A Polymorphism in the Cyclooxygenase 2 Gene as an Inherited Protective Factor Against Myocardial Infarction and Stroke

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Context  Myocardial infarction (MI) and ischemic stroke are thought to be caused by matrix digestion by metalloproteinases (MMPs) leading to rupture of atherosclerotic plaques. Production of macrophage MMP-2 and MMP-9 is induced by cyclooxygenase 2 (COX-2) and prostaglandin E2 synthesis. Although COX-2 expression may be genetically determined, the relation between COX-2 polymorphisms and the risk of MI and stroke is unclear.

Objective  To investigate the relationship between the −765G→C polymorphism of the COX-2 gene and clinically evident plaque rupture.

Design, Setting, and Participants  Prospective, matched case-control study conducted between March 2002 and October 2003 among 864 patients with first MI or atherothrombotic ischemic stroke and 864 hospitalized controls. The groups were matched for age, sex, body mass index, smoking, hypertension, hypercholesterolemia, and diabetes. The −765G→C variant of the COX-2 gene was genotyped by restriction endonuclease digestion of polymerase chain reaction products.

Main Outcome Measures  Presence of the −765G→C polymorphism of the COX-2 gene; COX-2, MMP-2, and MMP-9 expression and activity in plaques and in peripheral monocytes; urinary 6-keto PGF1α (marker of endothelial prostacyclin); and endothelium-dependent and -independent forearm blood flow vasodilation.

Results  The prevalence of −765GC was 2.41 times higher among controls than among cases (43.3% vs 17.9%; \(P<.001\)). The prevalence of −765CC homozygosity was 5.81 times higher (6.4% vs 1.1%; \(P=.04\)). Among participants carrying the −765GC and −765CC genotypes, the prevalence ratios for MI or stroke were 0.48 (95% CI, 0.36-0.68) and 0.33 (95% CI, 0.24-0.55), respectively. Expression of COX-2 and MMPs was significantly lower in atherosclerotic plaques from participants carrying the −765C allele, while the −765G→C polymorphism did not affect endothelial prostacyclin biosynthesis or endothelium-dependent vasodilation in vivo. In subgroup analyses (n=224 cases), serum high-sensitivity C-reactive protein was significantly lower in patients carrying the −765C allele (mean [SD], 0.78 [0.1] vs 2.56 [0.4] mg/L; \(P=.04\)).

Conclusions  We found that the −765G→C polymorphism of the COX-2 gene is associated with a decreased risk of MI and stroke. Detection of this genotype may be useful for predicting genetic risk of MI and stroke.
Table 1. Participant Characteristics

| Characteristics                        | Cases (n = 864) | Controls (n = 864) |
|----------------------------------------|----------------|-------------------|
| Age, mean (SD), y                      | 62.7 (10)      | 62.9 (8)          |
| Male/female                            | 579 (67)/285 (33) | 553 (64)/311 (36) |
| Hypercholesterolemia                   | 449 (52)       | 432 (50)         |
| Hypertension                           | 386 (44)       | 389 (45)         |
| Diabetes                               | 156 (18)       | 147 (17)         |
| Cigarette smoking                      | 311 (36)       | 302 (35)         |
| Body mass index, mean (SD)†            | 27 (6)         | 27 (7)           |
| NSAID or glucocorticoid treatment      | 0              | 0                |
| Aspirin treatment                      | 578 (67)       | 535 (62)         |
| Angiographically proven CAD (n = 750)  | 320 (76.2)     | 253 (76.8)       |
| 1-Vessel disease                       | 127 (30.2)     | 96 (25)          |
| 2-Vessel disease                       | 98 (23.3)      | 79 (23.9)        |
| 3-Vessel disease                       | 92 (22.2)      | 73 (22.1)        |
| ICA stenosis severity (n = 232), %     | 74 (5)         | 74 (5)           |
| Range                                  | 70-92          | 70-91            |

Abbreviations: CAD, coronary artery disease; ICA, internal carotid artery; NSAID, nonsteroidal anti-inflammatory drug.
*Data are expressed as No. (%) unless otherwise indicated.
†Body mass index was calculated as weight in kilograms divided by the square of height in meters.

individual's genetic makeup and environmental factors. Increasing evidence supports a central role for inflammation in the cascade of events that results in plaque fissuring. Lesion macrophages synthesize matrix metalloproteinases (MMPs); in particular, 72-kDa (MMP-2) and 92-kDa (MMP-9) gelatinase, proteolytic enzymes capable of degrading plaque constituents. Recently, Papafili et al and Cipollone and Patrone identified a new variant in the COX-2 promoter, a guanine to cytosine substitution at position −765 (−765G→C), and showed that this variant is located within a putative binding site for the transcription factor Sp1. Patients carrying the −765C allele had markedly (30%) lower promoter activity and lower plasma levels of C-reactive protein (CRP), a marker of low-grade inflammation, compared with patients homozygous for −765G. Because lower COX-2 activity could reduce generation of culprit MMPs in plaques, and because CRP is closely related to cardiovascular risk, we hypothesized that −765G→C might be protective against plaque instability, leading to lower risk of MI and stroke. We therefore prospectively performed a case-control study in which we genotyped this locus in 1728 individuals at high risk of cardiovascular events (864 patients with well-characterized previous MI or stroke and 864 controls).

METHODS

Study Participants

The study participants comprised 1728 unrelated Italian patients who were admitted to 4 participating hospitals (SS Annunziata University Hospital of Chieti, University Hospital of Palermo, S Chiara University Hospital of Pisa, and La Sapienza University Hospital of Rome, all in Italy) between March 2002 and October 2003 and who had at least 1 conventional risk factor for cardiovascular disease.

Cases (n=864) were patients who had had a first-event MI or ischemic stroke. We specifically focused on ischemic strokes that were atherothrombotic and related to ulceration of a culprit internal carotid artery lesion. We made every effort to exclude patients with other subtypes of ischemic stroke by clinical examination and laboratory and imaging analyses. In particular, strokes had to be in the territory of the mid cerebral artery as documented by computed tomographic scan and cerebral angiography. All patients had Doppler evidence of an ulcerated culprit lesion in the internal carotid artery as well as preprocedural transcranial Doppler analysis of cerebral blood flow strongly indicative of active thromboembolism. Finally, all study participants were in sinus rhythm at the time of hospitalization and had no history of atrial fibrillation.

Controls (n=864) were high-risk patients prospectively matched for risk factors and concomitant therapy, as shown in Table 1, who had no history of MI, unstable angina, transient ischemic attack, or stroke and were hospitalized for any clinical reason except MI, unstable angina, transient ischemic attack, or stroke. Controls were hospitalized within 1 year of case hospitalizations.

Among the 1728 patients enrolled in the study, 232 (102 cases [11.8%] and 130 controls [15%]) underwent carotid endarterectomy and 750 (420 cases [48%] and 330 controls [38%]) underwent coronary angiography during their hospitalization as part of their routine clinical care. Furthermore, serum high-sensitivity CRP (hsCRP) (Immulyte, Diagnostic Product Corp) was measured and compared in a subgroup of 224 cases with −765GG vs −765GC genotypes.

The protocol was approved by local ethics review committees. Written informed consent was obtained from all patients prior to study enrollment and before each examination.

Data Collection and Analysis

Blood and urine samples were collected from all participants after an overnight fast, and multiple aliquots of whole blood, plasma, serum, and urine were immediately stored at −80°C until analysis.

Genomic DNA was prepared from frozen whole blood with the use of a blood DNA isolation kit (Helix Fast
Blood DNA, DiaTech, Jesi, Italy). The −765G→C variant was genotyped by FAU I (Celbio, Milan, Italy) restriction endonuclease digestion of the polymerase chain reaction product (Figure 1). Genotypes were determined by independent investigators who were blinded to patients’ identities and phenotypes.

After carotid endarterectomy, plaque sections were prepared and analyzed by immunohistochemistry using a color image analysis system (AlphaEase 5.02, Alpha Innotech Corp, San Leandro, Calif) as previously described.6

As previously described,6 Western blot analysis on plaque extracts and plaque-derived macrophages was performed to detect COX-2, MMP-2, and MMP-9 expression; concurrent immunodetection of β-actin was performed to ensure equal gel loading. Bands were quantified by computer-assisted densitometry (AlphaEase 5.02) and expressed as densitometric units (DU). Zymography on plaque extract and plaque-derived macrophages was performed to detect MMP-2 and MMP-9 gelatinolytic activity, as previously described.6 Conditioned medium of human fibrosarcoma cell line HT1080 was used as a positive control with known gelatinolytic activity.

Sirius red polarization microscopy was used to detect type 1 and 3 interstitial collagen in plaque sections according to a previously described method.9

Macrophages were selectively extracted from plaques as described by de Vries et al.11 Flow cytometry analysis of purified cell preparations using a phycoerythrin-conjugated anti-CD68 monoclonal antibody (Clone Y1/82A, BD Biosciences PharMingen, San Diego, Calif) showed that more than 98% of the selected cells were positive for CD68. Then, immunocytochemistry, Western blot, and zymography were performed. Results are representative of 3 different analyses (intrasample tests for in vitro reproducibility).

Peripheral blood monocytes were purified and cultured from 10 randomly selected patients carrying the −765GG genotype, 10 carrying the −765GC genotype, and 10 carrying the −765CC genotype.9 In particular, to create 3 homogeneous groups, patients were selected not only on the basis of genotype but also on the basis of clinical features. Thus, for each genotype, 5 control patients and 5 case patients were selected (Table 2). Monocytes (20 × 10⁶/mL of Dulbecco modified eagle’s medium) were stimulated with lipopolysaccharide (1 µg/mL), oxidized low-density lipoprotein (LDL) (50 µg/mL), angiotension II (10⁻⁷ mol/L), and advanced glycation end products (800 µg/mL) as previously described.6,8,14 At the end of the incubation, COX-2 and MMP expression was evaluated by Western blot, MMP activity by zymography, and PGE₂ (Cayman Chemical, Ann Arbor, Mich) and MMP-9 (Amersham Biosciences, Piscataway, NJ) release by enzyme-linked immunosorbent assay. In vivo prostacyclin (PGI₂) production, a process largely dependent on COX-2 activity in endothelium,15,16 was analyzed in 8 randomly selected patients with the −765GG genotype, 8 with −765GC, and 8 with −765CC by measuring urinary excretion of 6-keto PGF₁α, the major urinary metabolite of renal PGI₂, according to a previously described radioimmunoassay.17 Strain-gauge plethysmography was used to measure forearm blood flow changes induced by intrabrachial acetylcholine and sodium nitroprusside according to a previously validated method18 in 6 randomly selected control participants with the −765GG genotype, 9 with −765GC, and 3 with −765CC to identify perturbations in endothelium-dependent vasodilation.

### Statistical Analyses

The significance of differences in biochemical marker expression and inflammatory cell infiltration among the 3 genotypes was analyzed by unpaired t test. Discrete data (including prevalence of the −765C allele in cases and controls) were analyzed by the χ² test.
with the allele frequency predicted on the basis of the Hardy-Weinberg equilibrium. We used logistic regression modeling to test the association of the COX-2 polymorphisms on the risk of MI and ischemic stroke after adjusting for age, body mass index, smoking status, hypertension, diabetes mellitus, and hypercholesterolemia. All analyses were performed using SPSS software, version 11.0.1 (SPSS Inc, Chicago, Ill). P < .05 was considered statistically significant.

RESULTS
Characteristics of the Study Participants
The characteristics of the 1728 study participants are shown in Table 1. There were no significant differences between cases and controls in age, body mass index, or prevalence of conventional risk factors for cardiovascular disease.

Table 3. Distributions and Results of Multivariable Logistic Regression Analyses of COX-2 Gene Variants Among Cases and Controls With MI or Atherothrombotic Ischemic Stroke

| Genotype | Distribution, % | Ratio, Controls/Cases | Prevalence Ratio (95% CI)* | P Value |
|----------|-----------------|-----------------------|---------------------------|---------|
| GG       | 50.7            | 81                    | 0.62                      | <.001   |
| GC       | 43.3            | 17.9                  | 2.41                      | <.001   |
| CC       | 6.4             | 1.1                   | 5.81                      | 0.33 (0.24-0.55) 0.04 |
| Allele frequency |     |                       |                           |         |
| C        | 0.252           | 0.104                 |                           |         |
| G        | 0.748           | 0.896                 |                           |         |
| Participants aged >70 y (n = 257; 112 controls and 145 cases) |     |                       |                           |         |
| GG       | 51.3            | 89.2                  | 0.57                      | <.001   |
| GC       | 41.2            | 9.7                   | 4.21                      | <.001   |
| CC       | 7.5             | 1.1                   | 6.81                      | 0.31 (0.21-0.63) .008 |
| Allele frequency |     |                       |                           |         |
| C        | 0.273           | 0.104                 |                           |         |
| G        | 0.727           | 0.896                 |                           |         |
| Participants with first-degree relative with previous acute MI or stroke (n = 187; 84 controls and 103 cases) |     |                       |                           |         |
| GG       | 47.9            | 88.9                  | 0.54                      | <.001   |
| GC       | 44.3            | 10.3                  | 4.31                      | <.001   |
| CC       | 7.8             | 0.8                   | 9.75                      | 0.30 (0.22-0.52) .004 |
| Allele frequency |     |                       |                           |         |
| C        | 0.279           | 0.089                 |                           |         |
| G        | 0.721           | 0.911                 |                           |         |

Abbreviations: CI, confidence interval; COX-2, cyclooxygenase 2; MI, myocardial infarction.
*The prevalence ratio is for the cumulative risk of MI and atherothrombotic ischemic stroke among patients who were heterozygous or homozygous for the −765G→C polymorphism compared with those who were not, calculated based on the odds ratio from logistic regression after adjustment for confounders.19

Prevalence of the −765G→C Polymorphism
TABLE 3 summarizes the genotype data for all study participants. The prevalence of the −765GC genotype was 2.41 times higher among controls than among cases (prevalence ratio, 0.48; 95% confidence interval [CI], 0.36-0.68). The prevalence of −765CC homozygosity was 5.81 times higher (prevalence ratio, 0.33; 95% CI, 0.24-0.55). The inverse association between −765G→C variant and cardiovascular events was even stronger in patients older than 70 years, suggesting that participants carrying the −765G→C variant have a higher probability of healthy aging. In the subgroup of 187 participants with 1 first-degree relative who had had MI or ischemic stroke, the prevalence of one −765C allele was 4.3 times higher among asymptomatic controls than among cases. There were no differences in the frequency of the −765GC genotype between the 2 subgroups defined according to diagnosis of MI or stroke (17.9% and 17.8%, respectively).

Inverse Association Between −765G→C Polymorphism and MI or Stroke
Homozgyosity as well as heterozygosity for the −765C allele was associated with a markedly lower risk of MI and stroke. Patients with the −765GC or −765CC genotype had a reduction in relative risk of MI and ischemic stroke of 52% and 67%, respectively, after adjustment for age, sex, smoking status, body mass index, hypercholesterolemia, hypertension, and diabetes (Table 3). The same was true when we analyzed patients who underwent carotid endarterectomy or coronary angiography as case-control substudies (carotid endarterectomy, adjusted prevalence ratio, 0.46; 95% CI, 0.34-0.69; P = .03; coronary angiography, prevalence ratio, 0.34; 95% CI, 0.26-0.58; P = .04).

To investigate the association of the COX-2 polymorphism with plaque growth, we analyzed the atherosclerotic burden as a function of the −765C allele in patients who had accurate quantification of atherosclerosis by carotid Doppler or coronary angiography. We did not find any association between the −765C allele and severity of atherosclerosis at either the carotid or the coronary level (Table 1). Nonetheless, in a subgroup of 224 cases, mean (SD) serum hsCRP was significantly lower in patients with the −765C allele (0.78 [0.1] mg/L vs 2.56 [0.4] mg/L; P = .04).

COX-2 Expression in Carotid Plaques of Patients With the −765G→C Polymorphism
COX-2 staining was significantly more abundant in −765G→C carotid plaques (Figure 1), as confirmed by quantitative analysis (mean [SD], 23% [2%], 16% [2%], and 10% [1%] for −765G→C [n = 145], −765GC [n = 78], and −765CC [n = 9], respectively; P < .001). COX-2 accumulated mainly in macrophages. Western blot confirmed higher COX-2 expression in −765G→C plaques (8576 [176] DU, 5132 [142] DU, and 3059
DU for −765GG, −765GC, and −765CC, respectively; \( P < .001 \) (Figure 2). These observations remained consistent when separate analyses for the case and control groups were performed. To exclude the possibility that observed discrepancies in COX-2 expression were merely a secondary effect of differences in inflammatory infiltration, we repeated immunocytochemistry and Western blot analyses on macrophages that had been selectively extracted from plaques. We again noted that plaque macrophages from patients carrying the −765G→C polymorphism had a lower COX-2 expression.

**MMP Expression and Activity in Carotid Plaques of Patients With the −765G→C Polymorphism**

Staining for MMP-2 and MMP-9 was significantly more abundant in −765GG lesions (n = 145) than in −765GC (n = 78) and −765CC plaques (n = 9) (mean [SD], 22% [3%] vs 15% [2%] and 9% [2%], respectively, for MMP-2; 24% [4%] vs 13% [2%] and 8% [1%], respectively, for MMP-9; all \( P < .003 \)) (Figure 1). Increased MMP immunoreactivity was also documented by Western blot in −765GG plaques compared with −765GC and −765CC plaques (n = 232) (mean [SD], 7165 [134] DU vs 5121 [78] DU and 3978 [67] DU for MMP-2; 8090 [187] DU vs 6012 [102] DU and 4765 [95] DU for MMP-9; all \( P < .003 \)) (Figure 2). Zymography demonstrated that extracts from −765GG plaques (n = 145) contained higher amounts of activated forms of MMP-2 and MMP-9 than −765GC (n = 78) and −765CC (n = 9) plaques (mean [SD], 3012 [134] DU vs 1898 [104] DU and 1342 [54] DU for MMP-2; 3867 [165] DU vs 2011 [89] DU and 1223 [65] DU for MMP-9; \( P < .001 \)) (Figure 2).

**Collagen Content in Plaques of Patients With the −765G→C Polymorphism**

Sirius red polarization showed considerably lower content of interstitial collagen in the tissue sections of patients carrying the −765GG genotype compared with patients carrying the −765GC and −765CC genotypes (n = 232) (mean [SD], 9% [1%] vs 15% [2%] [\( P = .002 \)] and 19% [3%] [\( P = .003 \)], respectively).

**Inflammatory and Proteolytic Response in Monocytes/Macrophages of Patients With the −765G→C Polymorphism**

In monocytes isolated from patients carrying the −765GG genotype, lipopolysaccharide, oxidized low-density lipoprotein, angiotensin II, and advanced glycation end products induced significant increases in COX-2, MMP-2, and

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**Figure 1. Immunohistochemical Analysis for Presence of COX-2 and MMP-9 in Atherosclerotic Carotid Plaques**

Cyclooxygenase 2 (COX-2) and matrix metalloproteinase 9 (MMP-9) protein expression by biotin–streptavidin immunohistochemical analysis using 2 monoclonal human antibodies to COX-2 and MMP-9, respectively, in plaques obtained by endarterectomy from patients carrying the −765GG, −765GC, and −765CC genotypes. Top and bottom panels show similar regions of the plaque. Brown chromogen indicates positive staining of COX-2 and MMP-9. Note the endothelium (arrows) in the −765 CC plaque, characterized by strong expression of COX-2, in contrast with the reduced expression of COX-2 in infiltrating macrophages beneath the endothelial layer (counterstain, hematoxylin; original magnification ×20).
MMP-9 protein expression (FIGURE 3) as well as in PGE2 (mean [SD], 4.25 [0.3], 2.86 [0.2], and 1.64 [0.2] ng/mL, respectively, after lipopolysaccharide, 1 µg/mL) and MMP-9 (mean [SD], 30.3 [4.0], 21.4 [3.0], and 12.6 [2.0] ng/mL, respectively, after lipopolysaccharide, 1 µg/mL) release compared with that detected in monocytes from patients carrying the −765GC and −765CC genotypes. Similar results were also observed in plaque-derived macrophages.

−765G→C Polymorphism and PGI2 Biosynthesis and Endothelial Function In Vivo

Urinary 6-keto PGF1α excretion was not different in patients carrying the −765GG genotype compared with patients carrying the −765GC and −765CC genotypes (mean [SD], 9.7 [1.0] ng/h vs 9.3 [2.2] ng/h [P=.65] and 10.5 [1.0] ng/h [P=.13]). Similarly, no differences in endothelium-dependent and endothelium-independent (sodium nitroprusside) vasodilation were observed among participants carrying the 3 genotypes (FIGURE 4).

COMMENT

We found that the −765G→C polymorphism of the COX-2 gene is associated with a reduction in the risk of MI and stroke, suggesting that this allele may offer protection against clinical events related to atherosclerotic plaque rupture.

The prevalence distribution of this polymorphism in our control group was significantly higher than that recently reported by Papafili et al10 in a smaller sample in the United Kingdom. Since the −765G→C polymorphism is associated with lower expression and activity of the COX-2 gene in plaque macrophages, the finding of a lower frequency of this polymorphism in the United Kingdom is consistent with the higher incidence of MI and stroke in that country compared with in Italy. In addition, it could explain, at least in part, the higher risk of MI observed in northern Europe compared with Mediterranean populations with similar levels of cholesterol and blood pressure.21

Both the −765GC and −765CC genotypes were significantly related to COX-2 expression and activity in circulating blood monocytes and carotid plaque macrophages, suggesting that the association between COX-2 genotype and MI and stroke may occur as a result of the modulation of COX-2 activity in inflammatory cells. Indeed, patients with the −765CC genotype, who had the lowest risk of MI and stroke, also had the lowest level of COX-2 expression and activity in plaque...
sections, in plaque-derived macrophages, and in circulating blood monocytes. Furthermore, the presence of at least one −765C allele was associated with a significant reduction in serum CRP, thus suggesting that this polymorphism may also influence systemic inflammatory status.

The precise mechanism by which the COX-2 polymorphism may affect the risk of MI and stroke is unclear. Both MI and atherothrombotic stroke are often triggered by rupture of vulnerable atherosclerotic plaques, with the propensity of rupture enhanced by PGE₂-dependent MMPs.22 Thus, the marked reduction in plaque MMPs observed in patients carrying the −765G→C polymorphism supports our hypothesis that reduction of MMP biosynthesis as a consequence of lower PGE₂ generation may explain the risk reduction we observed. The lower COX-2 induction observed in monocytes and macrophages of patients carrying the −765C allele after stimulation with oxidized low-density lipoprotein, angiotensin II, and advanced glycation end products (stimuli upregulated in the setting of hypercholesterolemia,23 hypertension,24 and diabetes,25 respectively) is also consistent with the possibility that the −765G→C polymorphism may be protective against MI and stroke. However, we cannot exclude that other important mechanisms—for example, the reduction of aspirin-insensitive thromboxane biosynthesis in circulating monocytes—may also contribute to the risk reduction associated with the −765C allele.26–28 Furthermore, because adjustment for baseline risk factors was not performed in these in vitro substudies, we cannot exclude a potential influence of selection bias.

We did not find differences in the severity of atherosclerotic lesions among patients carrying the 3 different genotypes. This observation confirms previous studies suggesting that COX-2 is involved in the evolution of atherosclerotic plaque toward instability rather than plaque growth.29,30 We noted that the −765G→C polymorphism was not associated with endothelial generation of PGI₂ and endothelium-dependent vasodilation. This is consistent with previous observations in epithelial cells11 and suggests that transcription factor Sp1 has differing roles regarding induction of COX-2 in monocytes and macrophages compared with endothelial and epithelial cells. Because PGI₂ is considered a potent antiatherogenic mediator,31 these data strengthen support for the protective role of the −765G→C polymorphism and point out a major difference with respect to COX-2 inhibition through COX-2 inhibitors (coxibs), which also inhibit COX-2 at the endothelial level.15,16

Our study also raises a number of provocative questions regarding the potential clinical importance of this genotype in asymptomatic patients taking selective coxibs. The individual pharmacodynamic response to COX-2 inhibitors may vary,32 as responders and nonresponders to coxibs have been described.33,34 Thus, patients with the −765GC and −765CC genotypes may represent subgroups of patients with different drug responses.

Further research will be needed to determine whether genotyping patients for the COX-2 polymorphism leads to better treatment outcomes.

In conclusion, consistent with the presence of genetic determinants of clinical atherosclerotic risk, we found that the −765G→C polymorphism of the COX-2 gene is associated with a lower risk of MI and ischemic stroke. The reduction in risk may be related to modification of COX-2 expression in plaque macrophages. Further studies are necessary to identify additional polymorphisms in the arachidonic acid pathway that are associated with risk of MI and stroke.

Author Contributions: Dr Cipollone had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Cipollone, Martinotti, F. Cuccurullo, Mezzetti. Acquisition of data: Cipollone, Toniato, Fazia, Iezzi, Cuccurullo, Pini, Ursi, Vitullo, Averna, Arca, Montali, Campagna, Uccchino, Spigonardo, Taddei, Virdis, Ciabattoni, Notarbartolo, F. Cuccurullo, Mezzetti. Analysis and interpretation of data: Cipollone, Martinotti, F. Cuccurullo, Mezzetti.

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ACKNOWLEDGMENT: We are indebted to Carlo Pa-
trono for his helpful suggestions in the design of the study and preparation of the manuscript and to Michele Scarano, Anna Maria Cupertino, Manfredri Rizzo, An-
tonio Castello, and Arian Frasheri for their contribu-
tion to patient recruitment and sample collection. Dr. Cipollone dedicates this work to the memory of his mother, Ines.

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