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## Abstract

**Background:** To examine the prevalence of an exon 12 polymorphism on the human Thromboxane synthase (CYP5A1) gene.

**Material/Methods:** Using sequence-specific PCR, we examined the allelic prevalence in 237 Greek patients with ischemic strokes and in 171 controls. In addition, we compared the CYP5A1 allelic prevalence in 71 patients with stroke recurrence despite Aspirin use, in comparison with patients who have not experienced recurrent stroke while taking Aspirin.

**Results:** The frequencies of the CYP5A1\*9 mutant (substitution of guanine by adenine near the heme-binding catalytic domain) and of the wild-type allele were 0.197 and 0.803, respectively; they did not differ significantly between stroke patients and controls. The wildtype allele was more frequent in the Cretan population compared to continental Greece (OR 1.80, 95% CI 1.19-2.74). The wild-type allele was more frequent among hypertensive and less frequent among diabetic stroke sufferers, respectively. The CYP5A1\*9 mutant was significantly more prevalent among stroke patients with history of previous cerebrovascular attacks (p

**Conclusions:** Allelic prevalence of the CYP5A1 exon 12 might differ between geographic areas within the same ethnic group, and is associated with particular characteristics of stroke patients. Allele mutations can abolish the enzymatic activity of thromboxane synthase, via impaired heme binding, associated with defective response to Aspirin used as secondary prevention, an effect independent from the conventional risk factors for cerebrovascular disease.

## Keywords

Aspirin, CYP5A1, arachidonic acid, single-nucleotide polymorphism, stroke, thromboxane synthase, transient ischemic attack

## Disciplines

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### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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### Background:

To examine the prevalence of an exon 12 polymorphism on the human Thromboxane synthase (*CYP5A1*) gene.

### Material/Methods:

Using sequence-specific PCR, we examined the allelic prevalence in 237 Greek patients with ischemic strokes and in 171 controls. In addition, we compared the *CYP5A1* allelic prevalence in 71 patients with stroke recurrence despite Aspirin use, in comparison with patients who have not experienced recurrent stroke while taking Aspirin.

### Results:

The frequencies of the *CYP5A1*\*9 mutant (substitution of guanine by adenine near the heme-binding catalytic domain) and of the wild-type allele were 0.197 and 0.803, respectively; they did not differ significantly between stroke patients and controls. The wild-type allele was more frequent in the Cretan population compared to continental Greece (OR 1.80, 95% CI 1.19–2.74). The wild-type allele was more frequent among hypertensive and less frequent among diabetic stroke sufferers, respectively. The *CYP5A1*\*9 mutant was significantly more prevalent among stroke patients with history of previous cerebrovascular attacks ( $p < 0.01$ ); and among those who failed secondary Aspirin prophylaxis even after adjusting for the common risk factors for cardiovascular disease (OR 1.49, 95% CI 1.06–2.11).

### Conclusions:

Allelic prevalence of the *CYP5A1* exon 12 might differ between geographic areas within the same ethnic group, and is associated with particular characteristics of stroke patients. Allele mutations can abolish the enzymatic activity of thromboxane synthase, via impaired heme binding, associated with defective response to Aspirin used as secondary prevention, an effect independent from the conventional risk factors for cerebrovascular disease.

### key words:

**aspirin • CYP5A1 • arachidonic acid • single-nucleotide polymorphism • stroke • thromboxane synthase • transient ischemic attack**

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## BACKGROUND

Cerebrovascular attacks are leading cause of mortality and morbidity worldwide [1]. In more than 80% of cases, they consist of ischemic events within the lumen of cerebral vessels [2], a process in which primary hemostasis and platelets play a cardinal role [3]. Antiplatelet agents, primarily Aspirin, remain the optimal treatment for primary and secondary prevention of ischemic strokes [4]. The beneficial action of Aspirin on strokes is affected via inhibition (irreversible acetylation) of the enzyme cyclo-oxygenase, resulting in reduced thromboxane A<sub>2</sub> formation. Thromboxane A<sub>2</sub> favors platelet aggregation and vasoconstriction [5]. The synthetic pathway that leads to thromboxane A<sub>2</sub> formation results in the metabolism of arachidonic acid to prostaglandin and leucotriene derivatives via the sequential activation of several enzymes [6].

One of the key enzymes in this process is the thromboxane synthase (CYP5A1). This is a unique member of family 5 in the cytochrome P<sub>450</sub> superfamily and catalyzes the conversion of prostaglandin H<sub>2</sub> to thromboxane A<sub>2</sub> [7]. A balance between thromboxane A<sub>2</sub> and prostacyclin production is very important for vessel integrity [6]. The mechanism, however, controlling the formation of these biofactors - formed by isomerization of their common precursor prostaglandin H<sub>2</sub> - remains unknown. Clinically, a deficiency of platelet CYP5A1 activity has been associated with moderate to severe bleeding disorders [8], but the molecular mechanisms underlying this remain unclear.

The *CYP5A1* gene encoding for thromboxane synthase has been characterised at the long arm of chromosome 7 [9]. It consists of 13 exons and 12 introns, and its size approximates 150Kb. Alternate splicing of the *CYP5A1* transcript has been described. The truncated *CYP5A1* mRNA lacks the entire exon 12 encoding the heme-binding domain of the enzyme; the truncated protein is lacking enzymatic activity [10]. In the only relevant study published so far, genetic polymorphisms of the *CYP5A1* gene have been described in a highly homogeneous French population of 200 healthy volunteers of Caucasian origin: almost 10% of subjects exhibited at least one out of the 8 missense point mutations detected [11]. Among the latter, the substitution of guanine by adenine at the position 1397 of exon 12 signifies the mutant allele designated as *CYP5A1*\*9; the mutation results in Glutamine replacing Arginine as the amino acid 466 of thromboxane synthase [11]. This position is related to the heme-binding domain that is important for the catalytic activity of the encoded enzyme [12]. A mutation has also been described in another (1425) position of exon 12, where substitution of cytosine by thymine leads to the silent (not causing any change in the encoded protein) polymorphic allele *CYP5A1*\*1C [11].

Genetic studies have previously suggested the role of CYP genes in defining genetically susceptible individuals at risk for tobacco-related cancers, due to impaired hepatic detoxification of tobacco substances [13]. In the present case-control study we examined the prevalence of the known *CYP5A1* exon 12 genetic polymorphism in stroke patients with and without Aspirin prophylaxis, as well as in healthy controls from the island of Crete in comparison with counterparts from continental Greece.

## MATERIAL AND METHODS

Recruitment of the study population was performed in two hospitals from different geographic areas of Greece: "Sotiria" University General Hospital in Athens; and the "Venizelion" General Hospital, Heraklion, Crete. Two hundred and thirty seven consecutively admitted patients with a history of documented cerebral ischemic attack, as well as 171 subjects without a history of cerebrovascular attack were included in the study. Controls from each centre were matched to local stroke patients, in terms of age, sex, and major modifiable risk factors for cerebrovascular disease (smoking, hypertension, diabetes, dyslipidemia). To examine the association between the *CYP5A1* SNPs and the protective effect of Aspirin, 71 patients with recurrent stroke while taking Aspirin were compared with the stroke patients who did not have any recurrent event while on aspirin secondary prophylaxis.

Recruitment study period was 1 year (September 2003–August 2004). Non-contrast computerized tomography was performed on all patients within 24 hours of admission, repeated in the following 72 hours or supplemented by MRI, as appropriate. Additional examinations (i.e. lumbar puncture, septic work-up, etc) were performed as necessary. Patients were excluded from the study if an alternative diagnosis (i.e. hematoma, encephalitis, tumour) was responsible for the neurologic findings; if there was radiologic evidence of intracerebral hemorrhage or hemorrhagic transformation; if they had a history of cancer or had received chemotherapy and/or radiotherapy.

Since we focused on the role of *CYP5A1* on the Aspirin pathway, patients were also excluded if they had atrial fibrillation (where the mechanism of stroke is different, and the treatment of choice does not entail Aspirin) or when the stroke was classified of cardioembolic origin, as well as if they were on oral anticoagulants or antiplatelet therapy other than Aspirin (i.e. dipyridamole, clopidogrel). Stroke patients were classified according to the TOAST diagnostic system [14]. Risk factors for cerebrovascular disease were assessed according to standard definitions [15]. Dyslipidemia was defined by pharmacological treatment, total serum cholesterol >6.2 mmol/L or LDL-cholesterol >4.1 mmol/L.

During the 12 months of the recruitment period, 511 patients were considered for inclusion in the study. Out of those, intracerebral hemorrhage was diagnosed in 58; for another 70 patients an alternative intracerebral pathology (i.e. hematoma, tumour, infection) accounted for the neurologic findings. A further 146 patients were excluded because of other reasons (cardioembolic stroke, atrial fibrillation and/or anticoagulation, use of other antiplatelet treatment, refusal to provide sample, etc). Main characteristics of patients and controls included in the study are shown in Table 1.

Peripheral blood from study subjects was collected in EDTA anticoagulated tubes. Genomic DNA was extracted from whole blood using proteinase K, followed by phenol extraction and ethanol precipitation according to standard procedures [16]. DNA was resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA purity was assessed by a UV/VIS spectrophotometer evaluating the

**Table 1.** Main characteristics of the study population.

|                               | Patients N=237 | Controls N=171 | p  |
|-------------------------------|----------------|----------------|----|
| Mean age (Range)              | 77.3 (47–99)   | 76.8 (43–96)   | NS |
| Sex                           |                |                |    |
| Male (%)                      | 114 (48.2)     | 83 (48.5)      | NS |
| Female (%)                    | 123 (51.8)     | 88 (51.5)      |    |
| Origin                        |                |                |    |
| Continental Greece (%)        | 173 (73)       | 125 (73.1)     | NS |
| Crete (%)                     | 64 (27)        | 46 (26.9)      |    |
| Risk factors <sup>1</sup>     |                |                |    |
| None (%)                      | 12 (5.1)       | 10 (5.8)       | NS |
| One (%)                       | 50 (21.1)      | 39 (22.8)      |    |
| More than one (%)             | 175 (73.8)     | 122 (71.4)     |    |
| Smokers (%)                   | 73 (30.8)      | 53 (30.9)      | NS |
| Hypertension (%)              | 193 (81.4)     | 133 (77.8)     | NS |
| Diabetes (%)                  | 92 (38.8)      | 56 (32.7)      | NS |
| Dyslipidemia <sup>2</sup> (%) | 83 (35)        | 62 (36.2)      | NS |
| Total chol. mmol/L (mean ±SD) | 6.6 (3.8)      | 6.4 (3.9)      |    |
| LDL-chol. mmol/L (mean ±SD)   | 4.4 (3)        | 4.1 (3.1)      |    |
| HDL-chol. mmol/L (mean ±SD)   | 1.1 (0.6)      | 1 (0.5)        |    |
| Previous CVA <sup>3</sup>     |                |                |    |
| No evidence (%)               | 108 (45.6)     | NA             |    |
| One (%)                       | 77 (32.5)      | NA             |    |
| More than one (%)             | 52 (21.9)      | NA             |    |
| Classification <sup>4</sup>   |                |                |    |
| TIA                           | 28 (11.8)      | NA             |    |
| LVD                           | 94 (39.7)      | NA             |    |
| SVD                           | 82 (34.6)      | NA             |    |
| IU                            | 33 (13.9)      | NA             |    |
| Daily Aspirin dose            |                | NA             |    |
| No                            | 166 (70)       |                |    |
| 100 mg/day                    | 15 (6.3)       | NA             |    |
| 160 mg/day                    | 27 (11.4)      | NA             |    |
| 325 mg/day                    | 29 (12.3)      | NA             |    |

<sup>1</sup> Denotes the major modifiable risk factors for cerebrovascular disease: smoking, hypertension, diabetes mellitus, dyslipidemia.

<sup>2</sup> Presence of one of the following: pharmacological treatment, total serum cholesterol >6.2 mmol/L, LDL-cholesterol >4.1 mmol/L.

<sup>3</sup> Includes ischemic strokes and/or documented transient ischemic attacks (TIA). CVA – cerebrovascular attack; NA – not applicable; NS – not statistically significant.

<sup>4</sup> According to the TOAST diagnostic system (see Ref. 14 for details): LVD – large-vessel disease; SVD – small-vessel disease; IU – ischemic stroke of undetermined etiology. Patients with cardioembolic disease, hemorrhage or acute stroke of etiology other (than ischemic) have been excluded from this study.

$A_{260}/A_{280}$  ratio. Working stocks were prepared by 10-fold dilution in double distilled  $H_2O$ .

An allele-specific polymerase chain reaction (AS-PCR) approach [17] was used to detect the presence of polymorphisms at the 1397 site of exon 12. We designed sequence specific primers with mismatches at the 3' end to identify each variant, which, in combination with a consensus primer, resulted in a PCR product of expected size if the specific allelic variant was present. A set of appropriate control primers confirmed PCR amplification in the reactions where specific allele amplification was absent: the presence of an allele-specific band of the expected size in conjunction with a control band was considered to be positive evidence for each particular allele. The absence of an allele-specific band with presence of a control band was considered to be negative indication for a particular allele. The allele-specific and control primers we used are shown in Table 2.

PCR assays were performed by introducing 100 ng of genomic DNA in a PCR reaction mixture containing 1X PCR buffer, 250  $\mu$ M dNTPs, 2.0 mM  $MgCl_2$ , 0.2 mM each allele-specific primer, 0.1 mM each control primer, and 0.2 U *Taq* DNA polymerase (Life Technologies Ltd., UK) to a 25  $\mu$ l total reaction volume. The cycling parameters were as follows: 2 minutes at 95°C, 34 cycles of 30 seconds at 94°C, 50 seconds at 60°C, and 25 seconds at 72°C, 21 cycles of 25 seconds at 96°C, 50 seconds at 65°C, and 5 minutes at 72°C. The PCR products were visualized by agarose gel (2%) electrophoresis after ethidium bromide staining.

To confirm results produced by AS-PCR, randomly selected samples (1 out of 10) from all variants (homozygous for each genotype and heterozygous) were analyzed by dye primer cycle sequencing on an ABI 377 automated sequencer (PE Applied Biosystems, Warrington, UK), using the same allele-specific and consensus primer.

Data were analyzed using SPSS for Windows, release 12.0.1 (SPSS, Chicago, IL, USA). To determine whether the distribution of the exon 12 *CYP5A1* genotypes fulfilled the Hardy-Weinberg equilibrium, the  $\chi^2$  test was used to compare the observed number of subjects with the expected number. In addition, continuous variables (i.e. age) were examined using one-way analysis of variance. Variables distributed in a non-parametric fashion were examined using the Mann-Whitney U or the Kruskal-Wallis test, as appropriate. Normality was tested using the Kolmogorov-Smirnov test.

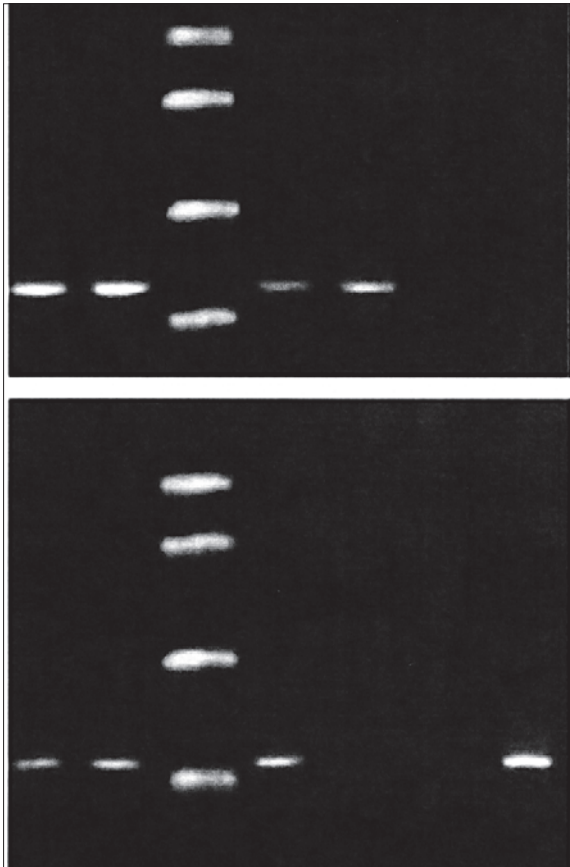
Our sample could provide us 90% power (with  $\alpha$  0.05) to detect at least 10% difference in the prevalence of the rare allele between stroke patients and controls, supposing the latter 15%. For the same power and statistical significance level, a sample of 133 patients would suffice for the detection of stroke recurrence on aspirin, should the mutant allele be overrepresented at an excess rate of 20% among stroke re-sufferers, in comparison to our reference stroke population.

In the stroke cohort, a logistic regression model was constructed to examine the relationship between *CYP5A1* allelotypes and stroke recurrence on Aspirin prophylaxis, using the following covariates: age, gender, stroke type, smoking status, diabetes mellitus, hypertension, dyslipidemia, use of statin and aspirin dose.

**Table 2.** Primers for polymerase chain reaction amplification of CYP5A1 exon 12 polymorphic sites.

| Gene         | Polymorphism | Annealing position | Primer sequence             | Product size |
|--------------|--------------|--------------------|-----------------------------|--------------|
| CYP5A1 ex.12 | +1397 G/A*   | 1375-1397          | F- gctgaggcccgagcagcaccg/A  | 120 bp       |
|              |              | 1495-1471          | R- gcagcacgtggagcagtgtaactt |              |
| GAPDH        |              |                    | F- GGAAGGTGAAGTCCGGAGTCA    | 101 bp       |
|              |              |                    | R- GTCATTGATGGCAACAATATCCA  |              |

\* Position referred to the ATG translation start site as +1.



**Figure 1.** Genotype analysis of CYP5A1 exon 12. Electrophoresis patterns for wild-type allele (top) and mutant allele (bottom). From left to right: Lanes 1, 2 and 4 – heterozygous cases; Lane 5 – CYP5A1 wild-type homozygosity; Lane 6 – CYP5A1\*9 homozygosity; Lane 7 – 100bp DNA ladder.

Approval to conduct this research was obtained from Ethics Committees in both participating hospitals.

## RESULTS

Representative examples of homozygous for the wild-type, CYP5A1\*9 homozygous and heterozygous cases are shown in Figure 1. The allelic frequencies of the wild-type CYP5A1 exon 12 and of the CYP5A1\*9 mutant among the study population were 0.803 and 0.197, respectively; these did not differ significantly between stroke patients and controls (Table 3). There was under-representation of the CYP5A1\*9 allele

among Cretan subjects, compared with those originated from continental Greece (Odds Ratio for the wild-type 1.80, 95% Confidence Intervals 1.19–2.74;  $p=0.005$ ). This difference was observed even when stroke patients were analyzed separately (OR 1.81, 95% CI 1.04–3.14;  $p=0.036$ ) (Table 3). Given this difference in allelic prevalence between mainland Greek and Cretan populations, the Odds Ratios for all following analysis in stroke patients were corrected for place of origin.

Among stroke patients, the presence of the CYP5A1 wild-type allele was more frequent among the hypertensives (OR 1.68, 95% CI 1.01–2.79;  $p=0.045$ ), and less frequent among the diabetics (OR 0.55, 95% CI 0.36–0.84;  $p=0.006$ ) (Table 3). Also among stroke patients, the CYP5A1\*9 mutant was significantly more prevalent among those who suffered a recurrent stroke while receiving Aspirin, compared to those with successful secondary Aspirin prophylaxis (OR 1.73, 95% CI 1.10–2.73;  $p=0.017$ ). After adjusting for aspirin dose, use of statins and conventional risk factors for cardiovascular disease (age, gender, smoking status, diabetes mellitus, hypertension, dyslipidemia) the overrepresentation of the CYP5A1\*9 mutant among stroke resufferers remained statistically significant (OR 1.49, 95% CI 1.06–2.11;  $p=0.038$ ) (Table 3). The adjusted allelic frequencies of the CYP5A1\*9 mutant among first-ever stroke sufferers, patients with one previous stroke, and those with history of multiple cerebrovascular attacks were 0.199, 0.272, and 0.280, respectively;  $p<0.01$  (Table 3). Regarding stroke types, the CYP5A1\*9 mutant was more prevalent among patients with small-vessel disease and (less so) among those with TIA compared with patients with large-vessel disease, but this difference was not statistically significant in multivariate analysis (Table 3).

There was no association between the genotype distribution of exon 12 alleles and age, sex and the presence of risk factors for cardiovascular disease other than those in Table 3 (i.e. family history, obesity, value of high-sensitivity C-reactive protein, etc) (data not shown).

## DISCUSSION

In this case-control study, we examined the allelic frequencies of a SNP on exon 12 of the thromboxane synthase (CYP5A1) gene in Greek stroke patients and controls. The allelic frequency of the mutant CYP5A1\*9 (that contains adenine instead of guanine at the position 1397) was 0.197 among our study population; this was higher than the frequency of CYP5A1\*9 (3.75%) found among another Caucasian (French) population in the only relevant study existing thus far [11]. Interestingly, the frequency of CYP5A1\*9 was significantly lower among the study subjects originating from the island

**Table 3.** Allelic frequencies of the *CYP5A1* exon 12 among the study population.

|                             | N   | All subjects (408)     |                       |                  | N   | Stroke patients (237) |          |                               |
|-----------------------------|-----|------------------------|-----------------------|------------------|-----|-----------------------|----------|-------------------------------|
|                             |     | CYP5A1 wt <sup>1</sup> | CYP5A1*9 <sup>2</sup> | OR (95% CI)      |     | CYP5A1 wt             | CYP5A1*9 | OR (95% CI)                   |
| All                         | 408 | 0.803                  | 0.197                 |                  |     |                       |          |                               |
| Status                      |     |                        |                       |                  |     |                       |          |                               |
| Patients                    | 237 | 0.812                  | 0.188                 | NS               |     |                       |          |                               |
| Controls                    | 171 | 0.792                  | 0.208                 |                  |     |                       |          |                               |
| Origin                      |     |                        |                       |                  |     |                       |          |                               |
| Crete                       | 110 | 0.855                  | 0.145                 | 1.80 (1.19–2.74) | 64  | 0.859                 | 0.141    | 1.81 (1.04–3.14)              |
| Continental Greece          | 298 | 0.765                  | 0.235                 |                  | 173 | 0.772                 | 0.228    |                               |
| Smoking                     |     |                        |                       |                  |     |                       |          |                               |
| Yes                         | 126 | 0.762                  | 0.238                 | NS               | 73  | 0.822                 | 0.178    | NS                            |
| No                          | 282 | 0.761                  | 0.239                 |                  | 164 | 0.750                 | 0.250    |                               |
| Hypertension                |     |                        |                       |                  |     |                       |          |                               |
| Yes                         | 326 | 0.773                  | 0.227                 | NS               | 193 | 0.782                 | 0.218    | 1.68 (1.01–2.79)              |
| No                          | 82  | 0.707                  | 0.293                 |                  | 44  | 0.682                 | 0.318    |                               |
| Diabetes                    |     |                        |                       |                  |     |                       |          |                               |
| Yes                         | 148 | 0.740                  | 0.260                 | NS               | 92  | 0.701                 | 0.299    | 0.55 (0.36–0.84)              |
| No                          | 260 | 0.769                  | 0.231                 |                  | 145 | 0.810                 | 0.190    |                               |
| Dyslipidemia                |     |                        |                       |                  |     |                       |          |                               |
| Yes                         | 145 | 0.755                  | 0.245                 | NS               | 83  | 0.732                 | 0.268    | NS                            |
| No                          | 263 | 0.764                  | 0.236                 |                  | 154 | 0.789                 | 0.211    |                               |
| Aspirin use <sup>3</sup>    |     |                        |                       |                  |     |                       |          |                               |
| Yes                         |     |                        |                       |                  | 71  | 0.711                 | 0.289    | 1.49 (1.06–2.11) <sup>6</sup> |
| No                          |     |                        |                       |                  | 166 | 0.810                 | 0.190    |                               |
| Previous CVA <sup>3,4</sup> |     |                        |                       |                  |     |                       |          | p<0.01 <sup>6</sup>           |
| No evidence                 |     |                        |                       |                  | 108 | 0.801                 | 0.199    |                               |
| One                         |     |                        |                       |                  | 77  | 0.728                 | 0.272    |                               |
| Multiple                    |     |                        |                       |                  | 52  | 0.720                 | 0.280    |                               |
| Classification <sup>5</sup> |     |                        |                       |                  |     |                       |          | NS <sup>6</sup>               |
| TIA                         |     |                        |                       |                  | 28  | 0.714                 | 0.286    |                               |
| LVD                         |     |                        |                       |                  | 94  | 0.782                 | 0.218    |                               |
| SVD                         |     |                        |                       |                  | 82  | 0.690                 | 0.310    |                               |
| IU                          |     |                        |                       |                  | 33  | 0.818                 | 0.182    |                               |

<sup>1</sup>Wild-type; <sup>2</sup>Allele that contains adenine instead of guanine at position +1397; <sup>3</sup>Among stroke patients only; <sup>4</sup>CVA – cerebrovascular attack; those include ischemic strokes and/or documented transient ischemic attacks (TIA); <sup>5</sup>LVD – large-vessel disease; SVD – small-vessel disease; IU – ischemic stroke of undetermined etiology; <sup>6</sup>Adjusted for age, sex, diabetes, hypertension, smoking, dyslipidemia, aspirin dose and statin use. NS – not statistically significant, OR – Odds Ratio (analysis for stroke patients was corrected for place of origin).

of Crete, compared with the continental Greek population; this difference was also observed when stroke patients were analysed separately. Different mutation frequencies among ethnic groups and subpopulations have been described for other polymorphic cytochromes P450 [18,19].

Prevalence of *CYP5A1* allelic forms in our study did not significantly differ between patients and controls. We

note, however, that stroke sufferers despite Aspirin prophylaxis were more likely to be carriers of the *CYP5A1*\*9 mutant than homozygous for the wild-type allele, even after adjusting for the common risk factors for cardiovascular disease. Similarly, the *CYP5A1*\*9 mutant prevailed among stroke patients with history of a previous cerebrovascular attack, compared to first-ever stroke sufferers (p<0.01).

Interestingly, correlations between the *CYP5A1*\*9 allele and both hypertension and diabetes (two of the major risk factors for stroke) were also recorded in our population. The significance of these relationships remains to be clarified; the fact, however, that overrepresentation of the mutant allele in patients resuffering from stroke while taking Aspirin remained after adjusting for the major risk factors for cardiovascular disease might imply for an independent role for this allele on the efficacy of the arachidonic acid metabolism pathway. Interestingly also, all correlations in stroke patients remained significant even after correction for place of origin, and this may signify for an effect of *CYP5A1* on stroke pathophysiology, additional to that conferred by ethnic predisposition.

Distribution of the mutated alleles did not differ among the different ischemic stroke subtypes in our study. This is particularly interesting for the case of the lacunar infarcts, whose pathophysiology is thought to differ from the rest ischemic strokes, and perhaps underlines the cardinal role of platelet-mediated thrombosis in the pathogenesis of brain infarcts [20].

A limitation to the interpretation of these results is that the metabolic activity of the genetic variants of thromboxane synthase has not been studied in depth as yet. Interestingly however, the *CYP5A1*\*9 allele denotes a substitution near the heme-binding area of the protein [11]. Mutations in this area can abolish the enzymatic activity of thromboxane synthase, via impaired heme binding [12]. The increased frequency of the mutant *CYP5A1*\*9 allele, interpreted in this context, may signify a population at risk for repeated ischemic strokes despite Aspirin prophylaxis, due to defective function of thromboxane synthase. The findings at the gene level presented herein may herald the functional studies required to dismiss or validate the hypothesis above. In this context, the frequency and significance of the alternative splicing that results in lack of the entire exon 12 need further investigation [21]. The effect of polymorphisms in other areas of the gene similarly merits further investigation. Finally, the validity of our results in other populations and ethnic groups remain to be determined.

The evaluation of the relationship between *CYP5A1* polymorphisms and stroke recurrence is certainly restricted by the cross-sectional nature of our data. Unfortunately, the duration of Aspirin use could not be accurately assessed for large proportion of the stroke cohort, and this precluded from utilizing a time-dependent statistical approach. We note, however, significant overrepresentation of the *CYP5A1*\*9 allele among patients with one, and multiple recurrences compared with those on "successful" Aspirin secondary prophylaxis. Another limitation to the interpretation of the results of this study is the absence of a validated surrogate marker for assessing compliance to aspirin. In addition, the degree of underlying cerebrovascular endothelial damage, potentially contributing to progression of the disease *per se*, could not be determined.

## CONCLUSIONS

This study provides evidence for high prevalence of the *CYP5A1*\*9 mutant among the Greek population. Our findings also imply that the presence of a mutant, affecting the heme-binding site of the enzyme, is possibly associated with

recurrent cerebrovascular attacks in patients who fail secondary prophylaxis with Aspirin. Additional genetic and functional studies of the other polymorphic variants described on the *CYP5A1* gene are expected to clarify the role of the thromboxane synthase enzyme in cerebrovascular disease.

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