CYP2A6 GENE SNPs ASSOCIATION WITH INTRACRANIAL ANEURYSMS AND SMOKING HABITS IN THE ITALIAN POPULATION.

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*Background*: Cigarette smoking is a long-known risk factor associated with the formation and rupture of intracranial aneurysms (IA), resulting in subarachnoid hemorrhage (SAH). The pathogenetic mechanism involves vascular endothelial damage triggered by oxidative stress products: some of these can be introduced into the body directly through smoking, while others derive from nicotine and xenobiotics catabolism due to cytochrome P450 activity. The goal of our study was to assess the relationship between IA and human cytochrome P450 family 2 subfamily A member 6 (CYP2A6) gene SNPs.

*Materials and Methods*: A case-control study was conducted on a population of 331 IA patients and 150 healthy controls. Three SNPs from the CYP2A6 gene, specifically responsible for nicotine metabolism, were analyzed on a biological sample (blood). Statistical analysis was performed to assess the presence of an association between these variants and IA natural history, with particular regard to smoking habits.

*Results*: In our cohort, the well-known correlation between ruptured IA and cigarette smoking habits was confirmed, regardless of SNPs pattern. A slight correlation between heterozygous allele \*14 and IA bleeding emerged from statistical analysis, that appears to be independent of smoking habits. A correlation between bleeding IA and smoking habits was also detected in patients presenting heterozygous \*B2 allele.

*Conclusions*: This study suggests that a gene variation coding for a fully active CYP2A6 enzyme determines an augmented production of oxidizing substances, compared to functionally less active proteins, leading to IA rupture. The enzymatic activity associated with the allele \*14 and its correlation with IA rupture, independent from smoking habits, needs further investigation.

*Keywords*: intracranial aneurysms – subarachnoid hemorrhage - cigarette smoking – cytochrome P450 – CYP2A6

INTRODUCTION

Despite its low prevalence (5-10% in the general population []), the impact of intracranial aneurysms rupture on public health is overwhelming. It’s been estimated that aneurismal subarachnoid hemorrhage (aSAH) has a mortality rate of nearly 65%, with most of deaths occurring during the first hours after the event []. Even early surgical intervention, proved to be effective in reducing mortality rates [], carries an intrinsic high risk of severe and moderate morbidity.

The pathophysiological mechanisms of intracranial aneurysms developing and evolution toward rupture are not yet clarified. Risk factors have been identified including cigarette smoking, female gender, vascular hypertension, increasing age, alcohol consumption and family history of intracranial aneurysm rupture []. Genetically determined diseases, as well as genetic polymorphisms, have been related to the developing of aSAH too [].

The prevalence of smoking habits in aSAH patients is around 20-35% (with variation from 45% to 75% in North America and Europe) []. The risk of aneurysms rupture in smokers is dose-dependent [] and has been estimated to be nearly 6 times greater than in nonsmokers []. The mechanisms triggered by cigarette smoking involve a reduction of flow-mediated dilation (Nitric Oxide production) and formation of a pro-oxidative and pro-inflammatory environment, as well as direct damage to vascular endothelium (cells contraction and death) and increased proliferation and migration of vascular smooth muscle cells []. Smoke contains more than 4’000 different substances: responsible for vascular damage is not only a single component or a single class (e.g. oxidants), but rather a commingling of them []. The most known is probably nicotine, a tertiary amine determining the activation of cerebral reward circuits that causes smoking addiction. Furthermore, studies have shown that nicotine plays an active role in vascular damage, causing alteration of flow-mediated vasodilation in large arteries []. Interaction of all these substances with genetic factors and with the environment determines the beginning, the location and the rate of progression of vascular disease. Being exogenous substances, the principal mechanism involved in the metabolism of smoke components is the microsomal liver cytochrome P450 enzymatic system (CYP). In vitro studies have shown that nicotine is mainly metabolized by the CYP2A6 isoform [], whose gene is located on chromosome 19q13.2. Numerous allelic variants and SNPs (Single Nucleotide Polymorphisms) of the gene have been identified, some of which have been associated with phenotypic variability in nicotine metabolism [].

This study proposes an analysis of three CYP2A6 gene SNPs, to evaluate whether a specific allele or genotype related to smoking habits would influence the presence of intracranial aneurysms and the occurrence of aneurismal SAH.

MATERIALS AND METHODS

*Patient selection*

We recruited 331 consecutive unrelated patients with IA documented by neuroimaging (brain angioCT scan and DSA), admitted to the Neurosurgery Department of Turin University between January 2001 and December 2011. Exclusion criteria were: death soon after diagnosis; angiographically negative SAH; patient carrying inherited genetic diseases associated with aneurysm formation (e.g. Marfan, Ehlers-Danlos type IV...); SAH related to rupture of other intracranial vascular malformations (AVMs, AVFs, …). In this group we collected detailed information about: 1) single (278 pts, 84%) versus multiple aneurysms (53 pts, 16%); 2) aSAH at diagnosis (255 pts, 77%); 3) smoking habit at the time of diagnosis, defined as more than 10 cigarettes smoked per day (84 pts, 25.4%).

The control group was formed by 150 healthy individuals selected from a cohort of blood donor patients, matched with patients group for sex, age and geographic features. The inclusion criterion was the absence of IA at neuroimaging. The study was approved by the Hospital Ethic Committee. Each participant provided written informed consent before the investigation.

*Genetic analysis*

A 2 mL blood sample was taken from each participant to the study and stored in EDTA tube. Cellular DNA extraction from the sample was performed using the QIAamp DNA Blood Mini Kit (QIAGEN S.p.A., Milan). Polymerase Chain Reaction was performed according to standard methods: initially denaturation was performed at 95 °C for 10 minutes, followed by 35 denaturation cycles at 95 °C for 1 minute, annealing at a specific temperature of 60 °C for 40 seconds for each couple of primers, then extension at 72 °C for 1 minute and a final extension at 72 °C for 5 minutes. The PCR products were loaded on 2% TBE agarose gel and stained with BrEt. All genetic analyses were performed at the Cytogenetics Laboratory of Turin University.

We examined the following CYP2A6 gene allelic variants:

* allele CYP2A6 \*2 (c.51G> A and 1799T> A) [] (c.479T> A, Yamano 1990, Hadidi 1997, Oscarson 1998), resulting in enzyme deactivation;
* allele CYP2A6 \*B2 (gene conversion adjacent to 3' region and -1031A> G) [] (Oscarson 1999b, Ariyoshi 2000, Pitarque 2004), a variant of the wild-type gene;
* allele CYP2A6 \*14 (51G> A and 86G> A) [] (Kiyotani 2002), whose functionality at phenotypic level has not yet been defined.

Combining different allelic variants, we divided the analyzed population into 8 different groups:

1. AA-AG-CC: \*2 not expressed, \*B2 heterozygous, \*14 not expressed;
2. AA-AA-CC: \*2 not expressed, \*B2 not expressed, \*14 not expressed;
3. AA-AG-CT: \*2 not expressed, \*B2 heterozygous, \*14 heterozygous;
4. AT-AG-CC: \*2 heterozygous, \*B2 heterozygous, \*14 not expressed;
5. AT-AA-CC: \*2 heterozygous, \*B2 not expressed, \*14 not expressed;
6. AA-GG-CC: \*2 not expressed, \*B2 homozygous, \*14 not expressed;
7. AT-AG-CT: \*2 heterozygous, \*B2 heterozygous, \*14 heterozygous;
8. AA-AA-CT: \*2 not expressed, \*B2 not expressed, \*14 heterozygous.

*Statistic analysis*

The Hardy-Weinberg equilibrium was verified with the χ2 test. Statistical analyses were performed using Genepop version 4.0 (http://wbiomed. curtin.edu.au/genepop) and SigmaStat version 3.1 (Jandel Corp., San Rafael, California). The distribution of allelic variants and their combinations was compared using the χ2 test. ANOVA (Fisher test) was used to analyze the differences in allelic expression between patients and control group, as well as in patients group according to different clinical features. We use Genetic Power Calculator to calculate the power of association of the study. According to the guidelines for genetic association studies, the level of statistical significance was set with *P* <0.01 []. Comparing clinical features, the level of statistical significance was set with *P* <0.05.

RESULTS

The population examined was not in Hardy-Weinberg equilibrium. Single allelic variants frequency in patients group compared to healthy people is showed in *Table 1*. There was no significant difference in the expression of the three SNPs between the two groups.

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| *Table 1. Allelic variants frequency between patient and control groups [n. (%)].* | | | | | | | |
|  | ***CYP2A6\*2*** | | ***CYP2A6\*B2*** | | | ***CYP2A6\*14*** | |
|  | **AA** | **AT** | **AA** | **AG** | **GG** | **CC** | **CT** |
| Patients | 318 (96,07) | 13 (3,93) | 44 (13,29) | 286 (86,40) | 1 (0,30) | 320 (96,68) | 11 (3,32) |
| Controls | 144 (96,00) | 6 (4,00) | 27 (18,00) | 121 (80,67) | 2 (1,33) | 146 (97,33) | 4 (2,67) |

Considering the 8 groups of allelic variants combinations, the frequency in case and control groups are shown in *Table 2*. No statistically significant differences were found between these groups, too. Of notice, AA-AG-CT and AT-AG-CC combinations (n°3 and n°4 respectively) are most represented in patients group (11 versus 1 in control group, for both combinations), although this difference has no statistical value.

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| *Table 2. Allelic variants combinations frequency in the analyzed population.* | | | |
| Combinations | **Patients [n. (%)]** | **Controls [n. (%)]** | **Total [n. (%)]** |
| *1 (AA-AG-CC)* | 264 (79.76) | 117 (78.0) | 381 (79.21) |
| *2 (AA-AA-CC)* | 42 (12.69) | 23 (15.33) | 65 (13.51) |
| *3 (AA-AG-CT)* | 11 (3.32) | 1 (0.67) | 12 (2.49) |
| *4 (AT-AG-CC)* | 11 (3.32) | 1 (0.67) | 12 (2.49) |
| *5 (AT-AA-CC)* | 2 (0.6) | 3 (2.0) | 5 (1.04) |
| *6 (AA-GG-CC)* | 1 (0.3) | 2 (1.33) | 3 (0.62) |
| *7 (AT-AG-CT)* | 0 (0) | 2 (1.33) | 2 (0.42) |
| *8 (AA-AA-CT)* | 0 (0) | 1 (0.67) | 1 (0.21) |

Frequencies of single polymorphisms and polymorphisms combinations related to the presence of clinical features are shown in Table 3. Considering single vs. multiple aneurysms, univariate analysis of the data regarding either single polymorphisms and polymorphisms combination did not found significant differences. Of notice is that all patients carrying the combination AT-AG-CC (11 patients) had a single aneurysm. Regarding clinical presentation with SAH, univariate data analysis showed no significant differences in the frequencies of single polymorphisms. Looking at polymorphisms combinations, instead, we found that all patients carrying AA-AG-CT combination (11 patients) had ruptured IA at diagnosis: this data is at the limit of statistical significance. In subjects with smoking habits the frequency of single polymorphisms, as well as polymorphisms combinations, was not significantly different compared to non-smokers in our study.

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| ***Table 3. Single polymorphisms and combinations frequencies among patients divided for clinical features [n. (%)].*** | | | | | | | |
|  | | **Single** | **Multiple** | **SAH** | **No SAH** | **Smoke** | **No smoke** |
| *CYP2A6 \*2* | ***AA*** | 265 (95,3) | 53 (100) | 246 (96,5) | 72 (94,7) | 83 (98,8) | 235 (95,1) |
| ***AT*** | 13 (4,7) | 0 (0) | 9 (3,5) | 4 (5,3) | 1 (1,2) | 12 (4,9) |
| *CYP2A6 \*B2* | ***AA*** | 35 (12,6) | 9 (17) | 34 (13,3) | 10 (13,2) | 10 (11,9) | 34 (13,8) |
| ***AG*** | 242 (87) | 44 (83) | 220 (86,3) | 66 (86,8) | 74 (88,1) | 212 (85,8) |
| ***GG*** | 1 (0,4) | 0 (0) | 1 (0,4) | 0 (0) | 0 (0) | 1 (0,4) |
| *CYP2A6 \*14* | ***CC*** | 268 (96,4) | 52 (98,1) | 244 (95,7) | 76 (100) | 80 (95,2) | 240 (97,2) |
| ***CT*** | 10 (3,6) | 1 (1,9) | 11 (4,3) | 0 (0) | 4 (4,8) | 7 (2,8) |
| ***1 (AA-AG-CC)*** | | 221 (66,77) | 43 (13,00) | 201 (60,72) | 63 (19,03) | 69 (20,85) | 195 (58,91) |
| ***2 (AA-AA-CC)*** | | 33 (9,97) | 9 (2,72) | 33 (9,97) | 9 (2,72) | 10 (3,02) | 32 (9,67) |
| ***3 (AA-AG-CT)*** | | 10 (3,02) | 1 (0,30) | 11 (3,32) | 0 (0) | 4 (1,21) | 7 (2,11) |
| ***4 (AT-AG-CC)*** | | 11 (3,32) | 0 (0) | 8 (2,41) | 3 (0,9) | 1 (0,30) | 10 (3,02) |
| ***5 (AT-AA-CC)*** | | 2 (0,60) | 0 (0) | 1 (0,30) | 1 (0,30) | 0 (0) | 2 (0,60) |
| ***6 (AA-GG-CC)*** | | 1 (0,30) | 0 (0) | 1 (0,30) | 0 (0) | 0 (0) | 1 (0,30) |

A strong correlation between smoking habits and aneurysm rupture emerges from our data (Table 4): 82 in 84 smoker patients with IA [*P = 0.9762* - 95% IC (0.916626, 0.997103); 99% IC (0.894267, 0.998761); Correct IC in terms of exact distribution] had SAH at clinical onset, while among 247 non-smoking patients with IA only 173 [*P = 0.7004* - 95% IC (0.639086, 0.756840); 99% CI (0.619900, 0.772984); Correct IC in terms of exact distribution] presented with SAH.

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| ***Table 4. Single polymorphisms and combinations correlation with IA rupture and smoking habits [n. (%)].*** | | | | | |
|  | | **Smoke + SAH** | **Smoke + No SAH** | **No smoke + SAH** | **No smoke + No SAH** |
| *CYP2A6 \*2* | ***AA*** | 81 (97,6) | 2 (2,4) | 165 (70,21) | 70 (29,79) |
| ***AT*** | 1 (100) | 0 | 8 (66,6) | 4 (33,3) |
| *CYP2A6 \*B2* | ***AA*** | 10 (100) | 0 | 24 (70,6) | 10 (29,4) |
| ***AG*** | 72 (97,3) | 2 (2,7) | 148 (69,8) | 64 (30,2) |
| ***GG*** | 0 | 0 | 1 (100) | 0 |
| *CYP2A6 \*14* | ***CC*** | 78(97,5) | 2 (2,5) | 166 (69,17) | 74 (30,83) |
| ***CT*** | 4(100) | 0 | 7 (100) | 0 |
| ***1 (AA-AG-CC)*** | | 67 (97,1) | 2 (2,9) | 134 (68,7) | 61 (31,3) |
| ***2 (AA-AA-CC)*** | | 10 (100) | 0 | 23 (71,9) | 9 (28,1) |
| ***3 (AA-AG-CT)*** | | 4 (100) | 0 | 7 (100) | 0 |
| ***4 (AT-AG-CC)*** | | 1 (100) | 0 | 7 (70,0) | 3 (30,0) |
| ***5 (AT-AA-CC)*** | | 0 | 0 | 1 (50,0) | 1 (50,0) |
| ***6 (AA-GG-CC)*** | | 0 | 0 | 1 (100) | 0 |

Single polymorphisms frequency within patients related to smoking habits and SAH appearance is shown in *Table 4*. We found that in patients carrying CYP2A6 \*2 AA polymorphism the probability of aneurysm rupture was statistically significant if related to smoking habit: in smokers the bleeding cases were 81 out of 83 [p = 0.975904 - 99% IC (0.893049, 0.998746)], while in non-smokers they were 165 out of 235 [p = 0.702128 - 99% IC (0.619556, 0.776294)]. Also in patients presenting CYP2A6 \*B2 AG polymorphism we found significant differences in SAH occurring: in this group smokers have a probability of IA rupture of 97.3% [72 of 74 ; p = 0.972973 - 99% IC (0.880677, 0.998593)], while non-smokers of 69.8% [148 out of 212; p = 0.698113 - 99% IC (0.610682, 0.776407)]. Finally, in our study patients with CYP2A6 \*14 CC polymorphism were found to present with SAH in 78 of 80 cases if smokers [p = 0.975000 - 99% IC (0.889220, 0.998699)], while if non-smokers only 166 of 240 patients had SAH [p = 0.691667 - 99% IC (0.609495, 0.766039)]. No significant differences in the probability of bleeding were found for the other polymorphisms analyzed. However, all patients in our cohort carrying CYP2A6 \*14 CT polymorphism had aneurysm rupture, regardless of the smoking habit.

Examining the bleeding rate of smokers based on polymorphisms combinations (Table 4), we detected a significant relation only in the group of patients presenting the AA-AG-CC combination. In this group, in fact, we found 67 cases of SAH in 69 smokers [p = 0.971014 - 95% IC (0.899185, 0.996470); 99% IC (0.872483, 0.998490)], compared to 134 cases of bleeding on 195 non-smokers patients [p = 0.687179 - 95% IC (0.617047, 0.751515); 99% IC (0.595223, 0.769748)]. Analyzing patients carrying polymorphisms combinations different from AA-AG-CC, no significant differences in bleeding occurrence were found based on smoking habit: on 52 patients with genetic combinations different from AA- AG-CC and non-smokers, 39 had a history of SAH [p = 0.750000 - 95% CI (0.610536, 0.859651], while all the patients (15 subjects) with non-AA-AG-CC combination and smokers had IA rupture [p = 1.000000 - 95% IC (0.818964, 1.000000)]. As previously described the only group in which 100% of bleeding cases occur, regardless of the smoking factor, is the one presenting the AA-AG-CT polymorphisms combination.

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| ***Table 5- χ2 test for variables “bleeding” and “smoking” in AA-AG-CC polymorphisms combination group.*** | | | | |
|  | **No SAH** | **SAH** | **Tot.** |  |
| **No smoke** | 61  (31,28)  46,53 | 134  (68,72)  148,47 | 195  (100,00) |
| **Smoke** | 2  (2,90)  16,47 | 67  (97,10)  52,53 | 69  (100,00) | *Pearson = 22,599*  *DF = 1; P-value = 0,000* |
| **Tot.** | 63  (23,86) | 201  (76,14) | 264  (100,00) | *Likelihood Ratio = 29,710*  *DF = 1; P-value = 0,000* |

DISCUSSION

The primary aim of our study was to find whether polymorphisms of the CYP2A6 gene, involved in nicotine metabolism, are related to intracranial aneurysms formation and evolution toward rupture in the presence of smoking habits.

The frequency of gene polymorphisms within the whole population enrolled in our study is in line with the results published in the literature [] (Http: //www.ncbi.nlm.nih. gov / SNP /).

We found no significant differences in single polymorphisms and polymorphisms combinations frequency between the IA patients and healthy controls. In our series, we noticed that AA-AG-CT and AT-AG-CC combinations, although rare, are more represented in patients group than in control group. Therefore, we suggest that CYP2A6 genetic pattern alone, limited to the examined polymorphisms, does not affect the formation of intracranial aneurysms according to our series. Since the CYP2A6 gene is mainly responsible for nicotine metabolism, there may be no direct association between its polymorphisms and the development of IA independently of smoking factor. Since no other studies about this relationship can be found in the current literature, further investigation expanding patients' series is needed to assess this statement.

Approximately 16% of our patients (53 out of 331) carry multiple aneurysms: these results are in line with those reported in the literature for sporadic multiple aneurysms [] (Pleizier et al. 2002, Crobeddu et al. 2014, Zhao et al. 2014). We found that the number of IA is not influenced by smoking habit or by the analyzed CYP2A6 genetic pattern, nor by the combination of these two. This data is in contrast with some studies demonstrating how chronic cigarette smoking appears to be one of the main risk factors for multiple IA appearance (Ho et al. 2015 []), together with the female sex (Qureshi et al. 1998 []) and age between 30 and 60 years-old (Juvela 2000 []). In all these studies smoking habit is defined as daily consumption, regardless of the number of cigarettes smoked per day, while the "non-smoker" have never smoked in the course of life. The difference between this definition and ours, considering as “non-smokers” also subjects who habitually smoke less than 10 cigarettes per day, could in part explain the differences between our results and the evidence in the literature.

In this series, 77% of subjects with IA had aSAH at onset (255 out of 331): this is reasonable considering that our subjects have been recruited within a Neurosurgery Department. Univariate analysis showed no significant differences in the incidence of IA rupture based on CYP2A6 single polymorphisms, nor on polymorphisms combinations. However, it is worth noting that the frequency of AA-AG-CT combination in patients group appears to be higher than in control group, albeit not significantly. Moreover, all of these patients presented with aSAH at onset, regardless of smoking habit, data placed at the limit of statistical significance. This fact relates to the observation that all subjects carrying CYP2A6 \*14 CT polymorphism (heterozygous allele) had aneurysm rupture regardless of smoking factor, as previously described in this article. Due to a lack of literature data regarding the phenotypic correspondence of CYP2A6 \*14 allele, it is not currently possible to make hypotheses on what could be the enzymatic activity in these subjects. However, since this association appears to be independent of smoking habit, the possibility that alternative metabolic pathways are involved cannot be excluded. It would also be possible that this allelic variant transcription results in a product with altered functionality that can trigger aneurysm rupture through an unknown mechanism. Further studies are needed, both to clarify the role of this allelic variant and to verify the correlation with IA rupture.

The incidence of smoking habit in our series is 25.4% (84 patients out of 331), slightly higher compared to that of the general population: according to 2012 WHO data, cigarette smoking prevalence was 19,8% in the Italian population ≥ 15 years-old, while 21% in the world population. Unfortunately, it was not possible to collect data on cigarette smoking in the control group. In the univariate analysis, there was no significant correlation between smoking habit and the frequency of single polymorphisms, nor polymorphisms combinations. In literature, the correlation between CYP2A6 gene polymorphisms and smoking habits has already been studied: Pianezza et al. 1998 [] found that carrying null alleles (CYP2A6 \*2 and \*3), both in homozygosis or heterozygosis, was associated with a lower risk of cigarette smoke addiction if compared to the presence of wild-type gene. Numerous studies were subsequently conducted to confirm this data, with conflicting results. The main confounders in these studies are essentially related to the variability in the definition of smokers' subgroups, and to the social determinants that can influence the development of smoking habits. In 2015 a meta-analysis by Pan et al. [] confirmed the relationship between CYP2A6 alleles and the number of cigarettes smoked per day, and that individuals with intermediate nicotine metabolism begin the chronic smoking habit later than subjects with normal metabolism.

As previously showed in literature (Juvela et al. 2001, Krex et al. 2001, Feigin et al 2005, Korja et al 2014, Backes et al. 2016) [], our data confirm that smoking habit is a risk factor for subarachnoid hemorrhage event from aneurysm rupture, independently of the CYP2A6 genetic pattern: in our series, 82 of 84 smokers patients (97.62%) with IA had subarachnoid hemorrhage, compared to 173 out of 247 (70.05%) non-smokers. Studies at histological and molecular level affirmed the key role of vascular wall chronic inflammation in the formation and evolution of IA: they found a greater infiltrate of macrophages and T -cells, augmented levels of pro-inflammatory products, cellular degeneration (endothelial damage, loss of wall cells, apoptosis) and an accumulation of oxidized lipids compared to the wall of non-ruptured aneurysms (Tulamo et al. 2010, Frösen et al. 2012) []. Cigarette smoke is a long known inducer of vascular inflammation: it contains numerous substances, nicotine in particular, that are able to increase the expression of pro-inflammatory molecules and subsequently induce the expression of endothelial adhesion molecules favoring chemotaxis and leukocyte infiltration in the vascular wall by VCAM-1 (Vascular Cell Adhesion Molecule 1), MCP-1 (Monocyte Chemotactic Protein 1) and IL-8 (Csordas et al . 2013, Norman et al. 2013) []. Oxidative stress induced by ROS (Reactive Oxygen Species), deriving directly from cigarette smoke or produced by endothelial cells exposed to smoke, also contributes to the expression of adhesion molecules, the release of pro-inflammatory cytokines and endothelial dysfunction by activating the transcription factor NF-κB. Furthermore, cigarette smoke, both directly and indirectly through endothelial damage, causes dysfunction in the coagulation system with the appearance of endoluminal thrombus at the aneurysm. Finally, the smoke acts by weakening the arterial wall by activating matrix metalloproteases (in particular MMP-2 and MMP-9) at the level of smooth muscle cells and endothelial cells, and by inhibiting the synthesis of collagen I and III (Messner et al. 2014) [].

Significant correlations with single CYP2A6 polymorphisms emerged from the multivariate analysis of smoking habits and aneurysm bleeding. For CYP2A6 \*B2 AG polymorphism (wild-type-analogous allele in heterozygosity), CYP2A6 \*14 CC polymorphism (absent allele) and CYP2A6 \*2 AA polymorphism (no null allele) the risk of aneurysm rupture is significantly greater in smokers than in non-smokers.

The significant association between IA rupture and smoking habit in the subgroup carrying AA-AG-CC combination, that is also the most represented in patients with IA, can explain the strong association found globally between smoking and bleeding. This hypothesis is supported by the observation that the subgroups with other-than-AA-AG-CC combinations have no significant correlation with IA rupture based on smoking habit. This association between genetic patterns and bleeding risk in smokers could simply be interpreted as a consequence of insufficient sample size. Alternatively, the presence of AA-AG-CC combination can affect cigarette smoking action. According to CYP2A6 alleles expressed, the population can be divided into normal (100% enzyme activity), intermediate (50-75%) and slow (0-50%) metabolizers []. In this case, the absence of the null allele CYP2A6 \*2 (AA) and of the CYP2A6 \*14 allele (CC), joint to the presence of the CYP2A6 \*B2 allele in heterozygosity (AG), could configure a phenotype with 100% enzymatic activity (rapid metabolizer). The hypothesis is that subjects with genetic patterns leading to normal nicotine metabolism have a greater capacity to trigger oxidative processes in the presence of smoking habits if compared to subjects with decreased enzymatic activity. This would lead to greater production of ROS (reactive oxygen species), which triggers the chronic inflammatory processes underlying aneurysms rupture.

An alternative but parallel explanation starts from the observations of Pianezza et al. 1998 [], according to which a genetic pattern producing a normal enzymatic activity is associated with an increased risk of addiction to cigarette smoke if compared to subjects carrying null alleles. In the first case, there is increased nicotine intake with a subsequent increase in damage from cigarette smoke that is dose-dependent. Following this hypothesis, some studies tried to find an association between CYP2A6 allelic variants and the risk of developing smoke-related tumors: a meta-analysis of Liu et al. (2013) [] showed that the risk of developing lung carcinoma is reduced in subjects with smoking habits carrying a null allele (both in homozygosity or heterozygosity), if compared to wild-type gene carriers. The results of our study seem to be partially in line with this hypothesis. However, we found polymorphisms combinations corresponding to a fully active enzyme even in patients group not significantly associated with aneurysm rupture, and this contrast with that hypothesis. Moreover, our analysis focus only on three CYP2A6 polymorphisms, but we don’t test the presence of other polymorphisms that can determine different phenotypic characteristics.

The main limitation of our study is the lack of statistical power, although the conspicuous sample size considering the low incidence of IA and aSAH in the general population. We suggest that the study should be repeated on a larger sample, obtainable only through a multicentre work. A more accurate analysis could be obtained with a more detailed stratification of the smoking habit, also taking account of the exposure to passive smoking, the age at which subjects started the exposure, and previous smoking habits (ex- smoking subjects).

CONCLUSION

The mechanisms that determine the formation and evolution of IA toward rupture are not yet fully understood. Cigarette smoking is one of the risk factors that contribute in many ways to vascular damage, a fundamental point in the pathogenesis of the disease. The evidence of a greater predisposition to IA rupture in subjects with a close relative with a history of SAH suggests, even in so-called sporadic aneurysms, the possibility of genetic determinants involved in the development and evolution of the disease. In this context we developed our study, searching for an explanation that can unify the numerous pieces that the international scientific community has gathered so far. In all cases, abstinence from cigarette smoking has to be recommended to all patients presenting with an unbroken intracranial aneurysm, given the strong association between this voluptuous habit and SAH event. The possible presence of a genetic predisposition could, in the future, provide the basis for modeling the therapeutic approach in the single patient. Further studies are needed to examine the hypothesis that certain genetic patterns of CYP2A6 may affect the evolution of IA towards rupture.

REFERENCES

Alg V.S., Sofat R., Houlden H., Werring D.J., “Genetic risk factors for intracranial aneurysms: a meta-analysis in more than 116.000 individuals”. Neurology, 2013 (80: 2154-65).

Backes D., Rinkel G.J., Laban K.G., Algra A., Vergouwen M.D., “Patient- and Aneurysm-Specific Risk Factors for Intracranial Aneurysm Growth: Systematic Review and Meta-Analysis”. Stroke, 2016 (STROKEAHA.115.012162).

Benowitz N.L., Jacob III P., “Individual differences in nicotine kinetics and metabolism in human”. NIDA Res Monogr, 1997 (173: 48-64).

Benowitz N.L., Jacob P.I., Fong I., et al., “Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine”. J Pharmacol Exp Ther, 1994a (268: 296-303).

Benowitz N.L., Jacob III P., “Metabolism of nicotine to cotinine studied by a dual stable isotope method”. Clin Pharmacol Ther, 1994b (56: 483-93).

Benowitz N.L., Jacob III P., Jones R.T., Rosenberg J., “Interindividual variability in the metabolism and cardiovascular effects of nicotine in man”. J Pharmacol Exp Ther, 1982 (221:368-372).

Cashman J.R., Park S.B., Yang Z..C, Wrighton .S.A, Jacob P., Benowitz N.L., “Metabolism of nicotine by human liver microsomes: Stereoselective formation of trans-nicotine N’-oxide”. Chem Res Toxicol, 1992 (5: 639-646).

Celermajer D.S., Sorensen K.E., Georgakopoulos D. et al., “Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation of in healthy young adults”. Circulation, 1993 (88: 2149–2155).

Crobeddu E., Panciani P.P., Garbossa D., Pilloni G., Fornaro R., Ronchetti G., Spena G., Tartara F., Ducati A., Fontanella M., “Cerebrovascular diseases in the elderly: the challenge of multiple aneurysms”. Int J Neurosci 2014 (124 (8): 573-6).

Crompton M.R., “The pathology of ruptured middle cerebral aneurysms with special reference to the differences between the sexes”. Lancet, 1962 (2: 421-25).

Csordas A., Bernhard D., “The biology behind the atherothrombotic effects of cigarette smoke”. Nat Rev Cardiol, 2013 (10:219-230).

Daigo S., Takahashi Y., Fujieda M., Ariyoshi N., Yamazaki H., Koizumi W., Tanabe S., Saigenji K., Nagayama S., Ikeda K., Nishioka Y., Kamataki T., “A novel mutant allele of the CYP2A6 gene (CYP2A6\*11) found in a cancer patient who showed poor metabolic phenotype towards tegafur”. Pharmacogenetics, 2002 (12: 299-306).

Feigin V.L., Rinkel G.J., Lawes C.M., Algra A., Bennett D.A., van Gijn J., Anderson C.S., “Risk factors for subarachnoid hemorrhage: an updated systematic review of epidemiological studies”. Stroke, 2005 (36: 2773–2780).

Fernandez-Salguero P., Hoffman S. M. G., Cholerton S., Mohrenweiser H., Raunio H., Rautio A., Pelkonen O., Huang J., Evans W. E., Idle J. R., Gonzalez F. J., “A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of variant CYP2A6 alleles”. Am J Hum Genet, 1995 (57: 651-660).

Fields W.A., “Aortao cranial occlusive vascular disease (stroke)”. Ciba Found Symp, 1974 (26 (4): 3 – 31).

Frösen J., Tulamo R., Paetau A., Laaksamo E., Korja M., Laakso A., Niemelä M., Hernesniemi J., “Saccular intracranial aneurysm: pathology and mechanisms”. Acta Neuropathol, 2012 (123:773-786).

Gu D. F., Hinks L. J., Morton N. E., Day I.N.M., “The use of long PCR to confirm three common alleles at the CYP2A6 locus and the relationship between genotype and smoking habit”. Ann Hum Genet, 2000 (64: 383-390).

Hanna S.T., “Nicotine effect on cardiovascular system and ion channels”. J Cardiovasc Pharmacol, 2006 (47: 348-58).

Ho A.L., Lin N., Frerichs K.U., Du R., “Smoking and Intracranial Aneurysm Morphology”. Neurosurgery, 2015 (77 (1): 59-66).

Ho M.K., Tyndale R.F., “Overview of the pharmacogenomics of cigarette smoking”. Pharmacogenomic J, 2007 (7: 81-98).

Iida M., Iida H., Dohi S. et al., “Mechanisms underlying cardio-vascular effects of cigarette smoking in rats in vivo”. Stroke, 1998 (29: 1656–1665).

Inoue K., Yamazaki H., Shimada T., “CYP2A6 genetic polymorphisms and liver microsomal cumarin and nicotine oxidation activities in Japanese and Caucasians”. Arch Toxicol, 2000 (73: 532-539).

Juvela S., Hillbom M., Numminen H., Koskinen P., “Cigarette smoking and alcohol consumption as risk factors for aneurysmal subarachnoid hemorrhage”. Stroke, 1993 (24: 639–646).

Juvela S., Porras M., Poussa K., “Natural history of unruptured intracranial aneurysms: probability and risk factors for aneurysm rupture”. Neurosurg Focus, 2000 (8: 1–9).

Juvela S., Poussa K., Porras M., “Factors affecting formation and growth of intracranial aneurysms: a long-term follow-up study”. Stroke, 2001 (32: 485-91).

Juvela S., “Prehemorrhage risk factors for fatal intracranial aneurysm rupture”. Stroke, 2003 (34: 1852–1857).

Juvela S., “Risk factors for multiple intracranial aneurysms”. Stroke, 2000 (31(2): 392-7).

Kitawa K., Kumgita N., Katoh T., Yang M., Kawamoto T., “The significance of the homozygous CYP2A6 deletion on nicotine metabolism: A new genotyping method of CYP2A6 using a single PCR-RFLP.” Biochem Biophys Res Commun, 1999 (262: 146-151).

Korja M., Kaprio J., “Controversies in epidemiology of intracranial aneurysms and SAH”. Nat Rev Neurol, 2016 (12(1): 50-5).

Korja M., Lehto H., Juvela S., “Lifelong rupture risk of intracranial aneurysms depends on risk factors: a prospective Finnish cohort study”. Stroke, 2014 (45(7):1958-63).

Krex D., Schackert H., Shackert G., “Genesis of cerbral aneurysms—an update”. Acta Neurochir, 2001 (143: 429–449).

Liu Y.L., Xu Y., Li F., Chen H., Guo S.L., “CYP2A6 deletion polymorphism is associated with decreased susceptibility of lung cancer in Asian smokers: a meta-analysis". Tumour Biol, 2013 (34(5): 2651-7).

London S. J., Idle J. R., Daly A. K., Coetzee G. A., “Genetic variation of CYP2A6, smoking, and risk of cancer”. Lancet, 1999 (353: 898-899).

McMorrow M.J., Foxx R.M., “Nicotine's role in smoking: an analysis of nicotine regulation”. Psychol Bull, 1983 (93: 302-327).

Messina E.S., Tyndale R.F., Sellers E.M., “A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes”. J Pharmacol Exp Ther, 1997 (282: 1608-1614).

Messner B., Bernhard D., “Smoking and cardiovascular disease. Mechanisms of endothelial dysfunction and early atherogenesis”. Arterioscler Thromb Vasc Biol, 2014 (doi: 10.1161/ atvbaha.113.300156).

Miyamoto M., Umetsu Y., Dosaka-Akita H., Sawamura Y., Yokota J., Kunitoh H., Nemoto N., Sato K., Ariyoshi N., Kamataki T., “CYP2A6 gene deletion reduces susceptibility to lung cancer”. Biochem Biophys Res Commun, 1999 (261: 658-660).

Murphy S.E., Jhonson L.M., Pullo D.A., “Characterization of multiple products of cytochrome P450 2A6-catalyzed cotinine metabolism”. Chem Res Toxicol, 1999 (12: 639-645).

Naik P., Fofaria N., Prasad S., Sajja R.K., Weksler B., Couraud P.O., Romero I.A., Cucullo L., “Oxidative and pro-inflammatory impact of regular and denicotinized cigarettes on blood brain barrier endothelial cells: is smoking reduced or nicotine-free products really safe?”. BMC Neurosci, 2014 (doi: 10.1186/1471-2202-15-51).

Nakajima M., Yamamoto T., Numoya K., Yokoi T. et al., “Characterization of CYP2A6 involved in 3’-idroxylation of cotinine in human liver microsomes”. J Pharmacol Exp Ther, 1996a (277: 1010-1015).

Nakajima M., Yamamoto T., Numoya K., Yokoi T. et al., “Role of human cytochrome P450-2A6 in C-oxidation of nicotine”. Drug Metab Dispos, 1996b (24: 1212-1217).

Norman P.E., Curci J.A., “Understanding the effects of tobacco smoke on the pathogenesis of aortic aneurysm”. Arterioscler Thromb Vasc Biol, 2013 (33:1473-1477).

Oscarson M., McLellan R. A., Asp V., Ledesma M., Ruiz M. L. B., Sinues B., Rautio A., Ingelman-Sundberg M., “Characterization of a novel CYP2A7/CYP2A6 hybrid allele (CYP2A6\*12) that causes reduced CYP2A6 activity”. Hum Mutat, 2002 (20: 275-283).

Oscarson M., McLellan R. A., Gullsten H., Yue Q.-Y., Lang M. A., Bernal M. L., Sinues B., Hirvonen A., Raunio H., Pelkonen O., Ingelman-Sundberg M., “Characterisation and PCR-based detection of a CYP2A6 gene deletion found at a high frequency in a Chinese population”. FEBS Lett, 1999 (448: 105-110).

Pan L., Yang X., Li S., Jia C., “Association of CYP2A6 gene polymorphisms with cigarette consumption: a meta-analysis”. Drug Alcohol Depend, 2015 (1 (149): 268-71).

Pianezza M. L., Sellers E. M., Tyndale R. F., “Nicotine metabolism defect reduces smoking”. Nature, 1998 (393: 750 only).

Qureshi A.I., Suarez J.I., Parekh P.D., Sung G., Geocadin R., Bhardwaj A., Tamargo R.J., Ulatowski J.A., “Risk factors for multiple intracranial aneurysms”. Neurosurgery, 1998 (43(1): 22-6).

Rubinstein I., Young T., Rennerad S.I. et al., “Cigarette smoke extract attenuates endothelium-dependent arteriolar dilatation in vivo”. Am J Physiol, 1991(261: H1913–H1918).

Schievink W.I., “Genetics and aneurysm formation”. Neurosurg Clin N Am, 1998 (9:485-95).

Stehbens W.E., “Etiology of intracranial berry aneurysms”. J Neurosurg, 1989 (70(6): 823-31).

Tromp G., Weinsheimer S., Ronkainen A., Kuivaniemi H., “Molecular basis and genetic predisposition to intracranial aneurysm”. Ann Med, 2014 (46: 597-606).

Tulamo R., Frösen J., Hernesniemi J., Niemelä M., “Inflammatory changes in the aneurysm wall: a review”. J Neurointerv Surg, 2010 (2:120-130).

Vlak M.H., Algra A., Brandenburg R., Rinkel G.J., “Prevalence of unruptured intracranial aneurysms, with emphasis on sex, age, comorbidity, country, and time period: a systematic review and meta-analysis”. Lancet Neurol, 2011 (10(7): 626).

Yamazaki H., Inoue K., Hashimoto M., Shimada T., “Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes”. Arch Toxicol, 1999 (72: 65-70).

Zhang W., Edvinsson L., Lee T.J., “Mechanism of nicotine-induced relaxation in the porcine basilar artery”. J Pharmacol Exp Ther, 1998 (284: 790–797).

Zhao L, Zhang L, Zhang X, Li Z, Tian L, Wang YX., “An analysis of 1256 cases of sporadic ruptured cerebral aneurysm in a single Chinese institution”. PLoS One, 2014 (doi: 10.1371/0085668).