# Cigarette Smoking and Intracranial Aneurysms: A Pilot Analysis of SNPs in the CYP2A6 Gene in the Italian Population

# Cigarette Smoking and Intracranial Aneurysms: A Pilot Analysis of SNPs in the CYP2A6 Gene in the Italian Population

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■ BACKGROUND: **Cigarette smoking is a modifiable risk factor associated with formation and rupture of intracranial aneurysms (IAs). Cytochrome P450 2A6 (CYP2A6) is the main enzyme implied in catabolism of nicotine and xenobiotics, giving rise to oxidative stress products. Our study investigated the associations between specific single-nucleotide polymorphisms (SNPs) in the CYP2A6 gene and the presence of sporadic IAs in a cluster of Italian patients, as well as their rupture regarding cigarette smoking habit.**

■ METHODS: **Three hundred and thirty-one Italian patients with sporadic IAs were recruited in a single institution. We recorded data on clinical onset with subarachnoid hemorrhage (SAH) and smoking habit. Genetic analysis was performed with a standard procedure on peripheral blood samples: CYP2A6 *1B2, CYP2A6 *2, and CYP2A6 *14 SNPs were analyzed in the study group along with 150 healthy control subjects. Statistical analysis was conducted according to genetic association study guidelines.**

■ RESULTS: **In the patient cohort, the frequency of aSAH was significantly higher in current smokers ($P < 0.001$; OR = 17.45), regardless of the pattern of CYP2A6 SNPs. There was a correlation between IA rupture and cigarette smoking in patients with the heterozygous CYP2A6 *1B2 allele ($P < 0.001$; OR = 15.47). All patients carrying the heterozygous CYP2A6 *14 allele had an aSAH event (100%), regardless of smoking habit, although this correlation was not statistically significant ($P = 1$).**

■ CONCLUSIONS: **According to our findings, a cigarette smoker carrying a fully active CYP2A6 enzyme (heterozygous *1B2 allele) may have an increased risk of IA rupture compared to those with functionally less active variants: further investigation on a larger sample is needed to verify this result. The role of the heterozygous CYP2A6 *14 allele in aSAH is yet to be clarified.**

## INTRODUCTION

The natural history of sporadic intracranial aneurysms (IAs) has been a subject of research for many years: their development and evolution toward rupture is influenced by features of both the aneurysm and patient. Regarding the latter, female gender, increasing age, cigarette smoking, blood pressure, and family history of aneurysmal subarachnoid hemorrhage (aSAH) are the main risk factors that have been identified.[1-3]

**Key words**
- Cigarette smoking
- CYP2A6
- Cytochrome P450
- Intracranial aneurysms
- Subarachnoid hemorrhage

**Abbreviations and Acronyms**
**aSAH**: Aneurysmal subarachnoid hemorrhage
**CTA**: CT angiography
**CYP450**: Cytochrome P450
**DSA**: Digital subtraction angiography
**EDTA**: Ethylenediaminetetraacetic acid
**EtBr**: Ethidium bromide
**IA**: Intracranial aneurysm
**MMP**: Matrix metalloprotease
**OR**: Odds ratio
**PCR**: Polymerase chain reaction
**ROS**: Reactive oxygen species
**SNP**: Single nucleotide polymorphism
**TBE**: Tris-borate-EDTA

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The smoking rate in patients with aSAH is 2–3 times higher than in the general population; the risk of IA rupture in current smokers is dose-dependent[4] and nearly 6 times greater than in nonsmokers.[5] Cigarette smoke contains nearly 7000 different chemicals: at a vascular level, they are responsible for reduction of flow-mediated dilation (through nitric oxide production), induction of a pro-oxidative and proinflammatory environment, direct damage to the endothelium (leading to cell death), and an increase in proliferation and migration of smooth muscle cells.[6,7] In particular, nicotine, a tertiary amine, impairs flow-mediated dilation in cerebral arteries[8] and activates cerebral reward circuits causing smoking addiction.

As exogenous substances, smoke components are metabolized by the microsomal liver cytochrome P450 enzymatic system (CYP). Nicotine catabolism is triggered mainly by the CYP2A6 isoform,[9,10] whose highly polymorphic gene is located on chromosome 19q13.2. Allelic variants of this gene are associated with differences in enzyme activity.[11,12]

Our purpose was to determine if there are links between CYP2A6 gene single-nucleotide polymorphisms (SNPs) and the presence of single or multiple sporadic IAs, as well as their rupture related to smoking habit.

## METHODS

The study was approved by the Hospital Ethics Committee (POLI2012 protocol n. 01,283) and follows the 1964 Helsinki Declaration and its later amendments. Each participant provided written informed consent before the investigation.

### Patient Selection

We recruited 331 consecutive unrelated patients with IAs documented by brain CT-angiography (CTA) and/or digital subtraction angiography (DSA) admitted to our Neurosurgery Department in Brescia University Hospital within 10 years. The exclusion criteria were as follows: 1) death within 48 hours after diagnosis; 2) angiographically negative SAH (documented by one CTA and 2 DSAs); 3) inheritable disorders associated with IAs (e.g., autosomal dominant polycystic kidney disease, Marfan, Ehlers-Danlos type IV, neurofibromatosis type 1); 4) SAH due to rupture of other intracranial vascular malformations (arteriovenous malformations and dural arteriovenous fistulas). Patients with either single or multiple IAs were included in the study. The presence of aSAH at clinical onset was registered. Current smoking habit was defined as more than 10 cigarettes smoked per day for at least 10 years (i.e. $>$ 5 pack-years).

A control group of 150 people, selected from a cohort of healthy blood donors, was matched with the study group for sex, age, and geographical background (**Supplementary Table 1**). The inclusion criteria were as follows: 1) absence of IAs at neuroimaging (CTA and/or DSA); 2) age over 18 years.

### Genetic Analysis

A 2 mL blood sample was taken from each patient and stored in ethylenediaminetetraacetic acid (EDTA). DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN S.p.A., Milan).[13] Polymerase chain reaction (PCR) was performed according to standard methods.[14] PCR products were loaded on 2% tris-borate-EDTA (TBE) agarose gel and stained with ethidium bromide (EtBr). All genetic analyses were conducted in the Cytogenetics Laboratory of our University.

Among the most frequent CYP2A6 gene polymorphisms in Caucasian populations,[12,15] we chose to analyze the following.

1) allele CYP2A6 *2 - missense mutation 1799T $>$ A resulting in enzyme inactivation both in vivo and in vitro.[11,16-18]
2) allele CYP2A6 *1B2 - a 58 bp gene conversion with CYP2A7 in the CYP2A6 3′ untranslated region and 1031A $>$ G, resulting in a variant of the wild-type gene.[19,20]
3) allele CYP2A6 *14 - missense mutation 86G $>$ A, which does not affect enzymatic activity.[21,22]

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

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

### Statistical Analysis

The Hardy–Weinberg equilibrium was verified with the $\chi^2$ test. We performed statistical analyses 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 assessed using Fisher's Exact Test. The univariate analysis was adopted to assess differences in allelic distribution between the study and control groups and to study the allelic distribution among chosen subgroups in the patient population. Multivariate analysis assessed the combination of smoking and aSAH variables with different allelic groups in the patient cohort. The odds ratio (OR) was calculated to deliver an effect size where there was a statistically significant association. We used the Genetic Power Calculator to calculate the power of association of the study. The level of statistical significance was set at $P < 0.05$.

## RESULTS

### Population Background and Genetic Distribution Analysis

Most of the patients in the study group had a single IA (278 pts, 84%) and presented with SAH at diagnosis (255 pts, 77%). Only 84 patients (25.4%) with IA were current smokers. Figure 1 shows the frequency of aSAH in current smokers and nonsmokers. Among current smokers with IA, 82 of 84 (98%) had aSAH at clinical onset, whereas 173 of 247 (70%) nonsmoking IA patients presented with SAH ($P < 0.001$; OR=17.45). Table 1 and Table 2 show the frequency of single allelic variants and the frequency of different haplotypes in the study group compared to the control group: there was no significant difference in the frequency of SNPs or the expression of haplotypes between the 2 groups.

### Clinical-Genetic Correlation Analysis

The frequencies of SNPs and haplotypes related to the clinical features are shown in Table 3. Considering single versus multiple aneurysms and current smokers versus non-smokers, univariate analysis did not show significant differences in either SNPs or haplotypes. Of note, all patients carrying the AT-AG-CC combination (11 patients) had a single IA. Regarding the presentation with SAH, univariate analysis showed no significant differences in the frequencies of SNPs. However, looking at haplotypes, all patients carrying the AA-AG-CT combination (11 patients) had a ruptured IA at diagnosis, although this data are at the limit of statistical significance ($P = 0.075$).

Table 4 shows the frequency of SNPs related to cigarette smoking habit and aSAH. In our series, the probability of IA rupture was significantly related to current smoking habit in patients carrying these SNPs.

- CYP2A6*2 AA: aSAH presentations were seen in 81 of 83 current smokers (98%), while in nonsmokers they were seen in 165 of 235 (70% - $P < 0.001$; OR=17.09).
- CYP2A6*1B2 AG: smokers carrying this variant had a 97% of IA rupture (72/74), versus 70% (148/212) in nonsmokers ($P < 0.001$; OR=15.47).
- CYP2A6*14 CC: smokers presented with aSAH in 78 of 80 cases (97%), while nonsmokers had 166 bleeding events out of 240 cases (69% - $P < 0.001$; OR=17.29).

No significant differences in the probability of bleeding were found for the other polymorphisms analyzed. Of note, 100% of patients carrying the CYP2A6*14 CT polymorphism (11 patients) had IA rupture regardless of smoking habit, although the correlation was not statistically significant ($P = 1$).

As for the bleeding rate of current smokers based on haplotypes, we found a significant correlation in the group of patients carrying the AA-AG-CC combination: in this group, aSAH at presentation occurred in 67 of 69 (97%) patients, compared to 134 of 195 in nonsmokers with the same haplotype (69% - $P < 0.001$; OR=15.25). No significant differences in aSAH occurrence were found in other haplotype groups based on cigarette smoking habit.

## DISCUSSION

In this study, we investigated the relationship between 3 CYP2A6 gene SNPs, cigarette smoking habit, and IA rupture. The

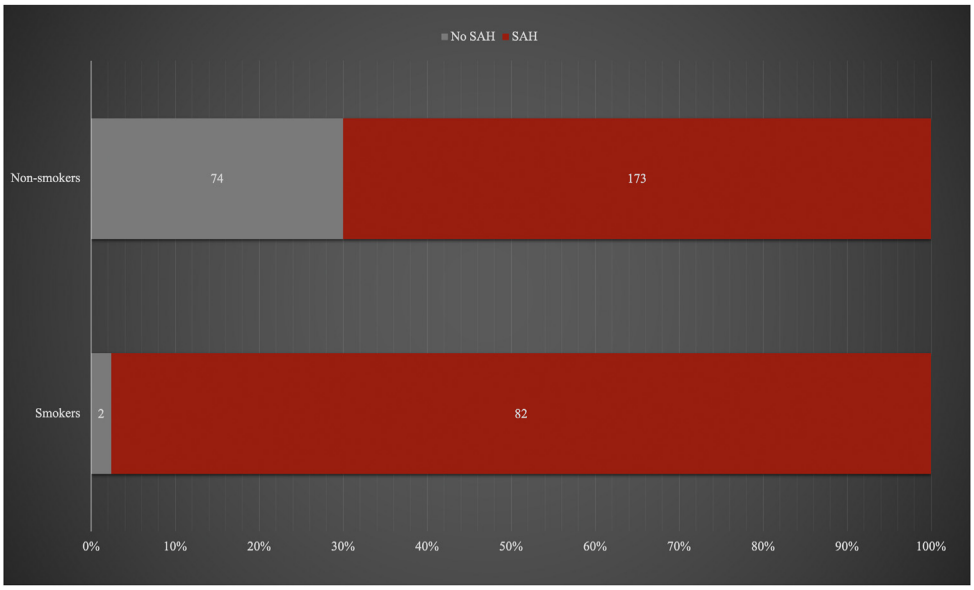


**Figure 1.** Frequency of aSAH in smokers and non-smokers.

**Table 1.** Allelic Variants Frequency in Case and Control Groups [n. (%)]

| | CYP2A6*2 | | CYP2A6*1B2 | | | CYP2A6*14 | |
|---|---|---|---|---|---|---|---|
| | AA | At | AA | AG | GG | CC | CT |
| Cases | 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) |

heterozygous CYP2A6 *1B2 genetic variation coding for a fully active CYP2A6 enzyme was significantly related to IA rupture.

## Population Background and Genetic Distribution Analysis

***Smoke and aSAH Prevalence.*** The prevalence of smoking habit in our series was 25.4% (84/331), which is slightly higher than in the general Italian population according to the most recent World Health Organization (WHO) data.[23] In our series, 82 of 84 smokers (98%) with IA had SAH, compared to 173 of 247 (70%) nonsmokers, rendering cigarette smoking as a plausible risk factor for aSAH regardless of the pattern of CYP2A6 SNPs, as previously reported in the literature.[24-28] Many studies focusing on histological and molecular patterns reported that cigarette smoking is an inducer of vascular inflammation, reactive oxygen species (ROS), thrombogenesis, and matrix metalloproteases (MMP-2 and MMP-9), all factors potentially contributing to rupture of an aneurysm.[7,29-32]

***CYP2A6 SNP Distribution.*** The frequency of the SNPs studied in the population in our study is in line with the results published in the literature.[33] The CYP2A6 pattern limited to the examined SNPs did not strongly correlate with the distribution of IAs. The AA-AG-CT and AT-AG-CC combinations were more represented in patients than in controls, although their presence was rare. Since CYP2A6 is notoriously responsible for nicotine metabolism, there may be no direct association between its polymorphisms and the development of IAs independently of smoking habit. However, further investigation in a larger series is needed to validate this statement.

## Clinical-Genetic Correlations

***Multiple IA Frequency.*** Approximately 16% of our patients (53 out of 331) carried multiple IAs in line with literature reports for sporadic multiple IAs.[34-36] The number of IAs was not influenced by smoking habit, genetic patterns of CYP2A6, or by their combination. Conversely, some studies have demonstrated that chronic cigarette smoking[37] is one of the main risk factors for multiple IAs together with female sex[38] and age from 30 to 60 years old.[39] However, these studies defined smoking habit as daily consumption regardless of the number of cigarettes smoked per day, whereas we considered subjects habitually smoking less than 10 cigarettes per day as "nonsmokers." This difference could in part explain the discrepancy between our results and previous studies.

***aSAH Frequency.*** In our series, 77% of patients with IA (255 of 331) presented with aSAH: this may be influenced by the recruitment within a Neurosurgery Department (bedside bias). Univariate analysis showed no significant differences in the incidence of IA rupture based on CYP2A6 SNPs or haplotypes. However, it is worth noting that all the patients carrying the CYP2A6*14 CT SNP (heterozygous allele) or the related haplotype AA-AG-CT had a hemorrhagic event regardless of smoking habit. Although this haplotype does not seem to affect enzymatic activity,[21,22] it could trigger aneurysm rupture through an unknown mechanism, regardless of smoking habit. Further studies are needed to clarify the role of this allelic variant and to verify a possible correlation with IA rupture.

***Cigarette Smoking Frequency.*** Univariate analysis showed no significant correlation between smoking habit and the frequency of SNPs or haplotypes. In 1998, Pianezza et al. found that carrying

**Table 2.** Haplotypes Frequency in Case and Control Groups [n. (%)]

| Haplotypes | Cases | Controls | Total |
|---|---|---|---|
| 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) |

**Table 3.** Frequencies of SNPs and Haplotypes Among Patients Divided for Clinical Features [n. (%)]

| | Single IA | Multiple IAs | SAH | No SAH | Smokers | Nonsmokers |
|---|---|---|---|---|---|---|
| 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*1B2 | | | | | | |
| 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) |

null alleles (CYP2A6*2 and *3), both in homozygosis and heterozygosis, was associated with a lower risk of cigarette smoking addiction compared to the presence of the wild-type gene.[40] Numerous studies were subsequently conducted to confirm this data with conflicting results. In 2015, a meta-analysis by Pan et al. reported that individuals with intermediate nicotine metabolism begin a chronic smoking habit later than subjects with normal metabolism.[41]

**Table 4.** SNPs and Haplotypes Correlation With aSAH and Smoking Habit [n. (%)]. Statistically Significant Correlations are Underlined

| | Smokers W/SAH | Smokers w/No SAH | Nonsmokers W/SAH | Nonsmokers w/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*1B2 | | | | |
| 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 |

***Genetic Determination of aSAH in Smokers.*** Significant genetic correlations emerged from multivariate analysis of smoking habits and aSAH. For CYP2A6*2 AA, CYP2A6*1B2 AG, CYP2A6*14 CC SNPs, and their corresponding haplotypes, the risk of aneurysm rupture was significantly greater in current smokers than in nonsmokers. The strong association found between smoking and aSAH can be explained by the significant association between IA rupture and smoking habit in the subgroup carrying the AA-AG-CC haplotype, which was the most represented in the study group.

The absence of the null allele CYP2A6*2 AA and the CYP2A6 *14 CC allele, together with the presence of the CYP2A6*1B2 allele in heterozygosity (AG), could give rise to a phenotype with high enzymatic activity (rapid metabolizer). This could lead to greater production of ROS that trigger a chronic inflammatory processes underlying aneurysm rupture. A possible adjunctive explanation comes from the observations of Pianezza et al. in 1998, according to which a genetic pattern producing normal enzymatic activity is associated with an increased risk of addiction to cigarette smoking compared to subjects carrying null alleles.[40] The increased nicotine intake would subsequently lead to increased damage from cigarette smoking in a dose-dependent manner. Following this hypothesis, some authors reported an association between wild-type CYP2A6 and the risk of developing smoking-related tumors, such as lung cancer.[42] Likewise, a subsequent meta-analysis highlighted a lower risk of lung cancer for CYP2A6 whole-gene deletion SNP carriers.[43]

### Limitations

Our study has limitations inherent to single-center retrospective studies. The main limit is the poor statistical power, considering the low incidence of IA and aSAH in the general population. A dedicated and matched control cohort for each of the factors studied in the patient population could have added statistical significance to the results, avoiding the influence of other possible confounding factors. A more accurate analysis should be carried out on a larger sample with a more detailed stratification of smoking habit, also considering passive smoking exposure, the age at which smoking exposure began, and ex-smoking subjects. Moreover, our analysis focuses only on 3 CYP2A6 SNPs, and others may determine different phenotypic characteristics.

Nonetheless, our study is the first to investigate the role of specific CYP2A6 SNPs relating to the presence of single or multiple IAs and their rupture concerning smoking habit. It may serve as a starting point for future studies.

## CONCLUSION

Our data align with the well-known correlation between smoking habit and aSAH, regardless of the pattern of CYP2A6 SNPs. Cigarette smokers with the heterozygous *1B2 genetic variation, coding for a fully active CYP2A6 enzyme, may be at increased risk of IA rupture; however, additional studies on a larger sample is needed to verify this result. The enzymatic activity associated with the CYP2A6*14 allele and its interplay with IA rupture, independently of smoking habit, must be clarified. Understanding specific genetic predispositions could provide the basis for further individualized management of patients with IAs.

## CRediT AUTHORSHIP CONTRIBUTION STATEMENT

**Erika Ferrari**: Term, Investigation, Writing — Original Draft. **Claudio Cornali**: Term, Investigation, Writing — Original Draft. **Alessandro Fiorindi**: Resources, Writing — Review & Editing, Visualization. **Edoardo Agosti**: Validation, Writing — Review & Editing, Data Curation. **Salvatore Gallone**: Methodology, Software, Formal analysis, Resources. **Elisa Rubino**: Methodology, Software, Resources. **Francesco Ponzio**: Methodology, Software, Formal analysis. **Marco Maria Fontanella**: Conceptualization, Methodology, Validation. **Lucio De Maria**: Validation, Supervision, Project administration.

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## SUPPLEMENTARY DATA

**Supplementary Table 1.** Sex, age, and geographical background of patients and healthy subjects. Controls were matched to cases to obtain similar ratios for each matching parameter

| | Cases | Controls |
|---|---|---|
| Total | 331 | 150 |
| Matching Parameter | | |
| Sex | | |
| Male | 112 | 51 |
| Female | 219 | 99 |
| Age | | |
| ≥80 | 90 | 41 |
| 60–79 | 165 | 75 |
| 40–59 | 65 | 29 |
| 20–39 | 9 | 4 |
| <20 | 2 | 1 |
| Geographical Background | | |
| White | 205 | 93 |
| Hispanic/Latino | 79 | 36 |
| Black/African Americans | 36 | 16 |
| Asian | 11 | 5 |