ORIGINAL ARTICLE

IMPACT OF CLOPIDOGREL PLASMATIC LEVELS, CYP2C19 POLYMORPHISMS AND DRUG-DRUG INTERACTIONS ON CLINICAL OUTCOME IN CORONARY ARTERY DISEASE PATIENTS

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Abstract

The metabolism of clopidogrel is driven by 3 main hepatic isoformes of cytochrome P450 enzymes: CYP3A4, CYP2C9 and CYP2C19. Genetic polymorphisms of CYP2C19 gene and the interaction of this cytochrome with other drugs have been reported to be associated with a reduced responsiveness to clopidogrel drug prescribed to patients with coronary pathologies. Therefore, the main objective of this study was to test the effect of two genetic variants (CYP2C19*2 and CYP2C19*17) and some non-genetic factors, such as co-administration of drugs, on the cardiovascular events and plasmatic levels of clopidogrel. The studied polymorphisms were determined in 213 coronary patients taking a 300 mg loading dose of clopidogrel using polymerised chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. The plasma levels of clopidogrel and its metabolites (the active form and two inactive-ones' carboxylic acid and acyl glucuronide derivatives) were determined by HPLC-MS/MS in 115 subjects (plasmatic levels could not be evaluated in all subjects due to personal and practical reasons at the time of sampling). Our results show that plasma levels of clopidogrel and its metabolites do not present significant differences between the wild-type homozygote and carriers of the CYP2C19 allele subjects. However, co-medications with simvastatin ($p = 0.018$), atorvastatin ($p = 0.006$), omeprazole ($p = 0.030$), metformin ($p = 0.044$) and insulin ($p = 0.018$) are the main factors affecting the clopidogrel plasmatic levels. The occurrence of death was significantly higher in patients with elevated acyl glucuronide plasma levels than those with lower plasma levels ($p = 0.036$). Also, patients receiving omeprazole treatment were at higher and statistically significant risk of developing cardiovascular events ($p = 0.035$). Based on the obtained results, we can conclude that only the non-genetic factors tested (i.e. co-medications with CYP3A4 metabolized statins, proton pump inhibitor (omeprazole), oral antidiabetic (metformin) and insulin) have a significant effect on the clopidogrel inefficiency in these patients.

Rezumat

Metabolizarea clopidogrelului este realizată cu ajutorul a trei izoforme hepatice ale citocromului P450: CYP3A4, CYP2C9 și CYP2C19. Polimorfismul genetic al genei CYP2C19 și interacțiunea acestui citocrom cu alte medicamente au fost raportate ca fiind asociate cu o reacție redusă la clopidogrel, prescris pacienților cu patologie coronariană. Prin urmare, obiectivul principal al acestui studiu a fost de a testa efectul a două variante genetice (CYP2C19*2 și CYP2C19*17) și al unor factori non-genetici, cum ar fi co-administrarea medicamentelor, asupra evenimentelor cardiovasculare și a nivelurilor plasmatice ale clopidogrelului. Polimorfismul studiat a fost determinat la 213 pacienți cu boală coronariană, care au luat o doză de încărcare de 300 mg clopidogrel. Nivelele plasmatice ale clopidogrelului și ale metaboliților săi (forma activă și cei doi derivați inactivi, acid carboxilic și acil glucuronid) s-au determinat prin HPLC-MS/MS la 115 subiecți (nivelele plasmatice nu au putut fi evaluate la toți subiecții din motive practice la momentul prelevării probelor). Rezultatele obținute arată că nivelele plasmatice ale clopidogrelului și ale metaboliților săi nu prezintă diferențe semnificative între genotipul de tip sălbatic și purtătorii alelei CYP2C19. Cu toate acestea, principalii factori care afectează concentrațiile plasmatice ale clopidogrelului sunt reprezentate de co-administrarea de simvastatină ($p = 0,018$), atorvastatină ($p = 0,006$), omeprazol ($p = 0,030$), metformin ($p = 0,044$) și insulină ($p = 0,018$). Apariția decesului a fost semnificativ mai mare la pacienții care realizează concentrații plasmatice crescute de acil glucuronid, decât la cei cu niveluri plasmatice mai scăzute ($p = 0,036$). De asemenea, pacienții cărora li s-a administrat omeprazol au prezentat un risc crescut, semnificativ statistic, de a dezvolta evenimente cardiovasculare ($p = 0,035$). Pe baza rezultatelor obținute, putem concluziona că numai factorii non-genetici testați (de exemplu co-administrarea de statine metabolizate de CYP3A4, inhibitori ai pompei de protoni (omeprazol), antidiabetice orale (metformin) și insulină) au un efect semnificativ asupra ineficienței clopidogrelului la acești pacienți.

Keywords: clopidogrel, coronary artery disease, drug-drug interactions, CYP2C19 polymorphisms

Introduction

Clopidogrel is an oral thienopyridine agent that is widely prescribed, in combination with aspirin, to reduce blood clot formation, by means of platelet aggregation inhibition, in patients with coronary artery disease (CAD). Despite the treatment with clopidogrel, platelet-dependent thrombosis could still occur in high-risk vascular patients, especially those who have experienced acute coronary syndromes [33, 36, 59]. The mechanism of this phenomenon, called "clopidogrel resistance", which leads to a reduced response to clopidogrel, is not yet fully understood. Many genetic and clinical factors as well as drug-drug interactions have been suggested as possible determinants of clopidogrel responsiveness [25, 59]. Among the most recognized non-genetic factors are diabetes mellitus, chronic kidney disease, smoking and obesity [18].

Clopidogrel is a pro-drug that requires activation in the liver by several cytochrome P450 (CYP) enzymes [13]. The known metabolic pathway of clopidogrel has two directions. On the one hand, there is the des-esterification to clopidogrel carboxylic acid (CCA) followed by conjugation to the inactive clopidogrel acyl glucuronide (CAG) through acyl glucuronidation reaction [42, 50]. On the other hand there is the oxidation to oxo-clopidogrel, which undergoes a further oxidation and leads to clopidogrel active metabolite (CAM) [41]. The active metabolite irreversibly inhibits the binding of adenosine diphosphate (ADP), to its platelet P2Y12 receptor preventing the activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation [41]. Due to the irreversible binding, the exposed platelets are affected for the rest of their lifespan (7 - 10 days).

Using cDNA-expressed human P450 isoforms, *in-vitro* tests carried out by Kazui *et al*. have shown that clopidogrel is metabolized firstly to 2-oxo-clopidogrel in a reaction catalysed by CYP1A2, CYP2B6 and CYP2C19, with an individual contribution of 35.8%, 19.4% and 44.9% respectively [31]. In the same system using 2-oxo-clopidogrel as a substrate, after the addition of glutathione, 2-oxo-clopidogrel was metabolized to CAM by CYP2B6, CYP2C9, CYP2C19 and CYP3A4 enzymes with an individual contribution of 32.9%, 6.76%, 20.6% and 39.8% respectively.

Many studies have shown that genetic polymorphisms of hepatic CYP enzymes are the most important contributors to clopidogrel responsiveness. Beside these findings, in addition to other studies, the Food and Drug Administration (FDA) in 2010 imposed on the clopidogrel label, a "boxed warning" about the diminished antiplatelet effect of the drug in patients metabolizing clopidogrel poorly due to deficiency in CYP system [20]. The warning also notes that tests are available to identify CYP2C19 poor metabolizers and recommend alternative treatment strategies to those subjects. The CYP2C19*2 loss of function (mutation of guanine to adenine at the position 681 in exon 5) allele is associated with higher levels of ADP-induced platelet aggregation values in clopidogrel-treated patients and therefore, with the occurrence of adverse cardiovascular events [45, 52]. On the contrary, an allelic variant recently reported, CYP2C19*17 (mutation of Cysteine to Tyrosine at the position 806 in the 5'-flanking region), has been associated with increased enzymatic function [51]. Therefore, individuals carrying this polymorphism are prevented from thrombotic events, but may have an increased putative risk of bleeding [51]. In a recent study conducted by Horenstein *et al*. it has been shown that dosages up to 4 times the normal-ones are necessary to overcome resistance in CYP2C19 poor metabolizers [28].

In the last years, several confirmations have emerged on drug-drug interactions, based on pharmacokinetics (PK), with important class of drugs such as statins [53] and proton pump inhibitors [22]. These studies concluded that extra attention must be put into the choice of medication, to get an optimal therapeutic effect [48]. This fact is particularly important in patients with cardiovascular diseases; they are indeed always treated with several drugs, which often modify the metabolic phenotype of the patients, to an extent that may completely alter the drug metabolism, as expected by the subject genotype. In such a situation the use of therapeutic drug monitoring looks quite interesting because this is the only way to really evaluate the patients´ PK characteristics resulting from this complex interaction between genotype and external factors (i.e. concomitant medications and clinical factors). Unfortunately, there is a paucity of studies dealing on therapeutic drug monitoring with clopidogrel; however, a work of Bouman *et al*., investigating PK/PD interaction for clopidogrel, is quite interesting [8]. In this last study, originally carried out to evaluate the appropriateness of different platelet aggregation tests, the authors correlated the PK of CAM with a series of these tests, some of which had a good correlation [8]. It is clear so far that the PK data of the active metabolite can be a good predictor of therapeutic activity. Further studies are needed to define an adequate threshold in the plasma level, for therapeutic drug monitoring. It is worth mentioning, that the determination of CAM, is quite critical from the analytical point of view, due to its instability. Besides, it would be interesting to see if

clopidogrel itself or other more stable metabolites could be used instead.

All studies mentioned above have shown critical elements in the outcome of patients treated with clopidogrel, but a global comparative estimation of genotype, individual plasmatic levels and drug-drug interaction relevance is still missing. Therefore, the objectives of the present study were the evaluation of the influence of CYP2C19 polymorphisms and the effect of co-medication interactions (i.e. statins, antidiabetic medication, etc.) on clopidogrel efficiency. Also, a follow-up has been planned in these patients to detect the occurrence of major adverse cardiac events (MACE) and to test their correlation with plasma clopidogrel and 3 relevant metabolites (including the active-one) levels in a group of patients hospitalized for major cardiovascular problems (stable angina, ST elevation myocardial infarction, non-ST elevation myocardial infarctions).

Materials and Methods

Study Patients

A total number of enroled 213 coronary patients were recruited between April 2010 and December 2012 for participation in this study; only 115 patients were selected to participate also in the plasmatic levels part due to different problems both personal and practical (i.e. poor vein situation, resident outside the city, work related issues, etc.).

Patients had been admitted to the Department of Cardiology of the Hedi Chaker University Hospital of Sfax, Tunisia and they had symptomatic coronary artery disease (CAD): acute coronary syndrome (ACS) or stable angina (SA) and underwent artery bypass graft (16 of them), thrombolytic treatment (69 of them) or percutaneous coronary intervention (PCI) with stent implantation (128 of them). Acute coronary syndrome was diagnosed when at least one of the following criteria was met: unstable angina (changes in the electrocardiogram, without evidence of myocardial necrosis and clinical symptoms), acute myocardial infarction (positive markers of myocardial necrosis), including ST-segment elevation myocardial infarction (STEMI). Patients with altered platelet aggregation were excluded from this study.

All patients took a 300 mg clopidogrel loading dose, within 12 hours from hospitalization in the emergency unit, followed by 75 mg/day clopidogrel in combination with 100 mg/day aspirin. After a coronarography, the doctor decided the type of procedure (PCI, artery bypass graft or thrombolytic treatment) that should be performed. Patients that underwent PCI, were treated with 75 mg clopidogrel daily for at least 3 months; the maintenance dose of clopidogrel was temporarily discontinued in patients who underwent artery bypass graft or thrombolytic treatment within 4 or 5 days.

Clinical parameters in patients were assessed including age, sex, body mass index and the presence of coronary risk factors (hypertension, dyslipidaemia, diabetes mellitus and smoking). Additional drug treatments were also recorded (angiotensin conversion enzyme inhibitors, beta-blockers, calcium channel blockers, proton pump inhibitors, anti-diabetics and statins).

Informed consent was obtained from all individual participants included in the study; the Local Committee of Medical Ethics (Hedi Chaker Hospital Ethics Committee, Sfax, Tunisia) approved the study protocol and the work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

Blood samples

Venous blood samples were collected in tubes containing 3.2% trisodium citrate at 6 hours after clopidogrel loading dose intake and centrifuged, within 15 minutes of collection, at 5000 rpm for 10 minutes. The plasma was separated and stored at -80°C for later analysis of clopidogrel and its metabolite levels.

Blood samples (5 mL) were also obtained from each patient for genetic analyses, using EDTA as anticoagulant.

Plasma levels analysis

Plasma levels of clopidogrel, clopidogrel carboxylic acid (CCA) and clopidogrel acyl glucuronide (CAG) were determined by a validated high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method, described by Silvestro *et al*., based on a previous study dealing with back-conversion problem of clopidogrel [49]. The plasma levels of CAM were separately assessed using a fully validated quantification method (unpublished data) with an analytical approach without sample derivatization, which is most commonly used [54], but with sample handling at low temperature (2 - 4°C) to minimize degradation. Samples were prepared by protein precipitation and then analysed by HPLC-MS/MS, following the fragmentation of protonated clopidogrel active metabolite ions (MRM transition 361.1/217.1); for separation reversed-phase columns were employed. Both the active metabolite (used for calibration curves and quality controls) and the internal standard were obtained *in vitro*, as described by Savi *et al*., starting from synthetic oxo-clopidogrel (d3-oxo-clopidogrel in the case of the internal standard) [41]. The validated quantitation range was from 5.0 (LLOQ) to 2000.0 (ULOQ) pg/mL, thereby obtaining linear calibration curves with correlation coefficients (r)

which were always > 0.995. At the lowest quality control (QC) level (3x LLOQ), the within-run accuracy (6 replicate) was 97.873% and the coefficient of variation (CV) 6.179%, while the between-run accuracy (18 replicates in 3 sequences), and the CV were respectively 97.631% - 5.711%. At the highest QC level (80% of ULOQ), the within-run accuracy (6 replicate) was 103.794% and the CV 6.766%, while the between-run accuracy (18 replicate in 3 sequences) and the CV were respectively 99.112% - 9.250%). This method selectively measured only the active form (4) of this metabolite; in particular, interferences from the isobaric endo isomer of clopidogrel active metabolite were excluded. Long term stability at -80°C was evaluated and it covered the whole period of time from the first sampling to the end of the measurements.

Genotyping

Genomic DNA was extracted from blood samples using the standard phenol/chloroform method. The CYP2C19*2 and CYP2C19*17 alleles were genotyped using PCR- restriction fragment length polymorphism method (RFLP) with the conditions described by Namazi *et al*. and Anichavezhi *et al*. respectively [2, 34].

Follow-Up

All patients underwent a follow up examination. Drug therapy compliance was monitored *via* phone calls and/or an out-patient clinic evaluation, every six months, for 3 years after their hospital discharge. From 213 patients, 209 were successfully followed.

The major adverse cardiac events included all-cause of mortality, myocardial infarction (MI), target lesion revascularization (LTR), heart failure and stroke, during the 36 months follow-up period; cardiovascular death is defined as death by acute MI, coronary CAD, or congestive heart failure. The diagnosis of MI was performed in the presence of any typical increase or decrease of cardiac biomarkers, along with clinical symptoms consistent with cardiac ischemia, following the American College of Cardiology definition [12]. Revascularization includes bypass surgery and PCI. Stroke was defined as a neurological deficit that persisted > 24 hours, as evaluated by a neurologist, according to the modified Rankin stroke scale.

Statistical analysis

The Kolmogorov-Smirnov test was performed to assess normality of the data. Normally distributed variables were compared using the unpaired two sided Student's t test or the ANOVA (Analysis of variance) test, when appropriate and are presented as mean ± standard deviation (SD). The Kruskal-Wallis test was used to compare abnormally distributed variables. Categorical variables are presented as counts or percentages. They were compared using the chi-square test or Fisher's exact test when the expected frequencies where less than 5; the classification of high/low metabolite levels was based according to the median values considered as threshold between the 2 groups.

In particular clopidogrel levels were defined as high using a median value of 492 pg/mL; while CAM levels were defined as high with a median value of 158 pg/mL. Finally, CCA levels were defined as high with a median value of 1950 ng/mL and CAG levels were classified as high with a median value of 1990 ng/mL. A binary logistic regression analysis was performed to estimate the influence of genetic factor, gender, age, hypertension, diabetes mellitus, dyslipidaemia, co-medication and incidence of MACE on the plasma concentrations of clopidogrel and its mains metabolites.

Survival estimates were analysed *via* the Kaplan-Meier method using the log-rank comparison test. Statistical analyses were performed using SPSS version 17 (SPSS Inc, Chicago, Il, USA). A two-tailed $p < 0.05$ was considered statistically significant.

Results and Discussion

In this study we evaluated the influence of genetic and non-genetic factors on plasmatic levels and cardiovascular events in CAD patients treated regularly with clopidogrel.

Clinical characteristics of the studied CAD patients according to their CYP2C19 genotypes are reported in Table I. The allele frequencies of the CYP2C19*1, CYP2C19*2 and CYP2C19*17 polymorphisms were 0.101, 0.655 and 0.244 respectively. According to the carriage of the CYP2C19 variant allele, all enrolled patients were classified into four groups: extensive metabolizers (EMs) (*1/*1, *1/*17 and *2/*17: 86%), intermediate metabolizers (IMs) (*1/*2: 9.8%), ultra-rapid metabolizers (UMs) (*17/*17: 3.3%) and poor metabolizers (PMs) (*2/*2: 0.9%). The frequencies of CYP2C19 allele were as follows: CYP2C19*1 (65.5%), CYP2C19*2 (10.1%) and CYP2C19*17 (24.4%). Due to the low number of homozygote subjects [(*2/*2) and (*17/*17)], we based most of the comparisons between CYP2C19*2 loss-of-function and CYP2C19*17 gain-of-function considered as alleles carriers and wild-type homozygotes (non-carriers).

Overall demographic and clinical variables were well balanced among genotype groups, apart from the prevalence of patients with MI, that was significantly different, with a higher prevalence of patients with MI in non-carriers of CYP2C19*17 allele ($p = 0.043$). Besides, a slightly higher proportion of patients with calcium channel blocker treatment in CYP2C19*2 loss-of-function carriers'

subjects (p = 0.035) and a higher proportion of dyslipidaemia in patients who were carriers of CYP2C19*17 gain-of-function (p = 0.012) were observed.

Table I

Baseline characteristics of the studied population according to genotype in the restricted group (n = 115)

Variable			CYP2C19*2 loss-of-function alleles		p value	CYP2C19*17gain-of-function alleles		p value
	Total (n = 213)	(n = 115)	Carriers*2 (n = 41)	Non Carriers*2 (n = 172)		Non Carriers*17 (n = 116)	Carriers*17 (n = 97)	
Age (years) (mean ± SD)	58.22 ± 11.7	58.01 ± 11.36	58.51 ± 11.9	58.15 ± 11.7	0. 860	57.99 ± 11.7	58.49 ± 11.7	0.756
BMI (Kg/m²) (mean ± SD)	25.85 ± 4.6	25.57 ± 4.85	25 ± 3.7	26 ± 4.7	0.602	26.1 ± 4.5	25.42 ± 4.8	0.605
Men/Women [N (%)]	169 (79.3)/ 44 (20.7)	91 (79.1)/ 24 (20.9)	36 (87.8)/ 05 (12.1)	133 (77.3)/ 39 (22.6)	0.293	96 (82.7)/ 20 (17.2)	73 (75.2)/ 24 (24.7)	0.178
Clinical presentation:								
Myocardial infarction	114 (53.5)	56 (48.7)	21 (51.2)	92 (54)	0.640	70 (60.3)	43 (44.3)	**0.043**
Unstable angina	77 (36.2)	45 (39.1)	17 (41.4)	60 (34.9)		36 (31)	41 (42.2)	
Stable angina	22 (10.3)	14 (12.2)	03 (7.3)	19 (11)		09 (8.7)	13 (13.4)	
Risk factors [N (%)]:								
Hypertension	89 (41.8)	54 (47)	15 (36.5)	74 (43)	0.453	48 (41.3)	41 (42.2)	0.896
Diabetes mellitus	85 (39.9)	37 (32.17)	15 (36.5)	73 (42.4)	0.494	43 (37)	45 (46.3)	0.169
Dyslipidaemia	90 (42.3)	50 (43.5)	16 (39)	74 (43)	0. 641	40 (34.4)	50 (51.5)	**0.012**
Current smoker	142 (66.7)	80 (69.6)	29 (70.7)	113 (65.6)	0.539	81 (69.8)	61 (62.8)	0.285
Familial CAD	40 (18.8)	23 (20)	10 (25)	30 (17.4)	0.306	20 (17.2)	20 (20.6)	0.530
Concomitant medication use [N (%)]:								
Aspirin	213 (100)	115 (100)	213 (100)	213 (100)	-	213 (100)	213 (100)	-
ACEI	98 (46)	52 (45.2)	21 (51.2)	77 (44.7)	0.456	52 (44.8)	46 (47.4)	0.705
Beta-blockers	128 (60.1)	69 (60)	22 (53.6)	106 (61.6)	0.349	67 (57.7)	61 (62.8)	0.447
CCB	14 (6.6)	4 (3.5)	05 (9.7)	09 (5.2)	**0.035**	07 (06)	07 (7.2)	0.069
PPI (omeprazole)	152 (71.4)	87 (75.7)	29 (70.7)	123 (71.5)	0.921	82(70.6)	70 (72.1)	0.813
Statins	135 (63.4)	69 (60)	26 (63.4)	109 (63.3)	0.996	72 (62)	63 (64.9)	0.664
Nitro-derivatives	79 (37.08)	47 (40.86)	17 (21.5)	62 (78.5)	0.519	39 (49.4)	40 (50.6)	0.252
Diuretics	32 (15.02)	19 (16.52)	07 (21.9)	25 (78.1)	0.683	14 (43.8)	18 (56.3)	0.187

ACEI: Angiotensin-Conversion enzyme inhibitor; CAD: Coronary Artery Disease; CCA: Calcium Channel Antagonist; N: Number; PPI: Proton Pump Inhibitor; SD: Standard Deviation; CCB: Calcium Channel Blocker

Effect of CYP2C19 polymorphisms on plasmatic concentrations of clopidogrel and its metabolites

The plasmatic levels of clopidogrel, CCA, CAG and CAM were not significantly different in carriers of the CYP2C19*2 and CYP2C19*17 allele, when compared to the wild-type homozygotes (p > 0.05) (Table II).

Table II

Plasma concentration of clopidogrel and its metabolites according to CYP2C19 polymorphism (n = 115)

	(CCA) (ng/mL)	Clopidogrel (pg/mL)	CAG (ng/mL)	CAM (pg/mL)
CYP2C19*2				
Carriers (n = 26)	2110 (182 - 8800)	297.5 (09 - 6800)	1470 (83 - 9090)	108.5 (05 - 8932)
Non carriers (n = 89)	1820 (88 - 9310)	465 (04 - 9010)	1970 (76 - 26200)	164 (07 - 11620)
p value	0.640	0.235	0.753	0.517
CYP2C19*17				
Carriers (n = 62)	1945 (88 - 9310)	461 (33 - 9010)	1810 (76 - 26200)	228 (0.0010 - 11620)
Non carriers (n = 53)	1630 (264 - 7760)	387 (4 - 6800)	1820 (101 - 15100)	129 (0.0010 - 8932)
p value	0.169	0.103	0.950	0.590

CCA: Clopidogrel carboxylic acid; CAG: Clopidogrel acyl glucuronide; CAM: Clopidogrel Active metabolite; n: number

It has been reported that CYP2C19*2 allele was associated with lower levels of active metabolite [10, 32, 57] and higher rate of adverse cardiovascular events [30] in cardiovascular patients under clopidogrel treatment. However, Fontana *et al*. supposed that there is no reason for "personalized" antiplatelet treatment based on CYP2C19 genotyping in clinical practice because of the controversial results of clinical and randomized studies [21]. Furthermore, some larger randomized clinical trials have shown that tailored anti-P2Y12 treatment is associated with a higher risk of stent thrombosis [16, 39, 55] contrary to prospective studies [6, 7, 47]. The plasmatic levels of clopidogrel and its metabolites seem not significantly impacted by the CYP2C19

characteristic most probably due to relevant drug-drug metabolic interactions influencing more profoundly the metabolizing phenotype.

Effect of the concomitant medications use and risk factors on plasmatic levels of clopidogrel and its metabolites

In our study co-medications with CYP3A4 metabolized statins (simvastatin or atorvastatin), proton pump inhibitor (omeprazole), oral antidiabetic (metformin) and insulin are the main factors affecting the plasma levels of clopidogrel and its metabolites (Table III).

Table III

Clopidogrel and its metabolites plasma levels according to co-medication and risk factors (n = 115)

	N (%)	CCA (ng/mL)	p value	Clopidogrel (pg/mL)	p value	CAG (ng/mL)	p value	CAM (pg/mL)	p value
PPI (omeprazole)									
Yes	87 (75.6)	1830 (88 - 9310)		456 (6 - 9010)		1970 (76 - 26200)		102 (0.0010 - 11620)	
No	28 (19.3)	1840 (264 - 5140)	0.807	401.5 (4 - 2490)	0.956	1735 (251 - 14500)	0.927	276 (15 - 7298)	**0.030**
Statins									
Yes	68 (59)	1940 (182 - 9310)	0.123	577 (9 - 9010)	**0.006**	1895 (83 - 14500)	0.982	228 (0.0010 - 8932)	0.057
No	47 (40.9)	1680 (88 - 8300)		252 (4 - 3820)		1610 (76 - 26200)		85 (0.0010 - 11620)	
CYP3A4 Metabolized Statins	43 (37.4)	1830 (182 - 9310)	0.290	605 (33 - 9010)	**0.019**	1970 (83 - 14500)	0.786	242 (0.0010 - 7298)	0.087
Non-CYP3A4-Metabolized Statins	25 (21.73)	2220 (409 - 8610)		506 (9 - 5390)		1330 (101 - 7680)		158 (0.0010 - 8932)	
Oral antidiabetic									
No	91 (79.13)	1830 (88 - 9310)	0.858	431 (4 - 7830)	0.453	1650 (76 - 26200)	0.807	209 (0.0010 - 11620)	**0.044**
Metformin	15 (13)	2160 (319 - 4990)		374 (39 - 2910)		1110 (128 - 9500)		26 (0.0010 - 7298)	
Sulfonylureas	09 (7.8)	1740 (701 - 3840)		715 (61 - 9010)		2610 (101 - 7210)		102 (0.0010 - 2148)	
Insulin									
Yes	10 (8.7)	1940 (997 - 4180)	0.616	1007.5 (343-5500)	**0.018**	2865 (816 - 15100)	0.138	375 (65-2269)	0.215
No	105 (91.3)	1780 (88 - 9310)		391 (4 - 9010)		1610 (76 - 26200)		129 (0.0010 - 11620)	
Hypertension									
Yes	54 (47)	1955 (264 - 8800)	0.449	499 (4 - 9010)	0.100	2570 (101 - 26200)	**0.001**	152 (0.0010 - 11620)	0.600
No	61 (53)	1780 (88 - 9310)		387 (9 - 7830)		1190 (76 - 15100)		129 (0.0010 - 8931)	
Diabetes mellitus									
Yes	37 (32.17)	2100 (319 - 4180)	0.214	615 (39 - 9010)	**0.027**	2610 (101 - 15100)	0.106	129 (0.0010 - 7298)	0.976
No	78 (67.8)	1685 (88 - 9310)		362 (4 - 7830)		1475 (76 - 26200)		158 (0.0010 - 11620)	

CCA: Clopidogrel Carboxylic Acid; CAG: Clopidogrel Acyl Glucuronide; CAM: Clopidogrel Active Metabolite; N: Number; PPI: Proton Pump Inhibitor; CYP3A4 Metabolized Statins: simvastatin and atorvastatin; Non-CYP3A4-Metabolized Statins: rosuvastatin and fluvastatin

The mean level of clopidogrel was increased in CAD patients taking insulin (p = 0.018) and statin treatments (p = 0.006), particularly with atorvastatin and simvastatin (p = 0.019) (Table III, Figure 1, Figure 2). In addition, CAM was decreased in the plasma of patients treated with omeprazole (p = 0.030) (Table III, Figure 1) and metformin treatments (Table III, Figure 2) (p = 0.044).

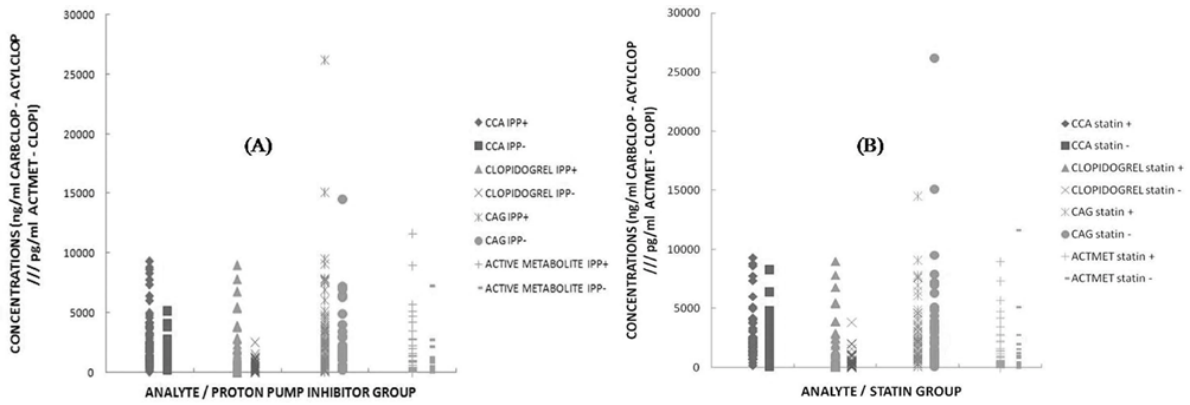

Figure 1.

Plasma levels of clopidogrel and its metabolites as scatter plots according to (A) pump proton inhibitor and (B) statin use

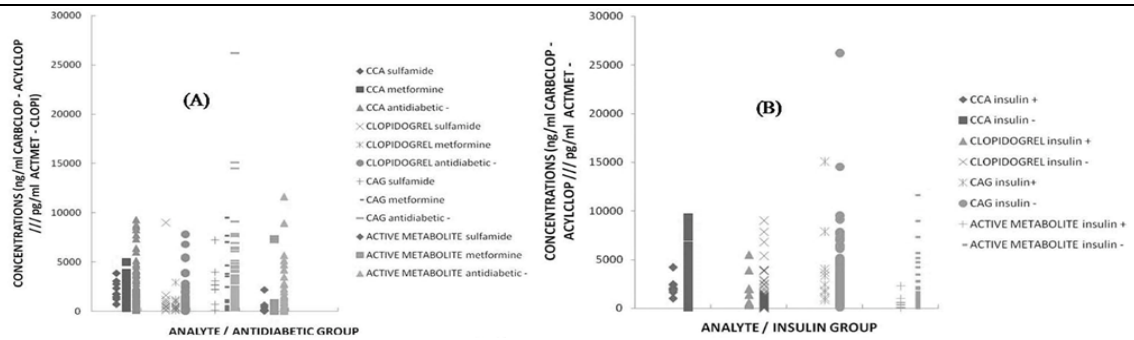

Figure 2.
Plasma levels of clopidogrel and its metabolites as scatter plots according to (A) antidiabetic and (B) insulin use

Higher plasmatic concentrations of CAG and clopidogrel were observed in hypertensive ($p = 0.027$) and diabetic patients, respectively ($p = 0.001$) (Table III). A difference was also observed when insulin was used as treatment. However in this case most probably the pathology itself (diabetes) and not the insulin co-medication was determining these significant plasmatic levels differences.

It is well known that CYP2C19 and CYP3A4 are the main effective enzymes in the two oxidative steps required in the formation of the active metabolite of clopidogrel; also, lipophilic statins (lovastatin, simvastatin, and atorvastatin) and omeprazole are metabolized by CYP3A4 and CYP2C19 respectively [3]. In this study, we found also that omeprazole treatment was associated with lower plasma active metabolite levels in patients. This finding is in accordance with several studies showing that concomitant omeprazole usage was associated with adverse cardiovascular outcomes [27, 37, 43] and reduced antiplatelet effect of clopidogrel [24, 46, 60] in patients treated with this drug. Conversely, some observational studies found no association between omeprazole use and adverse cardiovascular outcomes [9, 14, 19].

In addition, we found that patients under treatment with CYP3A4 metabolized statins (atorvastatin and simvastatin) have higher plasma clopidogrel and CCA levels than patients under non-CYP3A4-metabolized statins. This is in agreement with several *ex vivo* studies reporting the inhibitory effect of clopidogrel metabolism by atorvastatin [15, 35] and clinical studies showing decreased risk for MACE in CAD patients treated with clopidogrel compared with those treated with atorvastatin and clopidogrel [5, 11]. In contrast to these findings, other pharmacodynamic studies have shown that a dual therapy with atorvastatin and clopidogrel have no antagonistic effect. This result was observed when atorvastatin was further added to clopidogrel [38, 58] or when a higher clopidogrel loading dose (600 mg) was administered [23, 55].

This study provides, for the first time, data about the influence of metformin and insulin on clopidogrel active metabolite plasma levels. Relevant studies have reported that clopidogrel responsiveness was associated with the pathology itself (diabetes) and not with the co-medication. In fact, *ex vivo* studies and sub-analyses of clinical trials showed a diminished response to clopidogrel and increased stent thrombosis [1, 17, 29] in CAD patients with diabetes mellitus (DM). Furthermore, several studies reported that platelet alterations are associated with insulin resistance and DM, increasing CAD risk for acute coronary complications [26].

Our data show significant effects of several drugs, in particular metformin, omeprazole and statins, with the exception of rosuvastatin and fluvastatin on clopidogrel plasmatic levels. Luckily, not all drugs affected clopidogrel and its metabolites to the same extent, suggesting that some, in particular omeprazole, changing only the active metabolite, influence prevalently just one step of the metabolic process. It should also be noted that the inactive metabolites clopidogrel carboxylic acid and clopidogrel acyl glucuronide, both correlate with the co-medication, but the difference in concentration are opposite to the one in the active metabolite. These apparently paradoxical results, seem unrelated to a pharmacological/toxicological activity of these metabolites, while, most probably, derived from the accumulation of these metabolites, due to the lower rate of transformation to oxo-clopidogrel.

Cardiovascular outcomes

No significant differences between patients with and without MACE were observed in the univariate analysis for the two studied polymorphisms ($p > 0.05$) (Table IV). The CCA, clopidogrel, CAG and active metabolite plasma levels did not differ between CAD patients showing or no MACE (Table V, Figure 3). Also, no significant association between lower or higher levels of clopidogrel and its metabolites with MI, unstable angina and LTR was found ($p > 0.05$; data not shown). However, the incidence of cardiac mortality, with a frequency of

11.6% (13 of 112 patients), was significantly elevated in patients with a higher concentration of CAG (p = 0.033; data not shown).

Table IV

Incidence of Major Adverse Cardiovascular Events (MACE) at the 36-month follow-up after hospitalization in 209 CAD Tunisian patients, according to CYP2C19*2 and CYP2C19*17 polymorphism (4 from 213 patients were not followed)

Genotype	MACE (n = 64)	NO MACE (n = 145)	p value
CYP2C19*2			
Carriers (n = 40), n (%)	16 (25)	24 (16.55)	0.152
Non carriers (n = 169), n (%)	48 (75)	121 (83.45)	
CYP2C19*17			
Carriers (n = 94), n (%)	27 (42.18)	67 (46.2)	0.590
Non carriers (n = 115), n (%)	37(57.81)	78 (53.8)	

MACE: Major Adverse Cardiovascular Events; n: number

Table V

Plasma concentration of clopidogrel and its metabolites for 112 patients with and without MACE (3 from 115 patients were not followed)

	MACE (n = 45)	NO MACE (n = 67)	p value
CCA	1930 (88 - 8800)	1780 (182 - 9310)	0.341
Clopidogrel	615 (4 - 9010)	387 (9 - 7830)	0.067
CAG	2235 (76 - 15100)	1400 (83 - 26200)	0.087
CAM	108 (0.0010 - 8932)	209 (0.0010 - 11620)	0.539

CCA: clopidogrel carboxylic acid; CAG: clopidogrel acyl glucuronide; CAM: clopidogrel Active metabolite

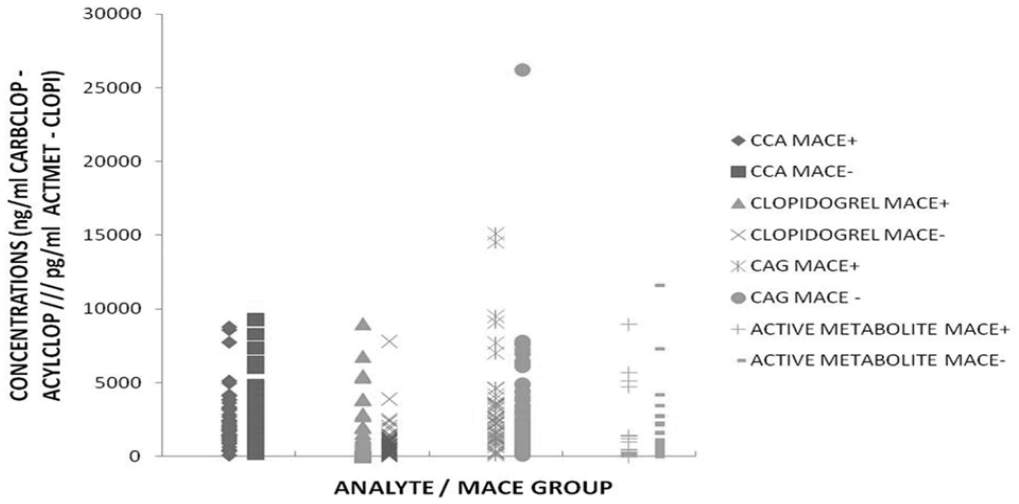

Figure 3.

Plasma levels of clopidogrel and its metabolites as scatter plots according to Major Adverse Cardiac Events (MACE)

The Kaplan-Meier survival analysis revealed that death rates were significantly higher in patients with elevated CAG plasma levels than the ones with lower concentrations (p = 0.036) (Figure 4).

Patients treated with proton pump inhibitors were at a higher and statistically significant risk of developing MACE (p = 0.035) (Table VI). No significant differences between patients with and without MACE were observed in the univariate analysis for the distribution of other co-medication variables. It should be pointed out that in the case of statins, despite the fact that a statistical significance was not reached (p = 0.240), a trend was clearly present. The same results are graphically presented as a mean, with error bars, according to MACE in Figure 5.

It is well established that the formation of acyl glucuronide metabolites is due to the metabolic conjugation of exogenous and endogenous carboxylic acid substrates with endogenous glucuronic acid, mediated by the superfamily of enzymes uridine diphosphoglucuronosyl transferase (UGT) [4]. The successive accumulation of glucuronides derivatives in the body was generally due to renal or liver diseases in aged patients [44].

Furthermore, the mechanism by which an acyl glucuronide could elicit a toxicological output is not well defined, with a suggestion of that the toxic behaviour could result by a potential immunogenic

response [40]. Despite these examples of potential toxicity, linked to glucuronide metabolites, it is highly possible that these elevated levels of CAG have no direct significant effects connected to MACE while, being CAG, a terminal metabolite deriving from the non-oxidative metabolism, it accumulates at higher levels in patients with a reduced oxidative metabolism and so far reduced formation of the active clopidogrel metabolite. Accepting this hypothesis, CAG looks like an interesting plasmatic levels marker, easier to measure then the active metabolite, to be evaluated in future studies. The fact that statistical correlations have been found between plasma levels of these molecules and the clinical outcome clearly shows that plasmatic levels determination could be useful in reducing the risk of clopidogrel based treatment being ineffective.

Table VI

Distribution of co-medication and other variables for patients with and without MACE (n = 209)

	Total (n = 209)	**MACE (n = 64)**	**NO MACE (n = 145)**	**p value**
Gender				
Men, n (%)	165 (78.94)	51 (79.68)	114 (78.62)	0.862
Women, n (%)	44 (21.05)	13 (20.31)	31 (21.37)	
Risk factors				
Hypertension				
Yes, n (%)	87 (41.62)	31 (48.43)	56 (38.62)	0.185
No, n (%)	122 (58.37)	33 (51.56)	89 (61.37)	
Diabetes mellitus				
Yes, n (%)	82 (39.23)	25 (39.06)	57 (39.31)	0.990
No, n (%)	127 (60.76)	40 (62.5)	87 (60)	
Dyslipidaemia				
Yes, n (%)	90 (43.06)	30 (46.9)	60 (41.37)	0.460
No, n (%)	119 (56.93)	34 (53.1)	85 (58.62)	
Current smoker				
Yes, n (%)	139 (66.5)	43 (67.2)	96 (66.2)	0.890
No, n (%)	70 (33.5)	21 (32.8)	49 (33.8)	
Co-medication				
ACEI				
Yes, n (%)	96 (45.93)	30 (46.9)	66 (45.51)	0.856
No, n (%)	113 (54.06)	34 (53.1)	79 (54.5)	
Beta-blocker				
Yes, n (%)	125 (59.80)	35 (54.68)	90 (62.1)	0.316
No, n (%)	84 (40.19)	29 (45.31)	55 (38)	
CCB				
Yes, n (%)	14 (6.7)	02 (3.12)	12 (8.27)	0.199
No, n (%)	195 (93.3)	62 (96.87)	133 (91.72)	
PPI (omeprazole)				
Yes, n (%)	149 (71.29)	52 (81.25)	97 (66.9)	**0.035**
No, n (%)	60 (28.70)	12 (18.75)	48 (33.10)	
Satins				
No, n (%)	74 (35.40)	27 (42.18)	47 (32.41)	
CYP3A4 Metabolized Statins, n (%)	86 (41.14)	21 (32.81)	65 (44.82)	
Non-CYP3A4-Metabolized Statins, n (%)	49 (23.44)	16 (25)	33 (22.75)	0.240
Oral antidiabetic				
Metformin, n (%)	37 (17.70)	13 (20.31)	24 (16.55)	
Sulfonylureas, n (%)	11 (5.26)	3 (4.68)	8 (5.51)	0.803
No, n (%)	160 (80.86)	48 (75)	112 (77.24)	
Insulin				
Yes, n (%)	28 (13.39)	9 (14.06)	19 (13.10)	0.866
No, n (%)	180 (86.12)	55 (85.93)	125 (86.2)	
Nitro-derivatives				
Yes, n (%)	77 (36.84)	27 (42.18)	50 (34.48)	
No, n (%)	132 (63.15)	37 (57.81)	95 (65.51)	0.287
Diuretic				
Yes, n (%)	5 (2.4)	0	5 (3.44)	
No, n (%)	204 (97.6)	64 (100)	140 (96.55)	0.520

MACE: Major Adverse Cardiovascular Events; n: number; PPI: Proton Pump Inhibitor; CYP3A4 Metabolized Statins: simvastatin and atorvastatin; Non-CYP3A4-Metabolized Statins: rosuvastatin and fluvastatin; ACEI: Angiotensin-Conversion enzyme inhibitor;

CCB: Calcium Channel Blocker; PPI: Proton Pump Inhibitor

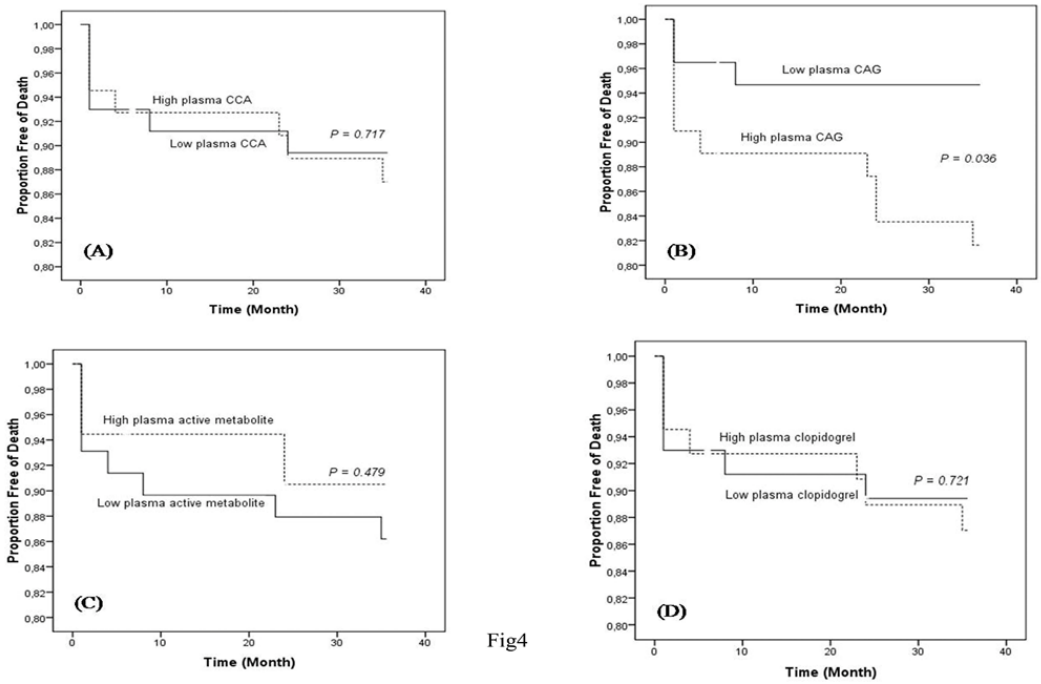

Fig4

Figure 4.

Kaplan Meier analysis of death during follow up period according to low and high plasma concentrations of (A) clopidogrel carboxylic acid, (B) clopidogrel acyl glucuronide, (C) clopidogrel active metabolite and (D) clopidogrel

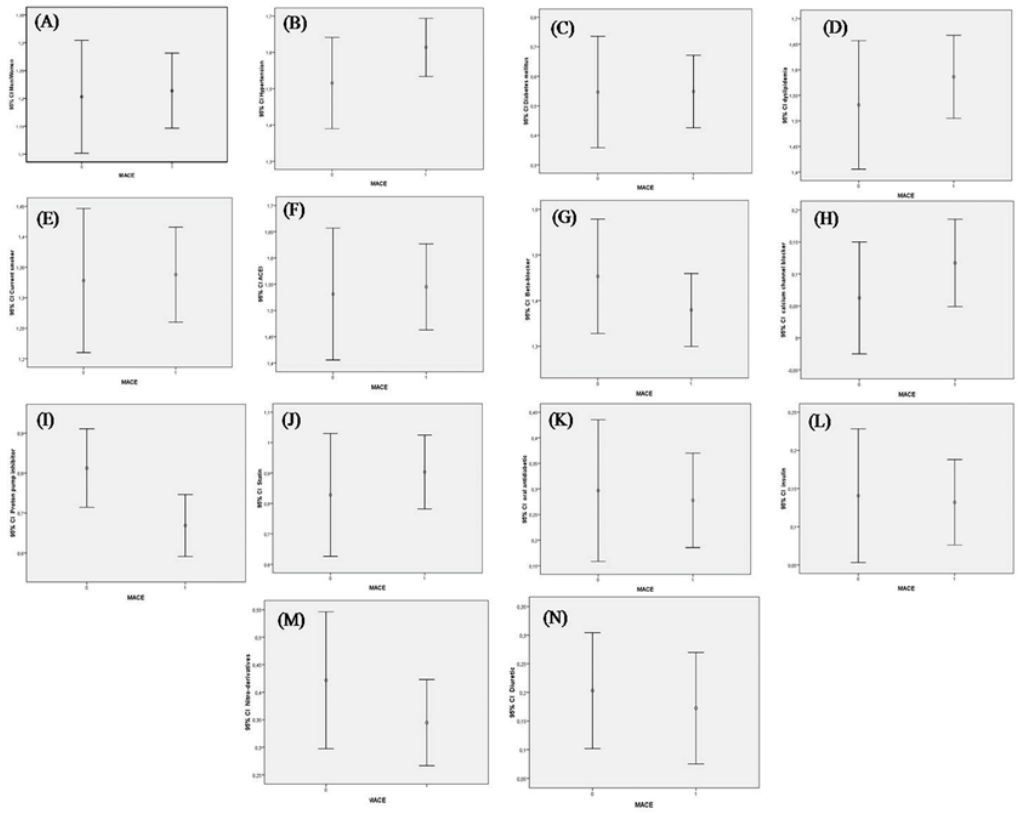

Figure 5.

Co-medication and other variables as means with error bar according to MACE

(A) Gender; (B) Hypertension; (C) Diabetes mellitus; (D) Dyslipidaemia; (E) Current smoker; (F) ACEI: Angiotensin-Conversion enzyme inhibitor; (G) Beta-blocker; (H) CCB: Calcium Channel Blocker; (I) Omeprazole; (J) Satins; (K) Oral antidiabetic; (L) Insulin; (M) Nitro-derivatives; (N) Diuretic

Interestingly, predictive results can also be obtained with inactive clopidogrel metabolites that can be more easily analysed, due to reduced stability problems and high plasma concentrations. However, some drugs, such as omeprazole, don't affect the concentrations of these metabolites. Further studies are needed to understand the potential of therapeutic drug monitoring with clopidogrel. In this context, another important point is to evaluate the optimal sampling time from the last administration. It has actually been observed, that the 6 hours interval employed in this study, is at the limit of the active metabolite PK curve due to the short half-life of this compound.

Multivariate analysis

Using logistic regression, no significant association was found for the two variants ($p > 0.05$), but incidence of MACE ($p = 0.030$) and diabetes mellitus ($p = 0.014$) were identified as an acquired risk factor of high plasma level of clopidogrel whereas, metformin treatment was found as an acquired risk factor for high CAM plasma level of CAM ($p = 0.040$) (Table VII).

Table VII

Logistic regression analysis of the contribution of genetic and non-genetic factors to the plasma concentrations of clopidogrel and its metabolites

Variables	CCA		Clopidogrel		CAG		CAM	
	OR (CI)	p value	OR (CI)	p value	OR (CI)	p value	OR (CI)	p value
Statins use	0.98 (0.751 - 1.279)	0.883	1.244 (0.972 - 1.593)	0.083	1.112 (0.846 - 1.462)	0.448	1.252 (0.980 - 1.6)	0.072
Incidence of MACE	0.576 (0.228 - 1.455)	0.243	0.403 (1.178 - 0.914)	**0.030**	0.599 (0.232 - 1.547)	0.290	1.110 (0.452 - 2.727)	0.820
Age	0.986 (0.946 - 1.028)	0.503	0.981 (0.942 - 1.021)	0.343	1.055 (1.015 - 1.097)	0.007	0.977 (0.938 - 1.017)	0.251
Gender	7.552 (1.228 - 46.43)	0.029	0.592 (0.113 - 3.096)	0.534	1.607 (0.270 - 9.557)	0.602	4.303 (1.476 - 12.55)	0.008
Hypertension	0.659 (0.259 - 1.679)	0.382	0.6 (0.242 - 1.491)	0.272	0.452 (0.194 - 1.054)	0.066	0.894 (0.356 - 2.247)	0.811
Diabetes mellitus	6.247 (0.861 - 45.31)	0.070	2.337 (1.187 - 4.603)	**0.014**	1.072 (0.250 - 4.601)	0.925	1.824 (0.369 - 9.014)	0.461
Insulin use	0.123 (0.002 - 9.239)	0.342	4 (0.112 - 143.7)	0.447	0.952 (0.037 - 24.44)	0.976	0.204 (0.006 - 7.336)	0.358
Oral antidiabetic (metformin)	0.341 (0.087 - 1.337)	0.123	1.163 (0.401 - 3.37)	0.781	1.5 (0.483 - 4.653)	0.483	0.477 (0.235 - 0.968)	**0.040**
Omeprazole	1.193 (0.448 - 3.177)	0.724	1.33 (0.494 - 3.622)	0.567	1.615 (0.581 - 4.491)	0.360	0.441 (0.163 - 1.192)	0.107
CYP2C19*2	2.319 (0.845 - 6.36)	0.102	0.851 (0.309 - 2.342)	0.755	1.193 (0.413 - 3.442)	0.744	1.101 (0.398 - 3.045)	0.853
CYP2C19*17	1.59 (0.657 - 3.746)	0.289	1.197 (0.515 - 2.78)	0.676	0.973 (0.401 - 2.357)	0.951	0.899 (0.386 - 2.094)	0.804

CI: Confidence interval; OR: Odds ratio

Conclusions

Based on these results, it seems clear that a genotyping focused only on CYP2C19, following the warning of FDA for clopidogrel, is not giving results correlated to the clinical outcome in patients treated with this drug. Instead it has been found that metabolic drug-drug interactions must be evaluated carefully, when selecting a therapeutic protocol, to avoid complex pharmacokinetic effects affecting the clopidogrel activity; it is noteworthy that drugs interacting with CYP3A4 played an important role in these interactions and genotyping for this cytochrome seems to be of interest for the future. Therapeutic drug monitoring looks quite promising to monitor the treatment in future studies. However larger groups and/or more homogenous patients are necessaries for conclusive findings.

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