Pannexin1 Single Nucleotide Polymorphism and Platelet Reactivity in a Cohort of Cardiovascular Patients

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ABSTRACT
Pannexin1 (Panx1), a membrane channel-forming protein permitting the passage of small-sized molecules, such as ATP, is expressed in human platelets. Recently, we showed that inhibiting Panx1 affects collagen-induced platelet aggregation but not aggregation triggered by other agonists. We also found that a single nucleotide polymorphism (SNP; rs1138800) in the Panx1 gene encoded for a gain-of-function channel (Panx1-400C) and was associated with enhanced collagen-induced platelet reactivity. Here, we assessed the association of this SNP with platelet reactivity in a cohort of 758 stable cardiovascular patients from the ADRIE study treated with aspirin and/or clopidogrel. We found that presence of the Panx1-400C allele was not associated with platelet reactivity in stable cardiovascular patients, irrespective of the platelet aggregation agonist used (collagen, ADP or arachidonic acid) or the anti-platelet drug regimen. Moreover, the Panx1-400A>C SNP did also not affect the re-occurrence of cardiac ischemic events in the same stable cardiovascular patient cohort.

INTRODUCTION
Given the risk of hemorrhagic events or recurrent ischemic events associated with the two most prescribed anti-platelet drugs (aspirin and clopidogrel) (Creager 1998), many efforts are made to find new regulators of platelet function. In this context, a new glycoprotein discovered in 2003 and called Pannexin1 (Panx1) has emerged as a potential target (Taylor et al. 2014; Molica et al. 2015).

Panx1 is a member of the small pannexin family of proteins that form membrane channels permitting the passage of ions and small molecules, such as ATP and ADP, from the intracellular to the extracellular space and inversely (Sosinsky et al. 2011; Penuela et al. 2014). Pannexins are produced in a cell type-specific fashion, with Panx1 being expressed in many tissues, including the vasculature and inflammatory cells (Lohman et al. 2012; Meens et al. 2015). Here, Panx1 has been found to promote ATP release, thus influencing several processes including neutrophil chemotaxis (Chen et al. 2006; Chen et al. 2010), activation of the caspase-1/IL-1β inflammasome (Pelegrin & Surprenant 2006; Pelegrin et al. 2008), or apoptotic cell clearance (Chekeni et al. 2010). Recently, human and mouse platelets were found to express Panx1 (Taylor et al. 2014; Molica et al. 2015). Given the crucial role of ATP and ADP in platelet function, Panx1 may play a role in platelet aggregation.

Interestingly, blocking Panx1 channel function with the FDA-approved drugs probenecid or mefloquine, or with a specific 10Panx1 peptide reduced collagen-induced aggregation of human platelets but not aggregation triggered by ADP or arachidonic acid (AA) (Molica et al. 2015). Similar results were obtained with platelets from Panx1-deficient mice. Finally, a mechanism was proposed in which GPVI receptor activation by collagen induced Src-mediated opening of Panx1 channels, ATP release and P2X1 purinergic receptor activation followed by Ca2+ influx into platelets promoting platelet aggregation (Molica et al. 2015). Src-mediated opening of Panx1 channels has also been implicated in
pathological depolarization of hippocampal neurons under ischemic conditions leading to neuronal cell death (Thompson et al. 2008; Weilinger et al. 2012). Interestingly, a peptide interfering with the SFK-interacting domain of Panx1 attenuated anoxic neuronal depolarization (Weilinger et al. 2012). This pharmacological strategy may not only prevent neuronal cell death upon stroke but may also be useful in the inhibition of arterial thrombosis.

Further support for a specific role of Panx1 in collagen-induced response of human platelets came from the fact that a single nucleotide polymorphism (SNP) in the Panx1 gene, Panx1-400A > C, was found to specifically affect collagen-induced reactivity of human platelets in a population of 96 healthy males (Molica et al. 2015). Moreover, in a small cohort of cardiovascular patients with platelet hyper- or hypo-reactivity while treated with aspirin (Zufferey et al. 2011), patients with a hyper-reactive phenotype were more frequently found to be homozygous for Panx1-400C, while heterozygous patients and patients homozygous for Panx1-400A more frequently displayed a hypo-reactive phenotype (Molica et al. 2015). The Panx1-400A > C SNP induces an amino-acid change from a Glutamine (Q) to a Histidine (H) at position 5 in the N-terminus of the Panx1 protein. When transfected in CHO cells, this SNP results in a gain-of-function Panx1 channel (Molica et al. 2015).

Altogether, these observations suggest that the gain-of-function channel encoded by Panx1-400A > C SNP is involved in the fine-tuning of platelet reactivity in healthy subjects. However, the phenotypic effect of this SNP in cardiovascular patients treated with various antiplatelet drugs remains to be investigated. In the present study, our goal was, therefore, to investigate a possible association between the Panx1-400A > C SNP and platelet reactivity in a cohort of 758 cardiovascular patients treated with aspirin and/or clopidogrel.

Material and methods

Study populations

The subjects in this study have been previously described in detail (Fontana et al. 2010). Briefly, the ADRIE study is a prospective study aiming to determine the association of platelet reactivity and recurrence of ischemic events in a stable population of cardiovascular patients treated with aspirin and/or clopidogrel during a follow-up of 3 years. Stable cardiovascular outpatients (n = 771) with symptomatic documented ischemic atherothrombotic disease such as coronary artery disease, ischemic cerebrovascular disease, and/or peripheral artery disease were included. The patients attended as outpatients at least 1 month after the last acute ischemic event and/or stenting and were recruited consecutively between June 2006 and December 2008 in the University Hospitals of Geneva, Switzerland (n = 416), Béziers, France (n = 327), and Montpellier, France (n = 28). Patients were treated with aspirin only (n = 223), clopidogrel only (n = 111), or both antiplatelet drugs (n = 437). During the follow-up, 15.6% had a recurrent major cardiovascular event (MACE), defined as acute myocardial infarction, unstable angina, hospitalization for revascularization, acute limb ischemia, ischemic stroke, transient ischemic attack, or cardiovascular death (Reny et al. 2012). The present study is a cross-sectional analysis of the ADRIE cohort.

Blood collection

Venous blood samples were collected as previously described (Fontana et al. 2010) in resting (>15 min) patients using a 21-gauge needle with no tourniquet, after an overnight fast, into tubes containing EDTA or lithium heparin or 0.105 M sodium citrate (1 vol/9 vol). Platelet aggregation was evaluated in citrated platelet-rich plasma adjusted to 250 × 10⁹ platelets/L with autologous platelet-poor plasma on an 8-channel aggregometer (TA-8V, SD Medical, Heillecourt, France) using one of the following agonists: 1 mM AA (Bio/Data Corp, Horsham, PA), 5 μM ADP (Sigma-Aldrich, Buchs, Switzerland), or 1 μg/mL Horm collagen (Nycomed, Linz, Austria) (Reny et al. 2012).

Phenotype-genotype association study

Seven-hundred and fifty-eight DNA samples out of the ADRIE patient cohort were available for genotyping. Polymerase chain reaction (PCR) was performed to amplify the relevant part of the Panx1 gene. Sequencing of PCR products was performed by Fasteris (Geneva, Switzerland). All sequences comprising parts of the Panx1 coding region were analyzed using the multiple sequence alignment program ClustalW to identify the Panx1 genotype. Allele frequency was assessed using the following formula: minor allele frequency = (WM +2 × MM)/((WW + WM + MM) × 2).

Statistical analysis

Data are reported as mean ± SD or as median and interquartile range (IQR). Categorical variables are expressed as percentages. Trends of platelet reactivity across genotype were tested with the non-parametric Kruskal–Wallis test for quantitative variables and the
Chi-square or Fisher’s exact test for qualitative variables. Statistical analysis was performed with the Stata 11.0 software package (Stata Corp, College Station, TX).

Results and discussion

The 758 patients of the ADRIE population were successfully genotyped for the Panx1-400A > C SNP (rs1138800). We found 421 (55.5%) homozygous for Panx1-400C, while 86 (11.3%) were homozygous for Panx1-400A and 251 (33.1%) were heterozygous. We first ensured the absence of any difference in the distribution of the Panx1-400A > C SNP with respect to geographical origin of the patients. Panx1-400C homozygosity was found in 196 patients from France and 219 from Switzerland representing, respectively, 58.8% and 52.4% of the patients in each country ($p = 0.18$). Of note, the ADRIE population is particularly interesting for testing the association of genetic variants and platelet reactivity in cardiovascular patients since the phenotype was performed in stable outpatients, thus circumventing confounding factors such as acute coronary events that can blur genotype-biological phenotype association (Fontana et al. 2010, 2011).

As the Panx1-400C affected collagen-induced platelet reactivity in healthy individuals (Molica et al. 2015), we paid particular attention to collagen-induced platelet reactivity in patients from the ADRIE population. We found no significant difference in collagen-induced platelet aggregation with respect to Panx1-400A > C SNP in patients with aspirin, clopidogrel or dual anti-platelet therapy (Table 1). In addition, we observed no association of Panx1-400A > C genotype and maximal aggregation response to other agonists such as ADP or AA (data not shown) with either anti-platelet drug treatment. Interestingly, we found that clopidogrel treatment (without aspirin) is associated with a significantly higher collagen-induced aggregation response compared with patients treated with aspirin alone in the ADRIE study (mean aggregation response 60.5%±16.3% versus 40.6%±16.2%, $p < 0.001$), in line with a previous study (Moshfegh et al. 2000), which is probably due to the fact that clopidogrel inhibits only one of the two ADP receptors while aspirin blunts the generation of thromboxane A2 (TxA2).

Since Panx1 channels are widely distributed in the vasculature, we also addressed the issue of the association of this Panx1 genetic variant and the recurrence of MACE in the ADRIE cohort. Seven patients out of 758 were missing for this latter analysis. Table 2 shows no association of the Panx1-400A > C genotype and recurrence of MACE during the 3-year follow-up.

Finally, as we previously observed a higher expression of homozygous Panx1-400C SNP among a subgroup of patients with platelet hyper-reactivity compared with hypo-reactivity (Molica et al. 2015), we tried to confirm this observation in the ADRIE population. We thus selected 26 out of the 223 ADRIE patients treated with aspirin only and with extreme platelet reactivity (13 hyper- and 13 hypo-reactive, as defined by the platelet reactivity index (PRI) (Zufferey et al. 2011), were studied). Table 3 shows no association between the presence of the Panx1-400C allele in the groups of patients.

We did not replicate the association between the Panx1-400A > C genetic variant and platelet reactivity that we found in healthy subjects (Molica et al. 2015) in this cohort of patients with various anti-platelet drug regimens. This is most likely due to the different populations studied; namely healthy unrelated Caucasian men aged from 18 to 40, non-smokers and who did not

### Table 1. Mean percentage of collagen induced-platelet aggregation according to Panx1-400A > C genotypes stratified by anti-platelet treatments.

| Anti-platelet treatment | Panx1-400A > C genotype | Mean | Standard deviation | Frequency | $p$ Value |
|-------------------------|-------------------------|------|--------------------|-----------|-----------|
| Aspirin                 | AA                      | 39.2 | 15.8               | 33        |           |
|                         | AC                      | 43.9 | 16.9               | 63        |           |
|                         | CC                      | 39.7 | 15.8               | 120       | 0.27      |
| Clopidogrel             | AA                      | 66.3 | 18.3               | 8         |           |
|                         | AC                      | 61.6 | 15.1               | 34        |           |
|                         | CC                      | 58.8 | 16.8               | 64        | 0.54      |
| Aspirin and clopidogrel | AA                      | 29.2 | 17.7               | 41        |           |
|                         | AC                      | 27.3 | 13.7               | 224       |           |
|                         | CC                      | 27.2 | 13.7               | 152       | 0.96      |

### Table 2. Association between recurrence of MACE in the ADRIE population and the Panx1-400A > C SNP.

| Recurrence of MACE | SNP   | No (%) | Yes (%) | $P$ Value |
|--------------------|-------|--------|---------|-----------|
|                    | AA    | 74 (12)| 11 (9)  |           |
|                    | AC    | 213 (34)| 38 (32) |           |
|                    | CC    | 346 (55)| 69 (58) | 0.67      |
take any medication during 10 d before the beginning of the study in the derivation study (Fontana et al. 2006; Molica et al. 2015) versus cardiovascular patients of the study in the derivation study (Fontana et al. 2006).

| PRI | AA and AC | CC | Total | Fisher's exact |
|-----|-----------|----|-------|---------------|
| High | 6 (1AA, 5AC) | 7 | 13 | 0.31 |
| Low  | 5 (3AA, 2AC) | 8 | 13 |     |

Finally, no association was found between \( Panx1-400C \) and MACE (Table 3) in cardiovascular patients. Here again, the different medications, together with traditional risk factors, may blur any functional effect of \( Panx1-400C \) on the occurrence of ischemic events.

Table 3. \( Panx1-400A > C \) SNP in 26 patients with extreme platelet reactivity (high or low PRI).

The limitations of this study include the fact that we investigated stable cardiovascular patients where platelet reactivity is not predictive of recurrence of ischemic events (Reny et al. 2012) and that patients with next-generation anti-P2Y12 inhibitors (prasugrel and ticagrelor) were not included. The impact of \( Panx1-400C \) in these patients remains unknown.

In conclusion, \( Panx1-400A > C \) (rs1138800) is not associated with modifications in platelet aggregation results using various agonists in a cohort of stable cardiovascular patients treated with various anti-platelet drugs (i.e., aspirin, clopidogrel, or both), neither does it affect the occurrence of cardiac ischemic events in the same stable cardiovascular patients.

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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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