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Increased clopidogrel response is associated with ABCC3 expression: A pilot study

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A B S T R A C T

Background: The aim of this study was investigate the relationship between ABCB1 and ABCC3 gene expressions in peripheral blood cells (PBC) and the response to clopidogrel in patients with coronary arterial disease (CAD).

Methods: Twenty-six male CAD patients (50–70 years) under treatment with clopidogrel (75 mg/day) for at least 5 days were selected. Blood samples were obtained to evaluate platelet reactivity and ABCB1 and ABCC3 mRNA expression. Platelet reactivity was measured in P2Y12 Reaction Units (PRU) using VerifyNow. RNA was extracted from PBC and mRNA levels were measured by qPCR, using GAPD as a reference gene.

Results: Platelet response to clopidogrel was categorized in to PRU quartiles. Individuals with PRU values within the first quartile (Q1, ≤151 units) were considered good responders, while those who had PRU within the fourth quartile (Q4, PRU > 260) were considered non-responders. ABCB3 was 1.7 times more expressed in Q4 than Q1 PRU group (p = 0.048). Moreover, CAD patients with low ABCB3 expression (Q1, ≤2.5 × 10−3) had higher probability to have a good response to clopidogrel (OR: 18.00, 95%CI: 1.90–169.99, p = 0.001). Univariate linear regression analysis demonstrated that low ABCB3 mRNA expression contributed with a reduction of 73 PRU in relation to the patients with expression value higher than 2.5 × 10−3 (p = 0.027). Neither ABCB1 mRNA levels nor clinical variables studied influenced PRU values.

Conclusions: Low ABCC3 mRNA expression in peripheral blood cells is associated with increased clopidogrel response, but further studies are needed to describe the functional relationship of clopidogrel with the ABC3.

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1. Introduction

Clopidogrel is a prodrug that requires hepatic biotransformation by cytochrome P450 to an active metabolite [1]. It is an antagonist of the platelet adenosine diphosphate receptor that prevents platelet aggregation [2]. Other important antiaggregation drug is aspirin (acetylsalicylic acid), that action is reduces the activation of platelets by irreversibly acetylating cyclooxygenase-1 (COX-1), and thereby reduces thromboxane A2 by platelets [3].

Treatment with clopidogrel in combination with aspirin can prevent recurrent ischaemic events after an acute coronary syndrome or a percutaneous coronary intervention [4]. However, the response to clopidogrel has substantial interpatient variability that has been associated with increased risk for recurrent cardiovascular events, including stent thrombosis [5,6].

This variability has been related, at least in part, to polymorphisms in genes involved in pharmacokinetic pathways that affects clopidogrel bioavailability and efficacy [7–9]. But other authors, suggest that variation in expression of ATP-binding cassette (ABC) membrane proteins, are a family of efflux transporters, that can limit intestinal absorption and intracellular concentrations of drugs [10,11]. Variants on genes encoding ABC transporters have been implicated in low drug efficacy and safety [12,13].

The ABCB1 is the first transporter membrane discovered in ABC family and it is responsible for the efflux of a broad range of different compounds including cytostatics, antiviral and lowering-cholesterol drugs [14]. However, the role of ABCB1 in the pharmacokinetic of clopidogrel is not well known. It has been shown that long exposure (12–48 h) to high concentrations of clopidogrel (> 10 μmol/l) down regulates ABCB1 in Caco-2 cells, indicating a possible relationship of this protein transport with clinical effectiveness of clopidogrel [7].
Another important member of ABC family is \textit{ABCC3} which plays an important role in the efflux of endogenous glucuronides and conjugated drug metabolites across cell membranes [15]. A recent study reported the detection of \textit{ABCC3} mRNA and protein expression in human platelets [16]. The authors suggested that \textit{ABCC3} and other ABC transporters may influence the transport of drugs on platelet membrane interfering with the drug availability and efficacy. The \textit{−211T>C} a variant located at the promoter region of the \textit{ABCC3} gene, that can influence their gene expression, was described to be associated with the clinical outcome of chemotherapy in lung cancer patients through alteration in the gene expression and consequently in the pharmacological response [17].

This study investigated the relationship between \textit{ABCB1} and \textit{ABCC3} mRNA expressions in peripheral blood cells (PBC) with clopidogrel response of patients with coronary arterial disease (CAD).

2. Subjects and methods

2.1. Subjects and clinical protocol

Twenty six, male individuals with CAD, aged 50 to 70 years, who underwent percutaneous coronary angioplasty (PCA) at the Hospital do Méxioeiro (Vigo, Spain) were enrolled in this study from July to December 2008. All individuals were under clopidogrel therapy (75 mg/day) at least for 5 days prior to PCA and received other medication such aspirin, betablockers, nitrates, statin and pump protons inhibitor.

Exclusion criteria: primary PCA; high bleeding risk; chronic renal failure; under thienopyridine, glycoprotein IIb/IIIa inhibitor or warfarin therapy; allergic reaction and also aspirin or clopidogrel contraindication.

Information on age, systolic and diastolic pressure, body mass index (BMI), diabetes, Dyslipidemia, tobacco smoking, family history of CAD, medications in use and time from last dose of clopidogrel (TLDC) were recorded. This study was approved by the local Ethics Committee and all subjects voluntarily provided written and informed consent in agreement with Helsinki declaration.

2.2. Determination of platelet reactivity

Platelet reactivity was evaluated before PCA by turbidimetric optical detection of platelet aggregation using the VerifyNow \textit{P2RY12} assay (Accumetrics, San Diego, USA) according to manufacturer’s instructions. Briefly, 2.0 ml of blood samples were drawn from an antecubital vein with 3.2% citrate tubes. After 30 min at room temperature, citrated-blood was transferred into standard cartridges containing fibrinogen-coated micro beads and 20 mmol/l of adenosine diphosphate an agonist of purinergic receptor P2Y, G-protein coupled, 12 (\textit{P2RY12}). Platelet aggregation occurs and the system converts luminosity transmittance of total blood in a result of platelet response to clopidogrel inhibition, this result is denominated \textit{P2RY12} reaction units (PRU).

2.3. \textit{ABCB1} and \textit{ABCC3} mRNA expressions

Total RNA was isolated from whole blood samples with the PAXGene Blood RNA system (PreAnalytiX, Hilden, Germany) by automated Qiacube system (Qiagen, Valencia, USA) following the manufacturer’s protocols including a RNase-free DNase (Qiagen, Valencia, CA, USA) step to remove genomic DNA. The quantity of RNA was measured using a Nanodrop ND-1000 Spectrophotometer to give the yield and a 260/280 ratio. Agilent Bioanalyzer Lab-on-a-chip RNA chips (Agilent Technologies, Waldbronn, Germany) were also used for each sample to check the quality by calculating RNA Integrity Number (RIN) scores, samples with RIN<5 were exclude. The RNA samples were stored at −80 °C until later cDNA conversion. Isolated RNA samples were converted to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, USA) in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, Foster City, USA) under conditions described by the supplier. cDNA samples were kept at −20 °C for further quantitative TaqMan® real-time PCR (qPCR) analysis. \textit{ABCB1} and \textit{ABCC3} mRNA levels were measured by qPCR using glyceraldehyde-3-phosphate dehydrogenase (GAPD) as a reference gene. Primers and probes sequences used for \textit{ABCB1}, \textit{ABCC3} and \textit{GAPD} mRNA expressions were provided as “Assay on demand” format (Applied Biosystems, Foster City, USA). qPCR assays was carried out in 96-well plates of a 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) using the following protocol: 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The relative quantification for each target gene was measured using a comparative Ct method using the 2$^{−\text{ΔΔCt}}$ formula, where \text{ΔΔCt} is the difference between the target gene Ct and the \textit{GAPD} Ct [18].

2.4. Statistical analysis

Statistical analyses were carried out using SPSS 15.0 for windows (SPSS, Chicago, USA). Categorical variables were compared by Chi-square test or Exact Fisher test and continuous variables were compared by Kruskal–Wallis test and t-test or Mann–Whitney test. Platelet aggregation response to clopidogrel and \textit{ABCB1} and \textit{ABCC3} mRNA expression values were categorized in quartiles for statistic comparison (Q1, Q2, Q3 and Q4 for PRU; and Qe1, Qe2, Qe3 and Qe4 for mRNA expression).

Univariate logistic regression analysis was performed to evaluate the variables associated with low mRNA expression levels of \textit{ABCC3} (model 1) and \textit{ABCB1} (model 2). Low mRNA expression was considered Qe1 group that was compared with the remaining groups (Qe2 + Qe3 + Qe4). In both models, the highest response to clopidogrel (Q1, PRU<151), age, BMI, hypertension, tobacco smoking, family history of CAD, and TLDC were introduced as independent variables. Diabetes and dyslipidemia had null value in one of the categorical groups, therefore, they were not included as independent variables in logistic regression analysis. Multivariate stepwise logistic regression was performed to evaluate the independent variables associated with low \textit{ABCC3} mRNA expression, including age, BMI, TLDC and good response clopidogrel (Q1, PRU<151), as covariates.

Univariate linear regression was performed the relationship between PRU values and other variables, such as low \textit{ABCC3} (Qe1$<2.50 \times 10^{-3}$) and \textit{ABCB1} (Qe1$<5.08 \times 10^{-3}$) expression values, age, BMI, hypertension, tobacco smoking, and family history of CAD/LTC and diabetes and dyslipidemia. For all these analyses the significance value was established at the p-value$=0.050$.

3. Results

3.1. Clinical data and response of clopidogrel

Clinical characteristics and response to clopidogrel data of the CAD patients are presented in Table 1. Dyslipidemia (88%), hypertension (62%) and patients with BMI$>30$ (23%) were the major risk factors for CAD in this sample. Platelet response to clopidogrel was categorized in to PRU quartiles. Individuals with PRU values within the first quartile (Q1, PRU<151) were considered good responders, while those who had PRU within the fourth quartile (Q4, PRU$>260$) were considered non-responders. Age, BMI, clinical data and TLDC of the CAD patients did not differ among clopidogrel response groups (p>0.05).

3.2. \textit{ABCB1} and \textit{ABCC3} mRNA expressions

\textit{ABCB1} and \textit{ABCC3} mRNA expression levels in PBC were compared among PRU quartile groups, as shown in Fig. 1. \textit{ABCC3} was 1.7 times less expressed in the Q1 PRU group (2.7±1.2$ \times 10^{-3}$) than in the Q4 PRU (4.5±1.7$ \times 10^{-3}$, p=0.048). On the other hand, no significant differences were observed in \textit{ABCB1} expression among PRU quartile values (p=0.864).
Variables from individuals with low ABCC3 mRNA levels (Qe1, mRNA<2.5×10^{-3}) were compared with those having mRNA expression within the other quartiles (Qe2 + Qe3 + Qe4). Interestingly, low ABCC3 expression (Qe1) was associated with low PRU mean values (p=0.039) (Table 2). Other clinical variables did not influence ABCC3 mRNA levels in PBC.

Univariate logistic regression revealed that CAD patients with low ABCC3 mRNA expression (Qe1, <2.5×10^{-3}) have 18 times more probability to be responsive to clopidogrel (OR: 18.000, 95%CI: 1.659–169.991, p = 0.011), as shown in Table 3. Others variables were not associated with PBC ABCC3 mRNA levels in CAD patients. No relationship was found between low ABCB1 mRNA expression (Qe1, <5.1×10^{-3}) and clinical variables and response to clopidogrel in CAD patients (Table 3).

Multivariate stepwise logistic regression confirmed that patients with low ABCC3 mRNA expression had high probability to have a good response to clopidogrel (OR: 30.755, 95%CI: 1.659–570.247, p = 0.021), whereas other variables included in this model (age, BMI and TLCD) were not related with ABCB3 mRNA levels in PBC (Table 4).

Univariate linear regression analysis demonstrated that low ABCB3 mRNA expression contributed with a reduction of 73 PRU in relation to the patients with expression value higher than 2.5×10^{-3} (p = 0.027) (Table 5). In this model, the variables low ABCB1 mRNA expression, age, BMI, hypertension, diabetes, dyslipidemia, tobacco smoking, and family history of CAD and TLCD did not influence platelet response to clopidogrel.

4. Discussion

Several risk factors for CAD such as hypertension, dyslipidemia, diabetes, tobacco smoking and family history of CAD have been found in this sample population, as it has been described previously [19]. However our results indicated that these risk factors did not influence the platelet response to clopidogrel. The categorization in quartiles of PRU values was a better approach to analyze the data of response to clopidogrel. Good responders (PRU>260), data that are in accordance with those previously reported [20].

Cuisset et al. have evaluated three different methods for determination of clopidogrel response (ADP-induced platelet aggregation, Platelet reactivity index Vasodilator-stimulated Phosphoprotein (VASP) and Point of care Verify Now Assay) [21]. Even though, they have shown a good correlation between the results from these methods, there was a poor agreement to identify clopidogrel non-responders. It is likely that the different cutoff values may had influenced these results.

In this study, diabetes was not associated with clopidogrel response in CAD patients. However PRU Q1 group did not contain diabetic patients suggesting association with low platelet response to clopidogrel. It has been reported that the relationship between diabetes and poor response to clopidogrel is probably due to an enhanced platelet reactivity found in hyperglycemic status [22].

We also evaluated the ABCB1 and ABCC3 mRNA expressions in PBC and its possible association with platelet response to clopidogrel in CAD patients. Low ABCB3 mRNA expresssors showed better response to clopidogrel, independently of ABCB1 mRNA expression and clinical variables.

The mechanisms involved in modulation of ABCC3 mRNA expression in leukocytes are not clear. Bohan et al. have shown that induction of ABCC3 is hepatoprotective in cholestasis and suggested that ABCC3 may be transcriptionally regulated by liver receptor homolog 1 dependent on an intact TNFalpha signaling pathway [23]. Hepatocyte

![Fig. 1. ABCC3 and ABCB1 mRNA levels in peripheral blood cells according to clopidogrel response. Patients were grouped in quartiles of PRU values (Q1, Q2, Q3 and Q4), mRNA expression was measured by qPCR using GAPD as reference gene (2^{-ΔΔCt} method). First PRU quartile (Q1) was compared with the others (Q2, Q3 and Q4) by Mann–Whitney test.](image-url)
nuclear factors (HNFs) have also shown to regulate ABCC3 mRNA in liver tissues from HNF4α−/− mice. Lu and co-workers have reported that mice lacking HNF4α expression in liver have selective loss of efflux transporters, including ABCC3 mRNA when compared with wild-type mice [24].

Linear regression analysis demonstrated a positive correlation between ABCC3 mRNA expression and response to clopidogrel suggesting that ABCC3 may be involved in clopidogrel bioavailability although we used whole blood cells as study model to analyze the molecular mechanisms of these cells in other cells such as hepatocytes and enterocytes [14,25–27]. Study of Arazzi et al., describe that atorvastatin, a drug metabolized in hepatocytes cells, has differential effects on SREBF1a and SCAP mRNA expressions in PBMC that are associated with baseline transcription levels, triglycerides response to atorvastatin. Therefore, mRNA expression of ABCB3 may play an important role in determining clopidogrel intracellular concentration and response.

The lack of relationship between ABCB1 mRNA expressions in leukocytes, response to clopidogrel and other clinical variables is suggestive that this membrane transporter is not involved in clopidogrel response. However more studies should be carried out to confirm this hypothesis. However, other author investigated the effect of ABCB1 3435C/T polymorphism on ABCB1 expression in PBMCs of 69 individuals treatment with atorvastatin and not found significant association between this polymorphism and ABCB1 mRNA expression [25].

One potential limitation of the present study is the small sample size of clopidogrel responder group to analyze its association with the change in mRNA expression. Therefore the effect of ABCB3 mRNA variants on clopidogrel response should be tested in larger populations for better evaluation of the pharmacogenomic role of these antiplatelet drug and its relationship with membrane transporter.

In conclusion, low ABCB3 mRNA expression can be indicative of better response to clopidogrel, independently of ABCB1 mRNA expression and clinical variables. However, the molecular mechanisms involved in regulation of ABCB3 expression and its effect on clopidogrel response or if clopidogrel can be modify directly this gene expression remains to be elucidated.

### Table 2

<table>
<thead>
<tr>
<th>Variables</th>
<th>Quartiles of ABCC3 mRNA expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCC3 mRNA levels</td>
<td>Qe1 (6)</td>
<td>Qe2 + Qe3 + Qe4 (20)</td>
</tr>
<tr>
<td>Age, years</td>
<td>59 ± 14</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>BML kg/m²</td>
<td>28 ± 3</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>67 (4)</td>
<td>60 (12)</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>0 (0)</td>
<td>35 (7)</td>
</tr>
<tr>
<td>Dyslipidemia, %</td>
<td>100 (6)</td>
<td>85 (17)</td>
</tr>
<tr>
<td>Family history of CAD, %</td>
<td>17 (1)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>Tobacco smoking, %</td>
<td>17 (1)</td>
<td>20 (4)</td>
</tr>
<tr>
<td>TLDC, hours</td>
<td>12 ± 11</td>
<td>11 ± 9</td>
</tr>
<tr>
<td>PRU, units</td>
<td>156 ± 75</td>
<td>229 ± 64</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Model</th>
<th>Predictor</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low ABCC3 mRNA expression</td>
<td>PRU Q1 (&lt;151 units)</td>
<td>30.755</td>
<td>1.659–570.247</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age</td>
<td>0.971</td>
<td>0.838–1.126</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BML kg/m²</td>
<td>1.105</td>
<td>0.723–1.688</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLDC</td>
<td>0.999</td>
<td>0.957–1.002</td>
</tr>
</tbody>
</table>

ABCC3 expression was introduced as dependent variables considering as low mRNA expression the first quartile for ABCB3 (Qe1 = 2.5 × 10⁻³), mRNA expression was measured by qPCR using GAPDH as reference gene (2⁻deltaCt method). PRU Q1 (<151 units) represent good platelet response to clopidogrel. PRU: P2RY12 reaction units; BML: Body Mass Index; CAD: coronary artery disease; TLDC: time from last dose of clopidogrel.

### Table 3

<table>
<thead>
<tr>
<th>Model</th>
<th>Predictor</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low ABCB3 mRNA expression</td>
<td>PRU Q1 (&lt;151 units)</td>
<td>18.000</td>
<td>1.9–169.991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age</td>
<td>0.994</td>
<td>0.905–1.092</td>
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<td></td>
<td></td>
<td>BML kg/m²</td>
<td>0.846</td>
<td>0.577–1.240</td>
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<td>Hypertension</td>
<td>0.750</td>
<td>0.110–5.109</td>
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<td>Tobacco smoking</td>
<td>1.000</td>
<td>0.600–14.640</td>
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<td>Family history of CAD</td>
<td>0.556</td>
<td>0.042–7.457</td>
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<td>TLDC</td>
<td>1.000</td>
<td>0.998–1.002</td>
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<tr>
<td>2</td>
<td>Low ABCB1 mRNA expression</td>
<td>PRU Q1 (&lt;151 units)</td>
<td>0.364</td>
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<td></td>
<td></td>
<td>Age</td>
<td>0.91</td>
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<td>BML kg/m²</td>
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<td>Hypertension</td>
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<td>Tobacco smoking</td>
<td>8.568</td>
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<td>Family history of CAD</td>
<td>0.259</td>
<td>0.008–7.88</td>
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<tr>
<td></td>
<td></td>
<td>TLDC</td>
<td>1.002</td>
<td>0.998–1.005</td>
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</table>

Univariate logistic regression analysis. ABCB3 and ABCB1 expressions were introduced as dependent variables considering as low mRNA expression the first quartile for ABCB3 (Qe1 = 2.5 × 10⁻³) and ABCB1 (Qe1 = 5.1 × 10⁻³). mRNA expression was measured by qPCR using GAPDH as reference gene (2⁻deltaCt method). PRU Q1 (<151 units) represent good platelet response to clopidogrel. PRU: P2RY12 reaction units; BML: Body Mass Index; CAD: coronary artery disease; TLDC: time from last dose of clopidogrel.
Acknowledgments

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References


