# Endothelial protein C receptor polymorphisms and risk of myocardial infarction

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## ABSTRACT

### Background

Haplotypes A1 and A3 in the endothelial protein C receptor gene are tagged by the 4678G/C and 4600A/G polymorphisms, respectively, and have been reported to influence the risk of venous thromboembolism. We assessed whether these haplotypes modify the risk of premature myocardial infarction.

### Design and Methods

We genotyped these polymorphisms in 689 patients with premature myocardial infarction and 697 control subjects. Activated protein C and soluble endothelial protein C receptor levels were also measured.

### Results

After adjustment for other cardiovascular risk factors, A1 and A3 haplotypes protected against premature myocardial infarction (odds ratio 0.7, 95% CI 0.4-0.8, $p$=0.044 and 0.5, 0.3-0.6, $p$<0.001, respectively). Moreover, the protective role of these haplotypes seemed to be additive, as carriers of both the A1 and A3 haplotypes had adjusted odds ratios of 0.3 (0.2-0.5, $p$<0.001) and 0.4 (0.2-0.8, $p$=0.006) compared to those carrying only the A1 or A3 haplotype, respectively. The presence of the A1 haplotype was associated with increased levels of activated protein C whereas individuals carrying the A3 haplotype showed the highest soluble endothelial protein C receptor levels.

### Conclusions

These results show that A1 haplotype carriers have a reduced risk of premature myocardial infarction via the association of this haplotype with increased activated protein C plasma levels. The study also shows that carriers of the A3 haplotype have a reduced risk of myocardial infarction, only in part due to increased soluble endothelial protein C levels.

Key words: endothelial protein C receptor, haplotypes, premature myocardial infarction, activated protein C, soluble EPCR.

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## Introduction

Myocardial infarction (MI) usually occurs following the development of an acute occlusive thrombus due to a ruptured coronary artery atheromatous plaque. The influence of coagulation factors and common polymorphisms in hemostasis/inflammation-related genes on the risk of MI has been studied extensively, with positive and negative results, although none of the polymorphisms studied has shown strong association with the risk of MI.[1-4] However, novel functional polymorphisms in hemostasis/inflammation genes remain good candidates for a role in the pathogenesis of MI,[5] especially in younger patients in whom angiographically normal coronary arteries are more frequent and a positive family history of premature coronary disease is more prevalent than in older patients.[6,7]

Inflammatory processes are a common underlying mechanism leading to coronary heart disease. Anti-inflammatory mechanisms may, therefore, constitute an important protection against MI. The protein C anticoagulant pathway plays a significant role in the regulation of inflammatory processes and displays anti-apoptotic and neuroprotective activities, reducing organ damage in animal models of sepsis, ischemic injury, endothelial cell injury, and stroke.[8-11] Protein C is activated on the surface of endothelial cells by the thrombin-thrombomodulin complex, a process that can be further enhanced when protein C binds to its membrane receptor, the endothelial-cell protein C receptor (EPCR). There is a preferential expression of EPCR on the endothelium of large blood vessels,[12] which may increase the concentration of protein C on the endothelium and overcome the relatively low thrombomodulin concentration in these vessels, thus providing efficient protein C activation. Autoantibodies against EPCR were found in antiphospholipid syndrome and have been reported to be a risk factor for fetal death and acute MI in young women.[13,14]

There are at least four haplotypes in the *EPCR* gene. Haplotype 3 (A3), tagged by the single nucleotide polymorphism (SNP) 4600A/G (rs 867186), has been reported to be associated with increased plasma levels of soluble EPCR (sEPCR)[15-19] and with increased EPCR shedding *in vitro*,[18,20] probably due to increased sensitivity to ADAM17,[21] but its association with the risk of venous thromboembolism is unclear. While two studies failed to show an association between the A3 haplotype and the risk of venous thrombosis in Spanish and Dutch subjects,[16,17] this haplotype was overrepresentated in male patients with venous thrombosis in a French study[15] and in carriers of the prothrombin 20210A allele in a Spanish study.[19] Ireland *et al.*,[18] in a pan-European case-control study of MI, reported that without the challenge of diabetes or metabolic syndrome, individuals carrying one A3 haplotype were protected from MI. However, when heterozygotes had factors constituting the metabolic syndrome or diabetes, the risk of MI increased. sEPCR has been reported to bind factor VII/VIIa[22,23] and block factor VIIa activity.[23] Haplotype 1 (A1), tagged by the 4678G/C SNP (rs 9574), is associated with increased levels of circulating activated protein C and with a reduced risk of venous thrombosis.[16,24]

The aim of the present study was to investigate whether these two functional EPCR haplotypes are associated with the risk of premature MI, whether the effect is mediated via sEPCR and activated protein C levels, respectively, and whether there are MI-risk interactions between genotype and the presence of diabetes or the metabolic syndrome.

## Design and Methods

### Study population

Our study included 689 subjects (601 men, 88 women) aged < 51 years (median age 42 years; men 42 years, women 42 years) at the time of the first acute MI. These patients were recruited via the files of the Departments of Cardiology of three hospitals from the east of Spain: *Hospital Universitario La Fe (Valencia), Hospital General Universitario* (Alicante), and *Hospital de la Santa Creu i Sant Pau* (Barcelona). MI was diagnosed as previously reported.[25] For measurement of activated protein C and sEPCR, samples were collected from patients in a clinically stable condition, at least 3 months after the acute event. Patients with unstable angina, malignancy, nephrotic syndrome, renal or hepatic dysfunction, inflammatory or infectious diseases or lupus anticoagulant were excluded.

The control group was recruited and studied along with the cases and comprised 697 unrelated Caucasian age- and sex-matched volunteers (608 men, 88 women) with a median age of 44 years (men 44 years, women 44 years) from the same geographical areas as the MI patients. The control subjects had no documented history of vascular disease, and no personal history of arterial or venous thrombotic disease. In both groups, cardiovascular risk factors were defined as indicated before.[26] Metabolic syndrome was defined by the presence of at least three of the following: body mass index ≥30 kg/m$^2$, triglycerides >175 mg/dL, HDL-cholesterol < 40 mg/dL, blood pressure ≥140/90 mmHg, fasting plasma glucose >126 mg/dL.

Informed consent was obtained from all study subjects. The study was approved by the local ethics committee, and performed in accordance with the Helsinki Declaration of 1975, as revised in 2000.

### Blood collection

Blood was taken after 12 hours of fasting, into a 0.1 vol of 0.129 M trisodium citrate and centrifuged at 1500 g for 30 min at 4 °C. Plasma was snap frozen in small aliquots, stored at –80 °C and used within 6 months.

### Activated protein C and sEPCR assays

Levels of circulating activated protein C were measured as previously reported.[24,27] The inter- and intra-assay variations were less than 12%.

Levels of sEPCR were measured using an enzyme-linked immunosorbent assay according to the instructions of the manufacturer (Asserachrom sEPCR, Diagnostica Stago, Asnieres, France). The inter- and intra-assay variations were less than 9%.

### Genetic analysis

The 4600A/G (rs 867186) and the 4678G/C (rs 9574) polymorphisms were assayed as indicated before.[16,24]

### Statistical analysis

The number of subjects was calculated with the two proportions comparison formula, with a bilateral contrast, and fixing type I and II errors as 5% and 20%, respectively, and the minimum difference between genotype frequencies to be detected as significant equal to 30% of the frequency in the control population. Considering the mean prevalence of the genetic variables, the final calculated sample size was 648 cases and 648 controls.

Other statistical analyses were conducted using the SPSS for Windows release 11.5 statistical software (SPSS Inc., Chicago, USA). The medians and 10$^{th}$ and 90$^{th}$ percentiles of activated protein C and sEPCR levels are reported. Allele frequencies were calculated by gene counting. The $\chi^2$ test was used to compare percentages. Parameter levels were compared using the Kruskal-Wallis test or the Mann-Whitney test. Correlations were assessed by Spearman's test. Logistic regression analysis was used to identify associations between genotypes and premature MI. For sEPCR levels we performed analysis by quartiles, according to the distribution among control subjects. The group with the lowest level (≤25$^{th}$ percentile) served as the reference category, relative to which risk was expressed. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated from the logistic model. A two-tailed *p* value <0.05 was taken as statistically significant.

## Results

Table 1 shows the main characteristics of the study subjects. There were no significant differences in age and sex distribution between the groups with or without MI. As expected, all cardiovascular risk factors analyzed were more frequent in patients than in control subjects. Frequencies of cardiovascular risk factors in control subjects were similar to those reported in our geographical area,[28] showing that our control group is a representative sample of the general population.

### *EPCR* polymorphisms and myocardial infarction risk

Allelic and genotypic frequencies of the 4600A/G and 4678G/C SNP in *EPCR* are shown in Table 2. The 4678C allele (A1 haplotype) and the 4600G allele (A3 haplotype) frequencies for the control group (0.468 and 0.103, respectively) were in the middle of the frequency range reported by other groups (0.480[15] and 0.392[17] for the A1 haplotype and 0.092[15], 0.127[17], 0.110[18] and 0.090[18] for the A3 haplotype). Both the A3 and the A1 haplotypes were more frequent in control subjects than in MI patients. Given that control subjects were recruited in three different geographical areas, we analyzed the haplotype frequencies in the three control populations. The frequencies of the A3 haplotype were 0.107, 0.105 and 0.097 in the control groups from Valencia, Murcia and Barcelona, respectively, whereas the frequencies of the A1 haplotype were 0.472, 0.452 and 0.493, respectively, with no significant differences between them. The expected frequency of the 4600GG genotype in control subjects is about 1% (six or seven carriers) but, initially using single-strand conformation polymorphism analysis, we did not find any control carrying the 4600GG genotype. To exclude genotyping errors we sequenced all patients and controls not carrying the A1 haplotype (ie, the 4678GG carriers), the only ones who could carry the 4600GG (A3A3) genotype, as well as all patients and controls with the 4600AG genotype. Only one control subject turned out to have the 4600GG genotype. Thus, the frequency of the 4600GG genotype in the control group was 0.001, which is similar to that observed in previous studies of healthy subjects (0.000).[16]

To identify associations between these genotypes and premature MI, we performed logistic regression analyses (Table 3). In univariate analysis, the presence of the A3 haplotype and, to a lesser extent, the A1 haplotype reduced the risk of premature MI. Subjects with cardiovascular risk factors such as smoking, dyslipidemia, hypertension, diabetes mellitus and obesity had odds ratios higher than 2.0. After adjustment for sex, age and all the other risk factors, both the presence of the A3

**Table 1. Characteristics of the study subjects.**

| *Characteristics* | *Patients (N=689)* | *Controls (N=697)* | *p* |
|---|---|---|---|
| Age at sampling (years)* | 44 (35-50) | 44 (34-51) | 0.582 |
| Age at MI (years)* | 42 (34-49) | – | – |
| Male, n. (%) | 601 (87.2) | 608 (87.2) | 0.962 |
| Smokers, n. (%) | 604 (87.7) | 329 (47.3) | <0.001 |
| Dyslipidemia, n. (%) | 432 (62.7) | 182 (26.1) | <0.001 |
| Hypertension, n. (%) | 240 (34.8) | 88 (12.6) | <0.001 |
| Diabetes mellitus, n. (%) | 101 (14.7) | 46 (6.6) | <0.001 |
| Body mass index ≥30 kg/m$^2$, n. (%) | 172 (25.0) | 83 (11.9) | <0.001 |
| Metabolic syndrome, n. (%) | 180 (26.1) | 70 (10.0) | <0.001 |

**Values are expressed as the median (10$^{th}$-90$^{th}$ percentile).*

**Table 2. Allelic and genotypic frequencies of the single nucleotide polymorphisms in the *EPCR* gene in MI patients and controls.**

| *Polymorphism* | *MI Patients (N=689)* | *Controls (N=697)* | *OR (95% CI)* |
|---|---|---|---|
| 4600A/G | | | |
| A allele | 1292 (93.8%) | 1250 (89.7%) | 1.0* |
| G allele | 86 (6.2%) | 144 (10.3%) | 0.53 (0.40-0.71) (*p*<0.001) |
| AA genotype | 606 (88.0%) | 554 (79.5%) | 1.0* |
| AG genotype | 80 (11.6%) | 142 (20.4%) | 0.53 (0.39-0.71)[a] (*p*<0.001) |
| GG genotype | 3 (0.4%) | 1 (0,1%) | |
| 4678G/C | | | |
| G allele | 786 (57.0%) | 741 (53.2%) | 1.0* |
| C allele | 592 (43.0%) | 653 (46.8%) | 0.85 (0.74-0.99) (*p*=0.044) |
| GG genotype | 235 (34.1%) | 201 (30.1%) | 1.0* |
| GC genotype | 316 (45.9%) | 339 (48.0%) | 0.80 (0.62-1.02) (*p*=0.073) |
| CC genotype | 138 (20.0%) | 157 (21.9%) | 0.75 (0.56-1.01) (*p*=0.060) |

**Reference group; [a]Includes the four individuals carrying the GG genotype.*

**Table 3. Association of the presence of *EPCR* A1 and A3 haplotypes and cardiovascular risk factors with myocardial infarction.**

| | Patients N (%) | Controls N (%) | OR (95% CI) Crude | OR (95% CI) Adjusted[a] |
|---|---|---|---|---|
| Presence of A3 (4600G allele) | 83 (12.0) | 144 (20.5) | 0.53 (0.40-0.71) | 0.51 (0.32-0.63) |
| Presence of A1 (4678C allele) | 454 (65.9) | 496 (71.2) | 0.85 (0.74-0.99) | 0.72 (0.44-0.84) |
| Smokers | 604 (87.7) | 329 (47.3) | 7.81 (5.96-10.34) | 5.43 (3.87-7.60) |
| Dyslipidemia | 432 (62.7) | 182 (26.1) | 4.80 (3.84-6.16) | 2.93 (2.10-4.03) |
| Hypertension | 240 (34.8) | 88 (12.6) | 3.62 (2.73-4.77) | 2.50 (1.86-3.74) |
| Diabetes mellitus | 101 (14.7) | 46 (6.6) | 2.43 (1.74-3.59) | 2.33 (1.40-3.77) |
| Body mass index ≥30 kg/m² | 172 (25.0) | 83 (11.9) | 2.56 (1.80-3.58) | 2.53 (1.74-3.88) |

[a]*Odds ratios adjusted for age, sex and the other six factors listed in the Table.*

allele and the A1 allele were associated with a reduction in the risk of premature MI (Table 3).

Ireland *et al.*[18] reported that the apparent protection against coronary heart disease in carriers of the A3 haplotype disappears in the presence of diabetes or metabolic syndrome. We, therefore, analyzed the subgroup of MI patients and controls with diabetes (101 patients and 46 controls). After adjustment for other risk factors, in this group of patients and controls the presence of the A3 haplotype increased the protection against premature MI (OR 0.40; 95% CI 0.15-0.80), but the A1 haplotype lost its protective role (OR 0.89; 95% CI 0.42-1.89). We also analyzed the subgroup of patients and controls with metabolic syndrome (180 patients and 70 controls). In this setting, both A3 and A1 haplotypes were associated with a reduction in the risk of premature MI (adjusted OR 0.44; 95% CI 0.24-0.75 and 0.87; 95% CI 0.56-0.98, respectively).

Given that the presence of both the A1 haplotype and the A3 haplotype seemed to reduce the risk of premature MI, we studied the effect on the risk of MI of the simultaneous presence of *EPCR* A1 and A3 haplotypes compared to the presence of only one or none of these haplotypes (Table 4). In both cases, the results suggest that the protective effects of A1 and A3 are additive.

**Table 4. Effect on the risk of myocardial infarction of the simultaneous presence of *EPCR* A1 and A3 haplotypes compared to the presence of neither or only one of these haplotypes.**

| Genotype combination | Patients N. | Controls N. | OR (95% CI) |
|---|---|---|---|
| 4600AA/4678GG (no A1 no A3) | 179 | 133 | 1.0* |
| 4600AG/4678GG (only A3 present) | 53 | 68 | 0.55 (0.76-0.86) ($p$=0.010) |
| 4600AA/4678GC or CC (only A1 present) | 427 | 421 | 0.73 (0.56-0.92) ($p$=0.027) |
| 4600AG/4678GC (A1 and A3 present) | 27 | 75 | 0.26 (0.18-0.39) ($p$<0.001) |
| 4600AG/4678GG (only A3 present) | 53 | 68 | 1.0* |
| 4600AG/4678GC (A1 and A3 present) | 27 | 75 | 0.44 (0.23-0.78) ($p$=0.006) |
| 4600AA/4678GC or CC (only A1 present) | 427 | 421 | 1.0* |
| 4600AG/4678GC (A1 and A3 present) | 27 | 75 | 0.34 (0.21-0.55) ($p$<0.001) |

**Reference group.*

### *Activated protein C and sEPCR and myocardial infarction risk*

Given that *EPCR* A1 and A3 haplotypes have been associated with increased plasma levels of activated protein C[16,24] and sEPCR[15-18], respectively, we determined these parameters in a subgroup of 260 control subjects and 260 MI patients from whom plasmas for these determinations were available. Haplotype and genotype distributions in these subgroups were similar to those observed in the whole group. Activated protein C levels increased with the number of 4678C alleles (A1) present, both in controls (GG=1.06, 0.73-1.56; GC=1.21, 0.84-1.88; CC=1.39, 0.86-1.98; $p$<0.001) and in MI patients (GG=1.02, 0.65-1.60; GC=1.07, 0.66-1.41; CC=1.15, 0.64-1.86; $p$=0.133), although in this case the difference did not reach statistical significance. The levels of sEPCR were influenced by the presence of the A3 haplotype. The level of sEPCR was significantly higher in carriers of the A3 haplotype than in those without this haplotype, both in controls (AA=103, 70-145; AG=281, 189-396; $p$<0.001) and in MI patients (AA=96, 66-144; AG=383, 231-491; $p$<0.001).

As reported before,[26] logistic regression analyses showed that when the level of activated protein C was considered as a continuous variable, the odds ratio for MI increased 50% for each standard deviation (0.40 ng/mL) decrease in activated protein C ($p$<0.001), and this association remained significant when the presence of the A1 haplotype was entered into the model. However, the protective effect of the A1 haplotype disappeared when activated protein C levels were entered into the model (adjusted OR for the presence of A1 haplotype 0.96; 95% CI 0.65-1.39), suggesting that the protective effect of the A1 haplotype is via activated protein C levels.

Stratification of sEPCR in quartiles according to the levels in controls showed that, compared to the first quartile, the odds ratio for subjects with values in the fourth quartile was 0.57 (95% CI 0.34-0.95). In this subgroup of 260 MI patients and 260 control subjects, the odds ratio for the presence of the A3 haplotype was

0.61 (95% CI, 0.43-0.94). When both the fourth quartile of sEPCR and the presence of the A3 haplotype were entered into the model, the adjusted odds ratios were 0.70 (0.33-1.43) and 0.83 (0.29-1.65), respectively, suggesting that the protective effect of the A3 haplotype is due, at least in part, to its association with increased sEPCR levels.

As sEPCR levels may inhibit protein C activation,[33] we calculated the correlation between activated protein C and sEPCR levels in patients and controls, both in the whole group and according to the 4600AG genotype. None of the correlations was statistically significant except that for the subgroup of healthy subjects carrying the 4600AG genotype (r=-0.248, $p$=0.041).

## Discussion

In the present study we found that the presence of one A3 haplotype (4600G allele) and, to a lesser extent, the presence of one A1 haplotype (4678C allele) in the *EPCR* gene were associated with a reduction in the risk of premature MI, and that these effects were additive. In a case-control study of MI, Ireland *et al.*[18] found that, in the absence of diabetes or metabolic syndrome, individuals carrying only one A3 allele were protected against premature MI. However, when heterozygous individuals were challenged by factors making up the metabolic syndrome or diabetes, the risk of MI increased. These results differ from ours, which show that after selecting individuals with diabetes or metabolic syndrome, heterozygous A3 carriers are still protected from premature MI. A major difference between the two studies is the age of onset. The first MI event in our patients occurred under 51 years of age, whereas patients in the other study were generally older.[18]

The mechanism by which the A1 haplotype reduces the risk of premature MI may be similar to that suggested in venous thrombosis: the increased circulating activated protein C levels identified in carriers of the A1 haplotype (4678C allele).[16,24] Besides its anticoagulant function, activated protein C exerts anti-inflammatory and anti-apoptotic effects via transmembrane receptors.[8-11,29-32] The anti-inflammatory activity of activated protein C depends on its ability to suppress the secretion of cytokines, such as tumor necrosis factor-α, by inflammatory cells, the activation and extravasation of leukocytes and the expression and function of adhesion molecules. Hence, activated protein C would protect the organism from vascular insult and prolong endothelial, cellular, and organ survival.[31] The present results support and extend those reported earlier[26] indicating that a reduced level of activated protein C is a risk factor for premature MI. Given that the A1 haplotype is associated with increased activated protein C levels, the association of A1 with a reduction in the risk of premature MI is most probably due to the increased activated protein C levels, since this association disappeared after adjusting for activated protein C levels.

The mechanism by which the A3 haplotype protects against premature MI is uncertain. The A3 haplotype is strongly associated with increased sEPCR levels in plasma[16-18,24] and shedding.[18,20] An explanation for the protective effect of the A3 haplotype might be the observation that sEPCR is able to bind factor VII and factor VIIa with an affinity similar to that of protein C/activated protein C[22,23] and that, upon complex formation, the ability of factor VIIa to activate factor X is reduced, even when complexed with tissue factor,[23] suggesting that sEPCR may play an anticoagulant role which is more prominent in carriers of the *EPCR* A3 haplotype. A number of remarkable *in vivo* anti-inflammatory effects of activated protein C require cell-bound EPCR.[11] Whether sEPCR is still able to preserve some of these activities remains to be elucidated. Nevertheless, the fact that the protective effect of the A3 haplotype partly disappears after adjusting for sEPCR levels might indicate that the effect is via increased sEPCR levels.

In conclusion, our results show that carriers of the A1 haplotype in the *EPCR* gene have a reduced risk of premature MI, most probably due to the association of this haplotype with increased activated protein C plasma levels. Our results further show that carriers of the A3 haplotype also have a reduced risk of premature MI, in part due to its association with high sEPCR levels, but the mechanism remains unknown.

## Authorship and Disclosures

FE designed the research; PM, SN, AE and FE performed the research; PM, SN, JC, EZ, AE, AS, RMB and FE analyzed the data; EZ, VR, AS, FM and JR took care of the clinical aspects of the study. FE and RMB wrote the paper; JC and AE substantially edited the manuscript. All authors revised and approved the final version of the manuscript.

The authors reported no potential conflicts of interest.

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