018
	AG	20	33.3	17	13.1	3.43	1.58–7.45	0.0026
	AA	2	3.3	2	1.5	1.86	0.26–13.57	n.s
	AG/AA	22	36.7	19	14.6	4.00	1.51–10.56	0.0048
Factor II SNP 20210G/A	GG	55	91.7	121	93.1	0.44	0.11–1.84	n.s.
	AG	4	6.6	7	5.4	1.26	0.35–4.46	n.s.
	AA	1	1.7	2	1.5	1.09	0.10–12.21	n.s.
	AG/AA	5	8.3	9	6.9	1.22	0.39–3.82	n.s.

**Table 2**

Evaluation of blood data (Mean ± D.S.) of 60 young patients affected by acute myocardial infarction (AMI) according to tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) –308G/A and prothrombin (factor II) 20210G/A genotypes.

Genotypes	CTnl	CK-MBm	AST	WBC	NEUT
TNF GG (38)	12.1 ± 5.4	23.5 ± 9.2	53.9 ± 43.8	9.2 ± 1.6	6.05 ± 1.7
TNF GA/AA (22)	20.8 ± 7.6	39.7 ± 11.1	61.7 ± 30.8	9.2 ± 5.2	6.9 ± 5.1
<i>p</i>	<0.0001	<0.0001	n.s.	n.s.	n.s.
Factor II GG (55)	11.2 ± 3.6	26.5 ± 8.7	44.33 ± 21.9	8.7 ± 4.8	5.7 ± 2.0
Factor II GA/AA (5)	19.9 ± 6.7	31.4 ± 9.2	75.9 ± 41.1	13.7 ± 3.2	9.33 ± 4.4
<i>p</i>	0.0456	n.s.	n.s.	0.0244	n.s.

**Table 3**

Evaluation of haematic coagulation marker profile (Mean ± D.S.) of 60 young patients affected by acute myocardial infarction (AMI) according to tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) –308G/A and to prothrombin (factor II) 20210G/A genotypes.

Genotypes	INR	APTT	Fibrinogen mg/dL	PLT × 10 <sup>9</sup> /L
TNF GG (38)	1.01 ± 0.07	29.1 ± 10.1	364.5 ± 41.1	181.1 ± 46.2
TNF GA/AA (22)	1.03 ± 0.16	23.8 ± 12.2	418.15 ± 113.0	275.0 ± 46.9
<i>p</i>	n.s.	n.s.	0.0110	<0.0001
Factor II GG (55)	0.89 ± 0.10	29.1 ± 15.6	315.7 ± 39.7	295.0 ± 56.3
Factor II GA/AA (5)	1.00 ± 0.23	29.0 ± 25.3	412.7 ± 95.2	250.0 ± 61.1
<i>p</i>	n.s.	n.s.	0.0017	n.s.

increased inflammatory response mirrored by a not specific increase of circulating leucocytes due to neutrophil mobilization. Inflammatory response is an important feature of acute coronary syndromes and myocardial infarction. In AMI signs of inflammation are well known and elevated levels of acute phase reactants have been shown to be associated with a worse short- and long-term prognosis [17,42]. Our data strongly suggest that a genetically determined higher production of TNF- $\alpha$  is associated in young subjects to a more severe cardiac damage in AMI as depicted by higher levels of troponin and mCK-MB.

A similar and probably additive effect might have a genetically increased production of prothrombin. On the other hand considering the small number of patients (5 subjects) positive for the 20210A allele and the relative variability of results obtained in this small group of subjects the definition of weight of this genetic variant on haematochemical marker necessitate further studies on larger group of subjects.

Fibrinogen is the major coagulation protein in blood by mass, the precursor of fibrin, and an important determinant of blood viscosity and platelet aggregation [24,43]. Our data indicate that subjects bearing proinflammatory or procoagulant genotypes have the high plasma fibrinogen. As well known starting from the seminal paper by Hashmi et al. [44], high fibrinogen levels are predictor of coronary artery lesions, more recently it has been reported that plasma fibrinogen level may predict critical coronary artery stenosis in young adults with myocardial infarction [45]. In this view our data seem to suggest that the typing of the studied SNP of TNF- $\alpha$  and of FII associated to plasma fibrinogen measurement might be a useful tool to the prognostic evaluation of IMA in young subjects.

## 5. Conclusions

All together the results of the present study, indicating the relationship among genetically determined TNF $\alpha$  and FII production

and increased levels of tissue damage markers of AMI as well central molecules for clotting processes, suggest that a complex genetic background, might be involved in susceptibility to AMI in young men and in the extension and severity of the disease.

In conclusion our data might prompt an approach to defining individual risk profiles that can be applied to healthy subjects to predict intrinsic risk of AMI with different ages at onset. Such risk profiles, when better established in a larger cohort of patients, can be used to trigger further diagnostic procedures and early therapeutic interventions aimed at preventing or significantly delaying the clinical manifestations of cardiovascular disease.

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