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Cytochrome P450 and inflammation : pharmacogenomic approach and molecular aspects of anti-inflammatory effects of theinopyridines

Payman Shahabi

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Par **Payman Shahabi**

**CYTOCHROME P450 ET INFLAMMATION;
APPROCHE PHARMACOGÉNOMIQUE ET ASPECTS MOLECULAIRES
DES EFFETS ANTI-INFLAMMATOIRES
DES THIÉNOPYRIDINES**

**CYTOCHROME P450 AND INFLAMMATION;
PHARMACOGENOMIC APPROACH AND MOLECULAR ASPECTS OF
ANTI-INFLAMMATORY EFFECTS OF THEINOPYRIDINES**

Le 12 Septembre 2013

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*My knowledge has reached the point,
in which,
I know that I know nothing*

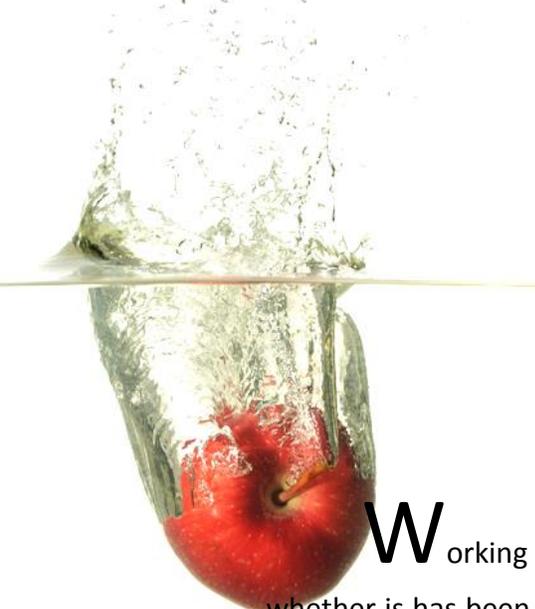
Pure-e-Sina (Avicenna) – (980 - 1037)

*To Atena, my dear wife and life
for her constant encouragement and genuine support through difficult times
and
for her endless love*

*To my parents
the primary reason for anything I achieve in my life*

And

*To IRAN
my beloved motherland
and
my heart song*



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Need for pharmacogenomic information also for generic medications; recommendation of the European Society of Pharmacogenomics and Theranostics (ESPT); *Equal contribution

http://www.esptnet.eu/downloads/espt_first_recommendation.pdf

Abbreviations

AA	arachidonic acid
AC	adenylyl cyclase
ACS	acute coronary syndrome
ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
AF	activation function
AhR	aryl hydrocarbon receptor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
Apo	apolipoprotein
AST	aspartate aminotransferase
AT ₂	angiotensin II type 2
AT ₂ R	AT ₂ receptors
Akt	protein kinase B (Akt is not an abbreviation)
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
BCG	bacillus Calmette–Guerin
BK _{Ca}	Ca ²⁺ -activated K ⁺ channel
BMI	Body mass index
BMS	Bare metal stent
BNP	brain natriuretic peptide
BRC	biological resource center
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CCRP	CAR retention protein
CNS	central nervous system
CNV	copy number variation
CO	carbon monoxide
CoA	coenzyme A
COX	cyclooxygenase
COUPTF	chicken ovalbumin upstream promoter transcription factor
cPLA ₂	cytosolic enzyme phospholipase A ₂
CREBP	cAMP-response-element binding protein
CRP	C-reactive protein
CS	current smoking
CVD	cardiovascular diseases

CYP	cytochrome P450
DBD	DNA binding domain
DBP	diastolic blood pressure
DES	drug-eluting stent
DHA	docosahexaenoic acid
DHET	dihydroxyeicosatrienoic acid
DME	drug-metabolizing enzyme
DMet	DNA methylation
DNA	deoxyribonucleic acid
EC	endothelial cell
EBP	enhancer-binding protein
EDHF	endothelium-derived hyperpolarizing factor
EDP	epoxydocosapentaenoic acid
EET	epoxyeicosatrienoic acid
EEQ	epoxyeicosatetraenoic acid
EGF	epidermal growth factor
Em	membrane potential
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide
Fe ²⁺	ferrous iron
Fe ⁺³	ferric iron
FMN	flavin mononucleotide
FXR	farnesoid X receptor
GGT	γ glutamyltransferase
GI	gastrointestinal
G protein	guanine nucleotide binding protein
GR	glucocorticoid receptor
GTF	general transcription factor
GU	genitourinary
GWAS	genome-wide association study
Hb	hemoglobin
HB-EGF	heparin-binding EGF-like growth factor
HDAC	histone deacetylases
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acid
HF	heart failure
H-FABP	heart-type FABP

HM	histone modification
HNF	hepatocyte nuclear factor
HO	heme oxygenase
Hp	haptoglobin
Hpr	haptoglobin-related protein
Hs-CRP	high sensitivity CRP
HSP90	heat shock protein 90
HTN	hypertension
HUVEC	umbilical vein endothelial cell
Hyper-apo B	hyperapolipoproteinemia B
ICAM	intercellular adhesion molecule
IFCC	international federation of clinical chemistry and laboratory medicine
IGE-PCV	interaction gène-environment en physiopathologie cardiovasculaire
I κ B	inhibitor of NF- κ B
I K_{CA}	intermediate conductance
IKK	I κ B kinase
IL	Interleukin
IM	intermediate metabolizer
INF	interferon
I/R	ischemic-reperfusion
IPA	Inhibition of platelet aggregation
ISR	in-stent restenosis
iv	Intravenous
JNK	c-Jun NH ₂ -terminal kinase
K _{ATP}	ATP-sensitive K ⁺ channel
K _{IR}	inward rectifying K ⁺ channel
LBD	ligand binding domain
LDL	low density lipoprotein
LETf	liver-enriched transcription factor
LOX	lipoxygenases
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex
LXR	liver X receptor
MACEs	major adverse cardiac events
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein -1
MFI	median fluorescence intensity
MKP-1	MAPK phosphatase-1
MMP	matrix metalloprotease
MiRNA	microRNA
MR	metabolic ratio

mRNA	messenger RNA
MS	metabolizing status
MSC	mesenchymal stem cell
NADP	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear transcription factor-kappa B
NIK	NF-κB-inducing kinase
NO	nitric oxides
NR	nuclear receptor
NSTEMI	non-ST segment elevation myocardial infarction
PAI-1	plasminogen activator inhibitor -1
PAPPA	pregnancy-associated plasma protein A
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PD	pharmacodynamics
PDGF	platelet-derived growth factor
PG	prostaglandin
PGx	pharmacogenetics/pharmacogenomics
PK	pharmacokinetics
PKA	protein kinase A
PKC	protein kinase C
PLA	platelet leukocyte aggregate
PLC	Phospholipase C
PM	poor metabolizer
PNS	peripheral nervous system
POC	point-of-care
POR	nicotinamide adenine dinucleotide phosphate (NADPH)-CYP oxidoreductase
PON-1	paraoxonase-1
PP-2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PPIs	proton pump inhibitors
PRI	platelet reactivity index
PXR	pregnant X receptor
PUFA	polyunsaturated fatty acid
RANTES	regulated on activation normal T-cell expressed and secreted
RBC	red blood cell
RCT	reverse cholesterol transport
RE	receptor element
Rel	reticuloendotheliosis
RHD	Rel homology domain
ROR	retinoid acid-related orphan receptor
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
RXR	retinoid X receptor

SAA	serum amyloid A
SBP	systolic blood pressure
S1P	sphingosine-1-phosphate
SC	STANISLAS cohort
SD	standard deviation
SE	standard error
sEH	soluble epoxide hydrolase
SFS	STANISLAS family study
SER	smooth endoplasmic reticulum
SHP	small heterodimer partner
SK-1	sphingosine kinase-1
SK _{Ca}	small conductance
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SRC-1	steroid receptor activator-1
SRS	substrate recognition site
STEMI	ST segment elevation myocardial infarction
TIF	transcriptional intermediary factor
TF	transcriptional factor
TF	tissue factor
TG	triglyceride
TGF	transforming growth factor
TNF	tumor necrosis factor
t-PA	tissue plasminogen activator
TRP	transient receptor potential
TRPV	vanilloid TRP
TX	thromboxanes
Ub	ubiquitin
UM	ultra-metabolizer
3'UTR	3' untranslated region
VASP	vasodilator-stimulated phosphoprotein
VCAM	vascular cell adhesion molecule
VDR	vitaminD receptor
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WBC	white blood cell

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AVANT PROPOS

The contribution of inflammation to the pathogenesis of diverse disorders, including cardiovascular diseases, has been extensively recognized. In addition, hepatic cytochrome P450 (CYP) is a superfamily of membrane-bound enzymes that plays critical roles in the overall metabolism and disposition of both endogenous compounds and numerous xenobiotics with anti-inflammatory properties. In this thesis, we focused on the potential influences of CYP polymorphisms on the inflammation-reducing effects of epoxyeicosatrienoic acids (EETs), as an endobiotics with known potent anti-inflammatory effects, and thienopyridines (clopidogrel and prasugrel), as anti-platelet medications which have been shown, in some studies, to exert pleiotropic anti-inflammatory effects. Accordingly, we have had a pharmacogenetic approach to the potential anti-inflammatory effects of thienopyridines.

Considering the fact that dual anti-platelet therapy including aspirin and a thienopyridine derivative is the standard of care in patients undergoing percutaneous coronary intervention and also in patients with acute coronary syndrome (ACS) and, therefore, these antiplatelet agents are currently widely prescribed, we decided to investigate the potential influences of thienopyridine pharmacogenomics on their anti-inflammatory effects in the context of a clinical trial. We also aimed to measure the plasma levels of EETs on a subset of the study population. Thus, we prepared a detailed proposal and made great efforts to find a clinical center to run a collaborative study. We have had a large amount of difficulties in this way which we do not intent to report them in detail. However, we have finally succeeded to test our hypotheses on an existing biobanks in Paris and Marseille and I am pleased that I can present my work based on my publications using the results obtained from these joint studies.

I am also delighted to mention that we are currently involved in GWAS on the anti-inflammatory effects of clopidogrel in patients with ACS. The results and relevant papares of this project will be hopefully released within the next few months.

CHAPTER 1 - INTRODUCTION

The introduction of our thesis consists of four main parts. The first part (I) is dedicated to the general definition of pharmacogenetics and personalized medicine and also to an overview of pharmacokinetics, pharmacodynamics and pharmacogenetics of clopidogrel and prasugrel. In this part, we also discussed about the anti-inflammatory properties of thienopyridines. In respect to EETs, in spite of our great efforts and due to the technical and financial problems, we failed to measure plasma levels of EETs in our study populations; however, in the second (II) and third (III) parts of the introduction and in the context of two review papers, we tried to discuss the mechanistic aspects of the potential interaction between CYPs and EETs. In the first article, we demonstrated how the inter-tissue, inter-individual and intra-individual variability in the CYP expression could influence on the EET formation and, subsequently, on the EET anti-inflammatory effects. The second article reviews the molecular aspects of inflammation-reducing effects of EETs and shows how inflammation can potentially modify the production of EETs. The last part of these two reviews, present hypotheses regarding reciprocal interactions between Inflammation and CYP-derived EETs. Further studies are needed to confirm the hypothesis.

The fourth and last part of the introduction (IV) describes how point-of-care genetic testing could be employed for the prediction of response to clopidogrel in the clinical settings.

I. Pharmacogenomics of Anti-inflammatory Effects of Thienopyridines

1. Pharmaco-genetics, -genomics and personalized medicine

The past decade, in particular after completion of the Human Genome Project (1,2), has witnessed an explosion of information regarding genetic susceptibility to complex diseases and genetic-related variability in drug response.

The term pharmacogenetics or pharmacogenomics (PGx) is the study of the associations between the genetics of individuals and their response to drug and deals with genetically determined variation in how individuals respond to medications (3). Indeed, the majority of phase I and phase II drug-metabolizing enzymes (DMEs) are polymorphic (4). These polymorphisms cause important inter-individual differences in drug and metabolite exposure and can influence drug response as well as the risk for adverse drug reactions (5). For instance, nortriptyline, an antidepressant drug, is metabolized in the liver mainly through a cytochrome P450 (CYP) 2D6-dependent pathway by which the drug is metabolized to its pharmacologically active metabolite. The effect of nortriptyline has shown to be markedly reduced in CYP2D6 poor metabolizers, determined by non-functional polymorphisms. Conversely, higher amounts of active metabolite are generated from nortriptyline in CYP2D6 ultra-metabolizers (6) (Figure 1A). In recent years, we have increased our understanding regarding the clinical importance of such variation and several databases containing PGx information regarding DMEs are readily

available (7). Using the PGx data, we anticipate that patient care will move from the one-size-fits-all approach to the treatment of each patient on the individual basis (5) (Figure 1B).

2. P2Y₁₂ receptor antagonists including thienopyridines

Adenosine diphosphate (ADP) plays an important role in the genesis of physiological platelet-rich hemostatic plugs as well as in the formation of pathological arterial thrombi (8). ADP is released from activated platelets, erythrocytes and endothelial cells, and induces platelet adhesion and aggregation. ADP activates platelets by binding to membrane-bound nucleotide receptors on the platelet surface called P2 receptors (9). Human platelets have two major G protein-coupled ADP receptors, the P2Y₁ and P2Y₁₂ receptors. P2Y₁ is a G_q-coupled receptor that initiates ADP-induced platelet aggregation through the stimulation of phospholipase C and phosphatidylinositol-signaling pathway. P2Y₁₂ is a G_i-coupled seven-transmembrane domain receptor, which mediates platelet activation by inhibiting the adenylate cyclase-mediated signaling pathway and decreasing intracellular cAMP levels. It also inhibits PI3K and induces Akt kinase activation (9,10) (Figures 2). The decrease in intracellular cAMP levels reduces the rate of phosphorylation of the vasodilator-stimulated phosphoprotein, thus inducing activation of the GPIIb/IIIa receptor and platelet aggregation (11). P2Y₁₂ plays a central role in amplification and stabilization of ADP-induced platelet aggregation. It is also involved in platelet secretion induced by strong agonists. The important role of the P2Y₁₂ receptor in platelet activation and stable thrombus formation has made it an important target in the management and prevention of arterial thrombosis (9).

Active metabolites of the thienopyridine pro-drugs (ticlopidine, clopidogrel and prasugrel) covalently bind to the P2Y₁₂ receptor and are irreversible, indirect platelet inhibitors. The newer, direct-acting P2Y₁₂ inhibitors (ticagrelor, cangrelor and elinogrel) change the

conformation of the P2Y₁₂ receptor, resulting in reversible, concentration-dependent inhibition of the receptor (9). The pharmacology of all P2Y₁₂ antagonists is summarized in Table 1, respectively.

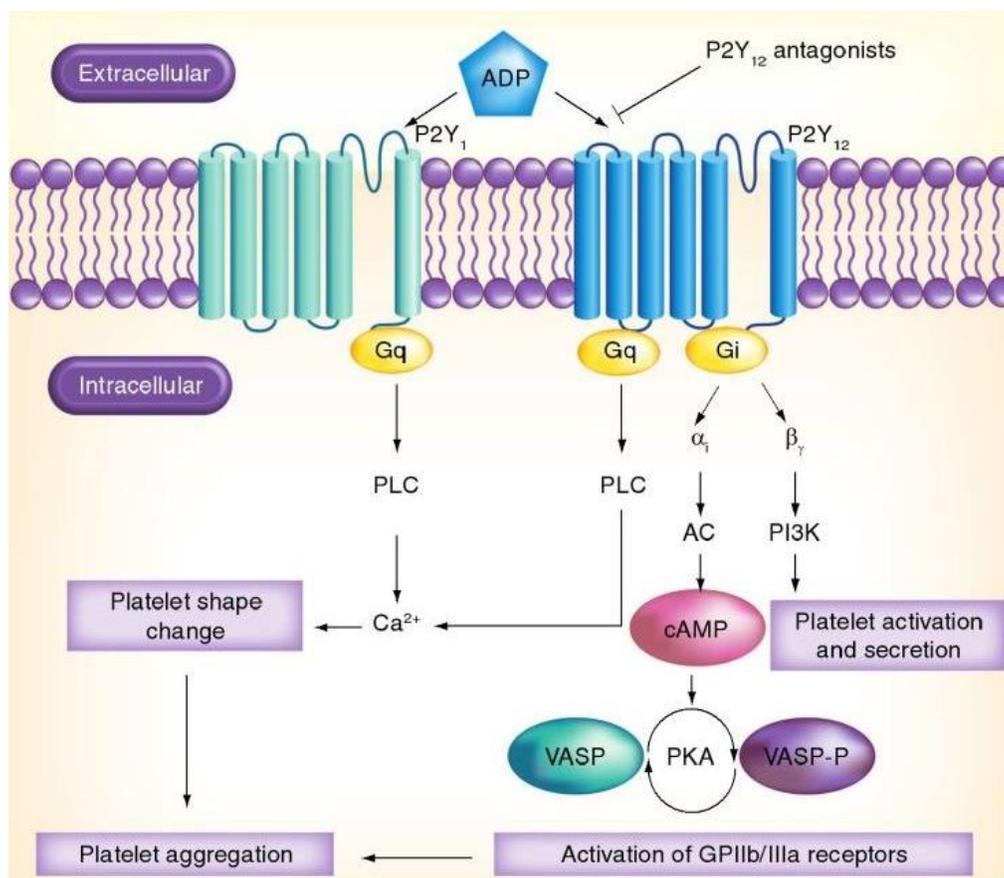


Figure 2. Mechanism of action of the P2Y₁₂ antagonists. P2Y₁ and P2Y₁₂ are G-coupled receptors, which utilize ADP as an agonist. P2Y₁ is a Gq-coupled receptor, which initiates ADP-induced platelet aggregation through the stimulation of PLC and phosphatidylinositol-signaling pathway. P2Y₁₂ is a Gi-coupled 7-transmembrane domain receptor, which mediates platelet activation by inhibiting an AC-mediated signaling pathway and decreasing the cAMP intracellular levels. It also inhibits PI3K and induces Akt kinase activation. The decrease in cAMP intracellular levels reduces the rate of phosphorylation of VASP, thus inducing activation of the GPIIb/IIIa receptor and platelet aggregation. AC: Adenyl cyclase; PLC: Phospholipase C; VASP: Vasodilator-stimulated phosphoprotein; VASP-P: Vasodilator-stimulated phosphoprotein phosphorylation.

Table 1. Characteristics of P2Y₁₂ antagonists.

P2Y ₁₂ antagonist	Type	Administration	Action	Time to peak IPA	Plasma half-life	Loading dose/maintenance	Drug interactions	Resistance
Clopidogrel	<u>Thienopyridine</u>	Oral	Hepatic activation, irreversible inhibition	6–12 h	7–8 h	300–600 mg/ 75 mg/day	Yes	Yes
Prasugrel	<u>Thienopyridine</u>	Oral	Hepatic activation, irreversible inhibition	2 h	7–8 h	60 mg/ 10 mg/day	Yes	Yes/no [†]
Ticagrelor	Cyclopentyl-triazolopyrimidine	Oral	Direct, noncompetitive, reversible inhibition	2 h	6–8 h	180 mg/ 90 mg twice-daily	Not reported	No
Cangrelor	ATP analog	iv.	Direct competitive reversible inhibition	Seconds	3–9 min	30 µg/kg bolus, 4 µg/kg/min, continuous infusion	Not reported	No
Elinogrel	N-[(5-chlorothiophen-2-yl)sulfonyl]N'-{4-[6-fluoro-7(methylamino)-2,4-dioxo-1,4-dihydroquinazolin-3(2H)-yl]phenyl}urea	Oral/iv.	Direct competitive reversible inhibition	4–6 h	12 h	80–120 mg bolus infusion/50–150 mg oral twice-daily	Not reported	No

[†]There are controversial data concerning prasugrel resistance, and there is a need for larger studies to confirm prasugrel resistance. IPA: Inhibition of platelet aggregation; iv.: Intravenous.

2.1. Clopidogrel

Currently, dual antiplatelet therapy with aspirin and clopidogrel is the accepted standard of care in the patients with ACS and those treated with PCI (12). Clopidogrel is a second-generation thienopyridine that has replaced ticlopidine owing to its equivalent efficacy, lower hematologic toxicity (neutropenia, thrombotic thrombocytopenic purpura) and for safety reasons (13). Clopidogrel is an intestinally absorbed prodrug that is converted *in vivo* to its active metabolite. Intestinal absorption of clopidogrel is limited by P-glycoprotein, an efflux pump also known as multidrug resistance protein 1 (MDR1), which is encoded by the *ABCB1* gene. The majority (85%) of the absorbed clopidogrel prodrug is metabolized into inactive metabolites by ubiquitous esterases (Figure 3). The remainder (15%) undergoes activation in the liver by the hepatic cytochrome P-450 (CYP) enzymatic pathway. Clopidogrel activation requires a two-step oxidative process conversion, firstly to an intermediate metabolite, 2-oxo-clopidogrel, which is still inactive, and then to its active thiol metabolite (R130964) (Figure 3). Both steps involve several hepatic CYP isoenzymes, including CYP2C19, CYP3A4/5, CYP2C9, CYP1A2 and CYP2B6 (9). More recently, it was demonstrated that the γ -thiobutyrolactone ring of 2-oxo-clopidogrel could be opened to the active thiol metabolite through hydrolytic cleavage catalyzed by the enzyme paraoxonase-1 (PON-1) and not by oxidation catalyzed by CYP2C19. Furthermore, it has been suggested that PON-1 is a key enzyme for clopidogrel-induced platelet inhibition, and for the drug's clinical efficacy (14,15), although other studies did not support this suggestion (16). The basis of this discrepancy still remains unclear (9). The clopidogrel-active thiol metabolite irreversibly inactivates P2Y₁₂ by forming a disulfide bond with two cysteine residues (Cys17 and Cys270) present in the extracellular domain of the

P2Y₁₂ receptor (9,10). P2Y₁₂ receptor blockade by clopidogrel-active metabolite potently inhibits ADP-induced platelet aggregation and also reduces platelet dense granule secretion. The maximum inhibition of P2Y₁₂ by clopidogrel is observed 4–5 days after daily administration of 75 mg clopidogrel and this delay is due to its need for metabolic activation. A higher clopidogrel dose of 150 mg has been associated with greater platelet inhibition (9,17).

A high inter-individual variability in platelet inhibition by clopidogrel has been observed, and it has been mostly attributed to differences in the extent of clopidogrel pro-drug metabolism. This variability is clinically important since many studies have demonstrated that patients exhibiting high on-treatment platelet reactivity (clopidogrel resistance or clopidogrel hyporesponsiveness) are not adequately protected from major adverse cardiac events (MACEs) (9). It is estimated that approximately one-third of clopidogrel-treated patients exhibit a diminished *ex vivo* platelet response to clopidogrel (8). Clinical, cellular and genetic factors have been associated with clopidogrel resistance. In this regard, the loss-of function *CYP2C19* alleles (*2, *3, *4, *5, *6, *7 and *8), especially the *CYP2C19**2 allele, can attenuate and *CYP2C19**17 can intensify the pharmacodynamic effect and clinical efficacy of clopidogrel (9). In a genetic sub-study of the TRITON-TIMI 38 trial, *CYP2C19**2 allele was associated with a 53% increased risk of the composite ischemic end point and a threefold increase in stent thrombosis in patients receiving clopidogrel (18). Through a collaborative meta-analysis, genomic data and clinical outcomes were extracted from nine clopidogrel trials involving nearly 10,000 patients who were treated predominantly with an invasive strategy. This retrospective analysis demonstrated a significantly increased risk of the composite end point of CV death, MI or stroke in both heterozygotes and homozygotes for reduced-function *CYP2C19* alleles. Similarly, there was an increased risk of stent thrombosis in both groups with the 'hazard' alleles (19).

CYP2C19 loss-of-function alleles do not account for all of the variability in clopidogrel response. Some studies have implicated variants in other genes associated with clopidogrel response (e.g., CES1, ABCB1, CYP2B6, CYP2C9, P2RY12 and PON1); however, these have not been consistently replicated (20). The clopidogrel pharmacogenomics has been comprehensively reviewed by several recently-published review papers (20-25).

However, genetic polymorphisms in genes, which are involved in clopidogrel metabolism and disposition, only partially explain the correlation with suboptimal clopidogrel response. Poor compliance to treatment has been suggested as an important cause of clopidogrel resistance (9,26), and obese or diabetic patients have an increased incidence of clopidogrel hyporesponsiveness and a corresponding increased sensitivity to ADP-induced platelet adhesion and aggregation. Furthermore, intracellular P2Y₁₂-dependent and -independent pathways may also be upregulated in clopidogrel poor responders (9,27). In contrary, several investigators have reported what may be termed a "smoker's paradox", whereby clopidogrel-treated nonsmokers appear to have either less or no CV-event reduction when compared to the substantial CV-event reduction in clopidogrel-treated smokers based on several large-scale trials (28) (see more in "*Discussion*").

The standard 75 mg daily maintenance dose of clopidogrel has proven to be clinically efficacious in reducing CV events in patients with an ACS and in those undergoing percutaneous coronary intervention (PCI) (9). Recently, it has been demonstrated that this clopidogrel dose differentially affects the platelet aggregation and platelet-derived pro-thrombotic and pro-inflammatory mediators in ACS patients undergoing PCI within the first month of the treatment, a phenomenon that is highly influenced by the drug response variability.

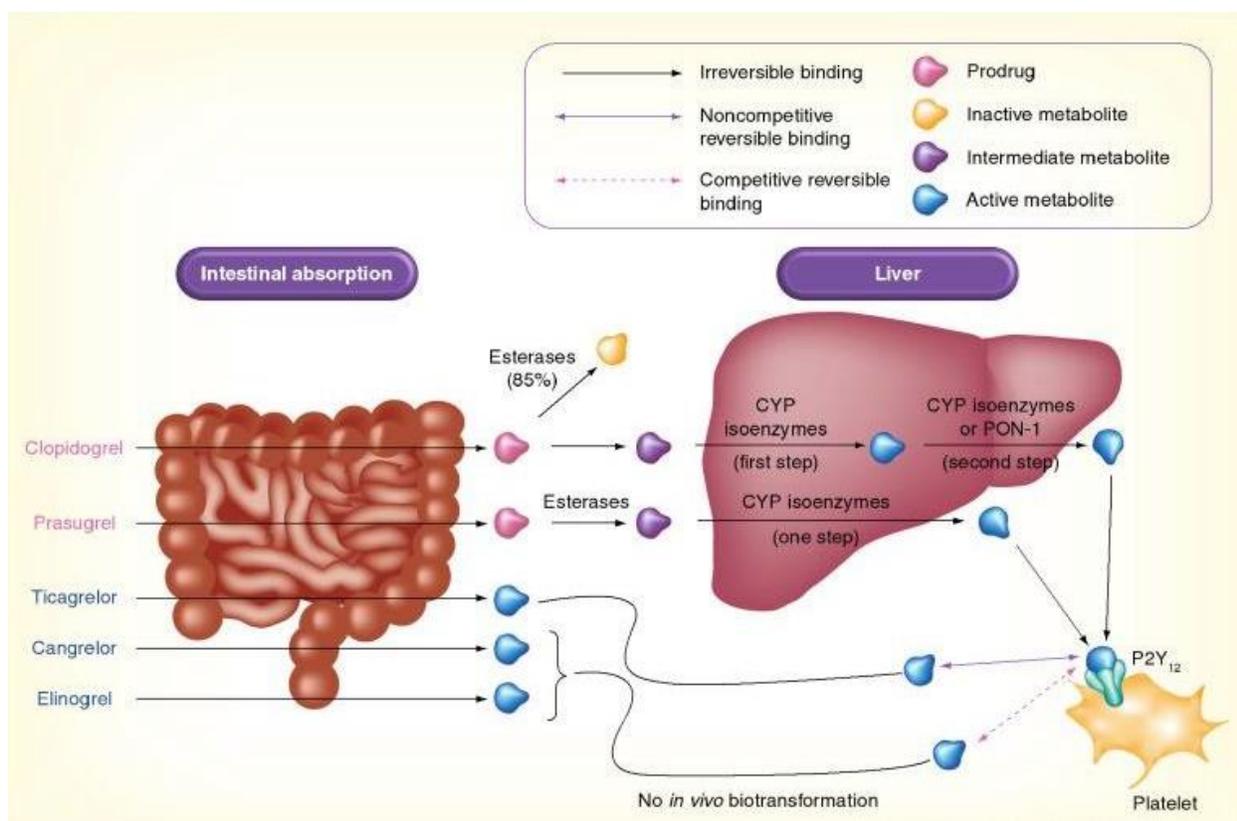


Figure 3. Metabolism of ADP P2Y₁₂ antagonists. The thienopyridines clopidogrel and prasugrel are prodrugs, requiring hepatic metabolism to form their active metabolites, which irreversibly bind to P2Y₁₂. After intestinal absorption, clopidogrel prodrug is metabolized into inactive metabolites by ubiquitous esterases. The remainder (15%) undergoes activation in the liver by the hepatic CYP450 enzymatic pathway. Clopidogrel activation requires a two-step oxidative process conversion, first to 2-oxo-clopidogrel and then to its active thiol metabolite. Both steps involve several hepatic CYP isoenzymes. PON-1 may also participate in the formation of clopidogrel's active thiol metabolite. Prasugrel is a prodrug that first undergoes a rapid de-esterification to an intermediate thiolactone, which is then converted in the liver to the active metabolite by CYP isoenzymes in a single CYP-dependent process. Clopidogrel and prasugrel are irreversible antagonists of the P2Y₁₂ receptor. Ticagrelor is a direct-acting, reversible, noncompetitive antagonist of the P2Y₁₂ receptor, which does not need metabolic activation. Cangrelor and elinogrel cause reversible inhibition of the P2Y₁₂ receptor as well. Both drugs directly and competitively antagonize ADP binding to the P2Y₁₂ receptor without the need for any metabolic activation. CYP: Cytochrome P450; PON-1: Paraoxonase-1.

Moreover, the platelet hyporesponsiveness to clopidogrel in these patients could be overcome within 1 month of treatment (29,30). Other studies have addressed whether doubling the clopidogrel maintenance dose of 75 mg/day could be associated with a lower rate of

hyporesponsiveness and increased clinical benefit (17). The results of the randomized CURRENT OASIS 7 trial in ACS patients showed a non-significant benefit of 150 mg/day over 75 mg/day clopidogrel in the whole population. However, subgroup analysis suggested a benefit of increased maintenance dose of clopidogrel in ACS patients treated with PCI (8). In a recent study involving patients with stable CV disease, it was demonstrated that tripling the maintenance dose of clopidogrel from 75 to 225 mg/day in *CYP2C19**2 heterozygotes achieved levels of platelet reactivity similar to that seen in non-carriers receiving the standard 75 mg/day dose. By contrast, in *CYP2C19**2 homozygotes, clopidogrel doses as high as 300 mg/day did not result in comparable degrees of platelet inhibition (9,31).

Based on the above results, recent studies have attempted to investigate whether tailored treatment of patients with clopidogrel based on the results of platelet function tests could help to overcome clopidogrel resistance (9). In a previous study performed in ACS patients scheduled for PCI who were resistant to 600 mg loading clopidogrel dose (evaluated with the VASP phosphorylation [VASP-P] test), additional loading doses of 600 mg clopidogrel reduced the rate of MACEs (32) or stent thrombosis at 30 days (8). However, the GRAVITAS study showed that although doubling the clopidogrel maintenance dose in poor responders may improve the measured response, it does not necessarily improve outcome (33). Likewise, in a recently performed ARCTIC study (34), no significant improvements in clinical outcomes was observed with platelet-function monitoring and treatment adjustment for coronary stenting, as compared with standard antiplatelet therapy without monitoring. Therefore, the utility of tailored treatment with clopidogrel in the daily clinical practice based on platelet function test results will have to be confirmed in further larger clinical trials. In fact, genetic testing before

starting clopidogrel therapy in high-risk patients, and platelet function testing in those who suffer adverse events, may facilitate the monitoring of clopidogrel treatment (9).

Several drugs that are metabolized by the CYP pathway can competitively inhibit CYP activation of clopidogrel, resulting in attenuated antiplatelet activity (9). Proton pump inhibitors (PPIs) are protective against gastrointestinal complications; however, several pharmacodynamic studies suggest that they may attenuate clopidogrel responsiveness when used concomitantly (9,35). Observational clinical studies, evaluating the possible impact of PPIs on clopidogrel's antiplatelet efficacy, have produced controversial results (36). The COGENT study, the only randomized trial investigating the interaction between clopidogrel and Omeprazole (37), has not shown increased CV risk with drug coadministration in patients with ACS undergoing PCI, whereas a significant reduction in gastrointestinal complications with PPIs use was observed. Since this study was terminated prematurely, it is underpowered to conclude definitive findings. Therefore, concurrent clopidogrel and PPI use appears safe, but coprescription is recommended only for patients at risk for gastrointestinal complications (38).

Early studies suggested an interaction between lipophilic statins and clopidogrel on CYP isoforms (39) and a possible negative effect on clopidogrel's efficacy from the use of these statins (40), possibly due to the CYP3A4 enzymatic pathway shared between statins and clopidogrel. However, it has also been demonstrated that clopidogrel's antiplatelet effectiveness is not influenced by atorvastatin (34) and it was subsequently supported by other studies using point-of-care methods, which indicated a lack of adverse effects during clopidogrel coadministration with lipophilic statins and clinical data (9).

2.1.1. *Anti-inflammatory effects of clopidogrel*

Although clopidogrel primarily inhibits platelet aggregation, it has also been shown to exert pleiotropic anti-inflammatory effects, including a reduction in serum C-reactive protein (CRP) levels as well as a decrease in platelet leukocyte conjugates, P-selectin and CD40L membrane expression, and platelet micro-particle formation, in patients with ischemic vascular diseases and also in post-PCI patients (16,27). The ability of clopidogrel and prasugrel in the reduction of inflammation would be particularly important in the clinical setting since it has been demonstrated that post-PCI inflammatory response is the primary causative mechanism of ISR (42-44), and that elevated inflammatory markers is associated with an increased incidence of adverse cardiac events in post-PCI patients (45). The anti-inflammatory properties of clopidogrel, however, have not been a persistent finding in all trials and the results of different studies in this field are sometimes contradictory (46-75) (Table 2). Looking to the Table 2, it could be realized that there is a decreasing trend in the levels of inflammatory markers in response to clopidogrel; analyzing the studies with followed up period of one/two months after clopidogrel therapy shows that certain studies reported a “decrease” and/or “no-change” in plasma level of CRP in response to clopidogrel. We can also observe in the results of others an increase in the level of CRP, one month after clopidogrel withdrawal. On the other hand, there are also some others indicating an increase in the level of CRP in short time and after one year. With a detailed analysis of CRP variations, we can observe that: 1) the numbers of recruited patients are often small 2) the period of treatment is different; however, often is one month 3) clopidogrel has been given usually as a single daily dose of 75 mg. Aspirin also often, but not in all studies, has been prescribed as a part of dual antiplatelet therapy.

Table 2. Anti-inflammatory effects of thienopyridines

Study	Population, treatment and duration of therapy	Markers assessed	Results
Kuzniatsova et al. (2012)⁴⁶	Patients with CAD (n = 32) taking Clopidogrel (75 mg/d) for two weeks and now discontinued	vWF hs-CRP IL-6 E-selectin P-selectin	Although soluble P-selectin increased when clopidogrel was stopped, there were no changes in plasma markers or vascular function
Smadja et al. (2012)⁴⁷	Healthy male volunteers (n = 28) treated with Clopidogrel 75 mg/d for 7 days	VEGF-A sVEGFR1 sVEGFR2 E-selectin vWF	The 7-day intake of Clopidogrel did not significantly modify the levels of the selected angiogenic factors or inflammatory markers.
Ostad et al. (2011)⁴⁸	Patients with CAD (n = 120) randomly allocated to either Clopidogrel 75 mg/d (n = 40) or ASA 100 mg/d (n = 38) or Clopidogrel + ASA (n = 39) For 28 days	hsCRP MCP-1 RANTES	The serum levels of Inflammatory markers remained unchanged in all treatment groups.
Patti et al. (2011)⁴⁹	PCI patients (n = 50) randomly allocated to either Clopidogrel 75 mg/d (n = 25) or Clopidogrel 150 mg/d (n = 25) for 1 month At this time-point cross-over was performed and treatment continued for 1 month	hs-CRP	hs-CRP values were significantly attenuated from baseline in both groups and plasma hs-CRP was significantly lower in patients receiving 150 mg/d of clopidogrel
Sambu et al. (2011)⁵⁰	PCI patients (n = 33) receiving ASA and Clopidogrel for 1 year after PCI and now discontinued Clopidogrel	hs-CRP IL-6 sCD40	Following Clopidogrel withdrawal, there was a decline in IL-6 and hs-CRP at 1 and 4 week respectively. Also, there was a non-significant increase in sCD-40.
Cekici et al. (2011)⁵¹	Patients with ACS who received a stent were divided into three groups: 20 patients with BMS receiving Clopidogrel for one month (BMS1 group) 20 patients with BMS receiving Clopidogrel for 6 months (BMS6 group) and 20 patients with DES receiving clopidogrel for 6 months (DES group). Hs-CRP was measured at baseline, and then at first, third and sixth post-operative month.	hs-CRP	The initial hs-CRP levels were similar and decreased significantly in all groups by one month of clopidogrel treatment. In the BMS1 group, after the first month, hs-CRP levels increased again at the third and sixth months. In the BMS6 and DES groups, hs-CRP levels continued to show a decrease at the third month and sixth months.
Palmerini et al. (2010)⁵²	STEMI patients undergoing PCI (n = 54) randomly allocated to either Clopidogrel 75 mg/d (n = 24) or Clopidogrel 150 mg/d (n = 24) for 1 month	hs-CRP	Serum hs-CRP decreased in both groups after 1 week and 1 month of treatment.

Husted et al. (2010)⁵³	NSTE-ACS patients (n = 990) randomly allocated to either Clopidogrel 75 mg/d (n = 327) or Ticagrelor 90 mg/BID (n = 334) or Ticagrelor 180 mg/BID (n = 323) for 4 weeks	hs-CRP IL-6 MPO sCD40	Inflammatory biomarker measurements were not significantly different among treatment groups at baseline, discharge and 4 weeks.
Akbulut et al. (2009)⁵⁴	STEMI patients (n = 175) randomly allocated to either Standard reperfusion therapy group without Clopidogrel (n = 90) or Standard reperfusion therapy + Clopidogrel (450 mg loading and 75 mg main.) (n = 85) For 48 hours	hs-CRP	The hs-CRP values were lower in Clopidogrel group
Wykrzykowska et al. (2009)⁵⁵	PCI patients (n = 98) receiving ASA and Clopidogrel for 1 year after PCI and now discontinued Clopidogrel	hs-CRP sP-selectin sCD40	Following Clopidogrel withdrawal, there was a significant increase in sCD-40 at 4 week and P-selectin at 1 and 2 week. Also significant decrease at 1 and 4 week in CRP.
Willerson et al. (2009)⁵⁶	Patients with metabolic syndrome (n = 181) randomly allocated either ASA (81 mg/d + Clopidogrel 75 mg/d) (n = 89) or ASA (81 mg/d + placebo) (n = 92) For 6 weeks	hs-CRP sP-selectin sCD40	There was a significant difference at 6 week in CD-40 levels in patients taking Clopidogrel. No significant change in hs-CRP, sP-selectin and proBNP.
Saw et al. (2008)⁵⁷	PCI patients (n = 26) treated with ASA (325 mg/d) and Clopidogrel (75 mg/d) for 1 year	sCD-40 sP-selectin hs-CRP IL-10 IL-18	sP-selectin remained unchanged at 1 day and 1 month, but became significantly higher at 6 and 12 months. Soluble CD-40 was stable at 1 day and 1 month, but became significantly lower at 6 and 12 months. Serum IL-18 was stable at 1 day and 1 month, but became significantly higher at 6 and 12 months but the 12-month IL-18 level was not significantly higher compared with 1 month. IL-10 levels were unchanged from baseline to all subsequent visits. Hs-CRP was significantly elevated 1 day after PCI but returned to baseline values at all subsequent visits.
Katsaros et al. (2008)⁵⁸	Stable CAD patients undergoing elective PCI (n = 51) 17 patients not pre-treatment with Clopidogrel before PCI were matched with 34 patients who received 300 mg Clopidogrel 12-24 h before PCI	PAI-1 t-PA hs-CRP	PCI induced a significant increase of PAI-1 levels in patients without pre-treatment but the procedure had no effects of PAI-1 in patients pre-treated with Clopidogrel. So, the PAI-1 levels were significantly lower in patients taking Clopidogrel at 24 h. No significant changes in CRP and t-PA.
Braun et al. (2008)⁵⁹	Aspirin-treated patients with stable CAD (n = 110) randomly allocated to either Prasugrel loading dose 60 mg and main. dose of 10 mg/ d or Clopidogrel loading 600 mg and main. dose 75 mg/d For 29 days	P-selectin	After 2 hours of loading doses, P-selectin was significantly lower in Prasugrel group than Clopidogrel. This effect remained through 24 hours and during the maintenance dose period.

Waehe et al. (2006)⁶⁰	<p>Patients with stable angina (n = 37) randomly allocated to either Clopidogrel 75 mg/d (n = 18) or placebo (n = 19) for 10 days 10 healthy controls also recruited</p>	<p>Gene expression of RANTES (in PBMC) MIP-1β (in PBMC) CD63 (in platelet) CD62P (in platelet) And the level of PMP</p>	<p>At baseline, the gene expression of RANTES and MIP-1β (in PMBC) and CD62P and CD63 (in platelets) and the level of PMPs were significantly higher in patients with angina compared with healthy subjects. Platelet markers: CD63 reduced and CD62P and PMP remained unchanged with Clopidogrel therapy. Clopidogrel significantly upregulated the gene expression of RANTES and MIP-1β in PBMC, while no changes were found in the placebo group.</p>
Solheim et al. (2006)⁶¹	<p>Patients on treatment with ASA (160 mg/d) for 7 days with stable CAD (n = 206) randomly allocated to either ASA 160 mg/d (n = 105) or Clopidogrel 75 mg/d (n = 101) for 1 year</p>	<p>hs-CRP IL-6 IL-10 MCP-1 P-selectin TNF-α CD-40 TGF</p>	<p>There were no differences in any variables including changes from baseline to 1 month and 1 year between two groups, but in both group, TNF-α reduced significantly after one year.</p>
Heitzer et al. (2006)⁶²	<p>Patients with symptomatic CAD and long-term ASA therapy (n = 103) allocated to Clopidogrel 75 mg/d (n = 77) or placebo (n = 26) For 5 weeks</p>	<p>hs-CRP sCD40 RANTES</p>	<p>The plasma levels of hs-CRP, sCD-40 and RANTES were reduced significantly in patients on additional treatment with Clopidogrel, but not in patients on placebo.</p>
Chen et al. (2006)⁶³	<p>NSTE-ACS patients (n = 115) randomly allocated to either ASA (300 mg loading dose + 100 mg/d) (n = 58) or ASA (300 mg loading dose + 100 mg/d) and Clopid (300 mg loading + 75 mg/d) (n = 57) For 30 days Also 30 healthy subjects were enrolled as control group.</p>	<p>hs-CRP TNF-α</p>	<p>The baseline level of inflammatory markers was higher in CAD patients compared with healthy subjects. After 7 days of treatment, hs-CRP and TNF-α level decreased significantly in both groups of CAD patients. Also after 30 days, the level of mentioned markers decreased further in both groups. However, after 30 days, significantly lower levels of hs-CRP and TNF-α were observed in Clopidogrel group.</p>
Montalescot G et al. (2006)⁶⁴	<p>NSTE-ACS patients (n = 103) randomly allocated to either Clopidogrel loading dose 300 mg or 600 mg or 900 mg For 24 hours</p>	<p>hs-CRP sCD40L PAI-1 vWF</p>	<p>At 24 h, there was a trend toward an increasing reduction from baseline in hs-CRP with greater Clopidogrel loading dose; although this effect was neither significant at 24 h nor apparent at 6 h. Levels of other markers were not significantly affected by the administration of increasing loading dose of Clopidogrel.</p>

Azar et al. (2006)⁶⁵	Stable CAD patients (n = 73) randomly allocated to either Clopidogrel (300 mg loading dose + 75 mg/d) (n = 37) or placebo (n = 36) for 8 weeks	hs-CRP sCD40L	The level of sCD-40 decreased from baseline at 8 week in the clopidogrel group and remained unchanged in the placebo group. Levels of hs-CRP were not affected by therapy and remained unchanged in both groups.
Angiolillo et al. (2006)⁶⁶	Diabetic patients on long term (12 months) therapy with ASA + Clopidogrel (n = 53) Levels of markers were measured before and 1 month after Clopidogrel withdrawal	hs-CRP P-selectin	A significant increase in both markers was observed following Clopidogrel withdrawal.
Vavuranakis et al. (2006)⁶⁷	NSTE-ACS patients (n = 86) randomly allocated to either ASA 325 mg/d for 1 week, followed by ASA (100 mg/d) + Clopidogrel (300 mg loading dose followed by 100 mg/d) for 36 weeks (ASA + Clopidogrel group) or ASA alone (325 mg/d for 1 week, followed by 75 mg/d for 36 weeks) (ASA group) Biomarker levels were determined on admission and at 8 hours, 48 hours and 6 days of treatment.	hs-CRP P-selectin CD-40	Mean P-selectin levels significantly lower in ASA + Clopidogrel recipients with elevated hs-CRP and sCD40 levels.
Quinn et al. (2004)⁶⁸	PCI patients (n = 74) non-randomly allocated to either Pre-treated with Clopidogrel (>24 h) (n = 31) or no pre-treated with Clopidogrel (n = 43) Markers compared before and after PCI and also 18-24 h later.	IL-6 CD40	Both IL-6 and CD-40 increased significantly at 18-24 h post PCI. Post-procedure serum CD40 were lower in patients pre-treated with Clopidogrel. Also, there was a trend toward lower IL-6 levels with Clopidogrel pre-treatment.
Vivekananthan et al. (2004)⁶⁹	PCI patients (n = 883) allocated to either Pre-treated with Clopidogrel 300-600 mg loading dose for 5 days (n = 280) or no pre-treated with Clopidogrel (n = 553)	hs-CRP	Despite a similar median baseline level of CRP, the mean increase in CRP after PCI was attenuated by 65% with Clopidogrel pre-treatment.
Xiao et al. (2004)⁷⁰	Patients with ACS (n = 23) treated with Clopidogrel 300 mg as loading dose. Markers were compared before and 24 h after Clopidogrel administration.	CD40 P-selectin	Plasma levels of sCD-40 and sP-selectin were reduced significantly (27% and 15%, respectively).
Woodward et al. (2004)⁷¹	Patients with acute MI within the previous 3-7 days (n = 184) randomly allocated to either ASA 75 mg/d (n = 90) or Clopidogrel 75 mg/d (n = 94) For 6 months	hs-CRP Fibrinogen vWF t-PA	By 1 month, vWF and CRP were reduced significantly from baseline in both treatment groups and t-PA in ASA group only. At 6 months, there were no differences between treatment groups for any of the markers.
Klinkhardt et al. (2003)⁷²	Patients with atherosclerotic vascular disease and peripheral occlusions (n = 44) receiving either Clopidogrel (n = 12) or ASA (n = 17) or combination (n = 8) or no treatment (n = 7) as well as in a group of healthy subjects (n = 9)	PLA P-selectin and MAC-1 expression And plasma level of ICAM-1	P-selectin and MAC-1 expression and PLA formation significantly reduced by Clopidogrel and Clopidogrel + ASA. Soluble plasma ICAM-1 levels significantly reduced by Clopidogrel, ASA, and Clopidogrel + ASA.

Klinkhardt et al. (2002) ⁷³	Healthy volunteers (n = 10)	PLA P-selectin expression	Expression of P-selectin and formation of PLA significantly reduced by Clopidogrel.
Chew et al. (2001) ⁷⁴	PCI patients (n = 860) allocated to either Pre-treated with Clopidogrel defined either as 300 mg loading dose on the day of procedure or 75 mg/d loading dose over several days or if Clopidogrel was part of the patient regular treatment (n = 565) no pre-treated with Clopidogrel (n = 295) For 30 days	hs-CRP	Clopidogrel pretreatment was not associated with a significant reduction in 30-day death or AMI within the overall population. However, by CRP quartiles, the association between increasing CRP and 30-day events remained evident among patients not receiving pretreatment but was attenuated in the pre-treated subgroup. Pretreatment with clopidogrel was associated with a 58% relative risk reduction in 30-day death or AMI in the highest quartile.

Abbreviation;

ACS: acute coronary syndrome; CAD: coronary artery disease; hsCRP: high sensitivity C - reactive protein; MCP-1: monocyte chemoattractant protein -1; NSTEMI: non-ST segment elevation; PAI-1: plasminogen activator inhibitor -1; PCI: percutaneous coronary intervention; STEMI: ST segment elevation MI; t-PA: tissue plasminogen activator; TNF- α : tumor necrosis factor alpha; vWF: Von Willebrand factor; PLA: platelet leukocyte aggregate; ICAM-1: intercellular cell adhesion molecule-1; ASA acetylsalicylic acid (Aspirin); IL: interleukin; RANTES: regulated upon activation, normal T-cell expressed, and secreted

Based on all mentioned above, it would be logical and makes sense to speculate that, potentially and theoretically, the same genetic variations in genes involved in clopidogrel PK/PD, are also responsible of the observed discrepancy in pleiotropic anti-inflammatory effects of clopidogrel in different studies; the hypothesis which has never been evaluated.

2.2. Prasugrel

Prasugrel is a new thienopyridine, which exhibits a more rapid antiplatelet effect *ex vivo* compared with clopidogrel owing to its faster metabolic activation (9). Like clopidogrel, prasugrel is a pro-drug that first undergoes a rapid de-esterification to an intermediate thiolactone, which is then converted in the liver to the active metabolite in a single CYP-dependent step involving CYP3A, CYP2B6, CYP2C9 and CYP2C19 (10) (Figure 3). Prasugrel's active metabolite appears in the circulation within 15 min after a 60-mg loading dose and reaches maximal plasma concentration at 30 min. In fact, among patients undergoing cardiac catheterization with planned PCI, loading with 60 mg prasugrel resulted in greater platelet inhibition than a 600-mg clopidogrel loading dose. Furthermore, a maintenance prasugrel dose of 10 mg/day results in a more potent and consistent inhibition of platelet activation than the standard clopidogrel maintenance doses of 75 or 150 mg/day (9,76).

The TRITON-TIMI 38 trial showed that in patients with ACS with scheduled PCI, prasugrel (60 mg loading dose followed by a 10 mg/day maintenance dose) compared with clopidogrel (300 mg loading dose and a 75 mg/day maintenance dose) significantly reduced rates of ischemic events, including stent thrombosis, but with an increased risk of major bleeding, including fatal bleeding (8). The SWAP study evaluated the pharmacodynamic response of switching patients on maintenance clopidogrel therapy after an ACS to prasugrel. This study assessed the pharmacodynamics and tolerability of a prasugrel 10-mg/day maintenance

dose administered immediately after clopidogrel 75 mg/day with or without prasugrel loading in patients treated with clopidogrel after an ACS event (9). Authors concluded that switching from 75 mg/day clopidogrel to 10 mg/day prasugrel did not influence the existing platelet inhibition in the first 24 h, but it significantly increased platelet inhibition 1 week later, as was determined by multiple assays including light transmission aggregometry, the VASP-P test and VerifyNow-P2Y₁₂ (9). Furthermore, when prasugrel was administered as a 60-mg loading dose, a rapid and marked decrease in platelet aggregation was observed within 2 h. Importantly, switching from clopidogrel to prasugrel was well tolerated without major safety events (77). The prasugrel-induced inhibition of platelet activation is not characterized by the interindividual variability observed for clopidogrel. Recent pharmacogenetic analyses in the TRITON-TIMI 38 trial showed that individuals with the *ABCB1* T3435T genotype exhibited reduced platelet inhibition and are at increased risk of recurrent ischemic events during clopidogrel treatment. By contrast, in patients treated with prasugrel, the *ABCB1* C3435T polymorphisms were not significantly associated with CV outcomes. Likewise, in healthy participants no associations between the C3435T variant and pharmacokinetic and pharmacodynamic outcomes were seen with prasugrel (78). These results suggest that although the intestinal P-glycoprotein efflux pump is involved in prasugrel absorption, the rapid metabolic activation of prasugrel may mitigate the genetic effect of *ABCB1* C3435T polymorphisms. Other studies have demonstrated that the *CYP2C19* polymorphism does not influence the pharmacodynamic or clinical efficacy of prasugrel (9,79). A recent study determined the antiplatelet effects of prasugrel versus high-dose clopidogrel in patients with high on-treatment platelet reactivity to clopidogrel after PCI as well as their relation to *CYP2C19**2 carriage. The results showed that in patients with high on-treatment platelet reactivity after PCI, prasugrel is more effective compared with high-

dose clopidogrel in reducing platelet reactivity, particularly in *CYP2C19*2* carriers (9,80). These findings are in concert with the minor role of CYP2C19 in prasugrel metabolic activation. Indeed the main contributors to the conversion of prasugrel to its active metabolite are CYP3A4 and CYP2B6. The CYP3A4 gene is not very polymorphic, whereas no clear association has been shown with CYP2B6 variants and prasugrel function (9,10).

Finally, a recent study suggested that although prasugrel significantly reduces thrombotic events in patients with ACS undergoing PCI because of its superior antiplatelet potency compared with clopidogrel, a significant number of patients exhibited high on-treatment platelet reactivity assessed with the VASP-P method. These patients have a higher risk for MACE during the 1-month follow-up period after PCI (81). Larger trials are necessary to confirm the existence of platelet resistance to prasugrel and to describe the possible underlining mechanisms (9).

2.2.1. Anti-inflammatory effects of prasugrel

In a few studies, prasugrel have also shown to have more potent inhibitory effects on inflammatory process (82,83); however, compared to clopidogrel, anti-inflammatory properties of prasugrel have been less explored. Moreover, any possible association between genetic variability in prasugrel PK/PD involved genes and its anti-inflammatory function have not been assessed.

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II. Influences of Inter-tissue, Inter-individual and Intra-individual Variability in the Expression of Human Cytochrome P450 Epoxygenases on Epoxyeicosatrienoic Acids-mediated Anti-inflammation

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Abstract

Beyond their contribution to the metabolism of xenobiotics, cytochrome P450 (CYP) epoxygenases actively are involved in the metabolism of endogenous substances such as arachidonic acid (AA). The main human CYP epoxygenases, i.e. CYP2C8, CYP2C9, CYP2C19 and CYP2J2, convert AA to four regioisomer epoxyeicosatrienoic acids (EETs). EETs possess a wide range of established protective effects on human cardiovascular system of which anti-inflammatory actions have gained great interest, recently. The expression of CYP epoxygenases is regulated through an extremely complex network of nuclear receptors, microRNAs and genetic/epigenetic factors. Accordingly, a large number of biological variables as well as xenobiotics and environmental factors can influence the expression of CYP epoxygenases, resulting in a significant intra- and inter-individual variability in the expression and activity of these enzymes and subsequently in the EET biosynthesis. Also, although human CYP epoxygenases are mainly expressed in the liver, most extrahepatic tissues are also capable to express epoxygenase isoforms. However, there is marked inter-tissue variability in the expression of epoxygenase enzymes and the EET formation. The variability in the expression of CYP enzymes may lead to differences in the relative abundance of EETs between tissues, in a given individual under various conditions and also between individuals of a population and/or different ethnics which may explain, at least in part, the inter-tissue and inter-individual difference in the prevalence of inflammation-related disorders, like cardiovascular diseases. Likewise, this may provide a reasonable explanation as to why in a given individual, various conditions can contribute to the development of the diseases of which inflammation has known to occur.

Key words: Inflammation, cytochrome P450, epoxygenase, epoxyeicosatrienoic acids, inter-tissue variability, intra-individual variability, inter-individual variability

II. Influences de la variabilité intertissulaire, interindividuelle et intraindividuelle dans l'expression des époxygénases du cytochrome P450 humain sur l'action anti-inflammatoire médiée par les acides époxy-eicosatriénoïques

Les époxygénases du cytochrome P450 (CYP), au-delà de leur contribution au métabolisme des xénobiotiques, sont activement impliquées dans celui de substances endogènes telles que l'acide arachidonique (AA). Les principales époxygénases du CYP humain – à savoir, CYP2C8, CYP2C9, CYP2C19 et CYP2J2 – convertissent l'AA en quatre acides époxy-eicosatriénoïques (EET) régioisomères. Les EET possèdent un large éventail d'effets protecteurs établis sur le système cardiovasculaire humain, parmi lesquels les actions anti-inflammatoires ont suscité beaucoup d'intérêt dernièrement. L'expression des époxygénases du CYP est régulée via un réseau extrêmement complexe de récepteurs nucléaires, micro-ARN et facteurs génétiques / épigénétiques. Un grand nombre de variables biologiques, ainsi que de xénobiotiques et de facteurs environnementaux, peut dès lors influencer l'expression des époxygénases du CYP, ce qui se traduit par une variabilité intra- et interindividuelle significative dans l'expression et l'activité de ces enzymes et ultérieurement dans la biosynthèse des EET. Et aussi, bien que les époxygénases du CYP humain soient principalement exprimées dans le foie, la plupart des tissus extrahépatiques ont également la capacité d'exprimer des isoformes d'époxygénases. Il existe cependant une nette variabilité intertissulaire au niveau de l'expression des enzymes époxygénases et de la formation d'EET. La variabilité dans l'expression des enzymes du CYP peut être à l'origine de différences d'abondance relative des EET d'un tissu à l'autre, chez un individu donné dans diverses conditions ainsi également qu'entre individus d'une population et/ou d'ethnies différentes, ce qui expliquerait peut-être – du moins en partie – la différence intertissulaire et interindividuelle de la prévalence des troubles liés à l'inflammation, comme les maladies cardiovasculaires. De même, ceci pourrait fournir une explication raisonnable de pourquoi, chez un individu donné, diverses conditions peuvent contribuer au développement de pathologies où la présence d'une inflammation est connue.

Mots clés : Inflammation, cytochrome P450, époxygénase, acides époxy-eicosatriénoïques, variabilité intertissulaire, variabilité intraindividuelle, variabilité interindividuelle

1. Introduction

Inflammation is a complex and highly orchestrated process that has a domino-like effect on the initiation and development of a wide range of systemic illnesses including cardiovascular disease (CVD) (1). Our knowledge about the contribution of inflammation to the development of CVDs has progressed to the point that, currently, coronary artery disease (CAD) and arterial hypertension (HTN) are believed to be diseases of inflammation characterized by interaction among platelets, leukocytes and endothelial cells (ECs) (2-4).

Growing body of evidence suggests that, beyond their well-established contribution to the metabolism of xenobiotics, cytochrome P450 (CYP) epoxygenase enzymes play an indispensable role in the regulation of inflammation thorough the biosynthesis of endogenous bioactive lipid mediators that modulate inflammation, such as arachidonic acid (AA) derivatives, epoxyeicosatrienoic acids (EETs) (5,6).

Even though human CYP epoxygenases are predominantly expressed in the liver, they are also expressed, on a smaller scale and in a heterogeneous pattern, in extrahepatic tissues (7), resulting in an inter-tissue variability in the CYP epoxygenase expression in a given individual. In addition, a large number of factors have been demonstrated to affect the expression pattern of CYP genes, leading to significant inter- and intra-individual differences in the expression levels of CYP epoxygenases. These heterogeneities could potentially give rise to a marked inter-tissue, inter- and intra-individual variability in the production and relative abundance of EETs and, subsequently, in the EET-mediated inflammation-reduction. Given the importance of inflammation in the pathophysiology of a wide range of CVDs, variability in the anti-inflammatory effects of EETs may have clinical consequences.

In this review, with particular emphasis on human epoxygenases, we first provide an overview of the structure, activity and regulation of expression of human CYPs as well as the

mechanism of synthesis and action of CYP-dependent AA metabolites, i.e. EETs. The review then presents in some detail, the factors affecting the expression of CYP epoxygenase genes and resulting in an inter- and intra-individual heterogeneity in the epoxygenase-mediated EET formation. Next, we discuss the potential influence of variation in the relative expression of CYP epoxygenase genes in various human organs on the local/systemic abundance and biological functions of EETs in a given individual. Lastly, the possible clinical implication of the inter-tissue, intra-individual and inter-individual variability in the epoxygenase expression and the EET production will be discussed.

2. Cytochrome P450, Epoxyeicosatrienoic acids; General considerations

CYP enzymes belong to the ubiquitous superfamily of hundreds of closely related hemoproteins found throughout the phylogenetic spectrum, from animals, plants and fungi to bacteria (8). According to their amino acid sequence homology, CYPs are classified into families and subfamilies, that is, if the similarity in the full length of their amino acid structure is more than 40%, the CYPs belong to the same “family” and if two CYPs have more than 55% of the cDNA-derived amino acid in common, they are thought to be in the same “subfamily” (9); two “isoforms” have 97% similarity in their general sequence (10). The nomenclature for cytochrome P450s is based on naming “cytochrome P450” with CYP followed by a number indicating the gene family (such as CYP1, CYP2, CYP3, etc.), a letter indicating the subfamily (i.e. CYP1A, CYP2A, CYP2B, CYP2C, etc.) and a number for the gene that identify the so named isoform (i.e. CYP1A1, CYP1A2) (10). Of more than 6500 genes and 780 CYP families which have been found in nature in total, only about 115 CYP genes, including 57 putatively active genes and 58 pseudogenes have been identified in human (11), and are

grouped into 18 and 44 families and subfamilies, respectively (<http://drnelson.uthsc.edu/human.P450.table.html>).

2.1. CYP enzyme; structure

Most mammalian CYPs are anchored in the lipid membrane of smooth endoplasmic reticulum (SER) microenvironment by a single N-terminal transmembrane α -helix, being partially embedded in the lipophilic membrane of the SER and their access channels are actually positioned inside the membrane ready to receive substrates, which are normally lipophilic (9,12).

The structure of CYP has been described in detailed, previously (9,13). Briefly, CYPs share a common overall fold and topology (Figure 1); CYP enzymes possess an “active site” consisting of 1) a “heme group” (also known as ferriprotoporphyrin-9), which is located deep inside the protein and is crucial for its catalytic activity, and also of 2) more superficially “substrate recognition sites” (SRS) (also known as binding areas) which holds the substrate in a proper orientation capable to present the molecule to the structure of the enzyme that catalyzes the reactions (13,14).

The iron-contained heme group of the active-site area is a quite rigid and highly conserved part of their structure and is hold in place by a conserved structural core which is formed by a four- α -helix-bundle composed of three parallel helices labeled D, L, and I and one anti-parallel helix E (13). The heme moiety has a highly specialized lattice structure that supports an iron molecule, which is the core of the enzyme and is the responsible of the substrate oxidation. The iron atom is normally bound to five other molecules which keep it secured; four of them are pyrrole nitrogens and hold it in the horizontal plane while the fifth group, a sulphur atom from a cysteine amino acid residue links the iron in a vertical plane. This

structure, known as the pentacoordinate state, represents the resting state, whilst the hexacoordinate state occurs when the iron binds another ligand. In this latter configuration, the iron atom appears to move 'upwards' and draws level with the nitrogens to bind a water molecule which is hydrogen bonded to an amino acid residue and is involved in the movement of protons during the catalytic activity (14).

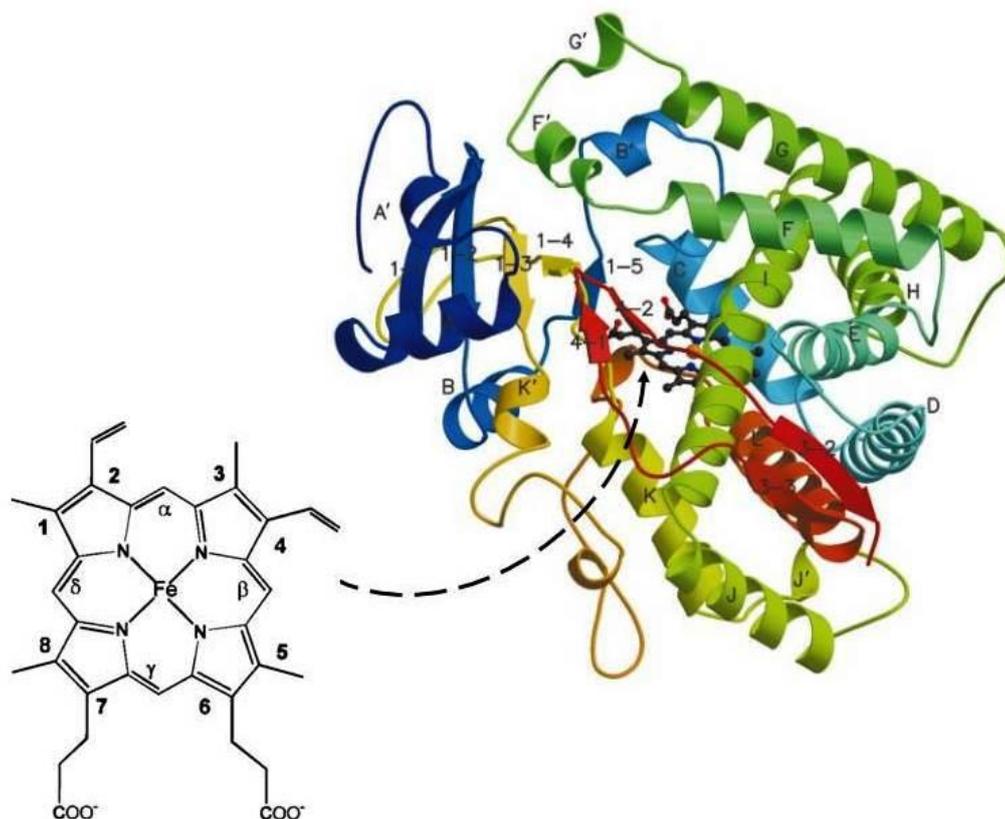


Figure 1. Ribbon representation (distal face) of cytochrome P450s fold (α -Helices are labeled with capital letters) and heme group chemical structure.

On the other hand, using state-of-the-art crystallographic methods, dimensions and other structural characteristics of the SRSs of different CYPs have been determined. It has been demonstrated that, unlike the heme-iron group area, thanks to the hydrophobic pockets in the active sites as well as the partial flexibility of the α -helices covering the SRSs, CYPs can undergo significant conformational changes and/or internal rearrangement in their active

areas which make them able to accommodate various substrates of different sizes, simultaneously; a phenomenon called “allotropism” (9,15,16). The hydrophobic pockets contain numerous amino acid residues which can bind a substrate molecule using hydrogen bindings and van der Waals forces and also by forming pi-pi stacking interactions with substrate phenyl groups (9). A light local rearrangement and also, in some cases, major conformational changes in the residues’ position, may provide a new binding pocket allowing the CYP to accommodate multiple ligands, concurrently (9); this can be best exemplified by CYP2C9 (17) and CYP3A4 (9,18), respectively. CYP2C9 metabolizes S-warfarin to inactive metabolites (19). The relevant crystallographic studies revealed that S-warfarin lies in a predominantly hydrophobic pocket lined by residues Phe 476, Phe 100 and Pro 367 (Figure 2A) (17). Although the binding of S-warfarin to CYP2C9 appears not to induce major conformational changes within the protein, some local rearrangements could be observed. For example, the presence of the compound displaces slightly the loop containing residues 474–478 by 0.5–1.5 Å. This trivial displacement, however, raises the intriguing possibility for additional small molecules, either another molecule of S-warfarin (Figure 2B) or a different molecule such as fluconazole, to simultaneously bind within the active site (17). The flexible α -helices of CYP3A4, on the other hand, can be induced to make a significant conformational change to extent the active site to bind a larger substance (9,18); the SRSs of CYP3A4 can be increased by 80% to accommodate erythromycin (9). This kinetic behavior is a typical property of the enzymes representing multiple SRSs, as CYPs, and is basically due to the conformational or chemical changes that occur in the active site of the enzyme after binding a first substrate (the effector). These changes in the CYP tertiary and quaternary structure may result in the alteration of enzyme catalytic activity which can consequently affect the

metabolism of the second substrate. In addition to CYP2C9 and CYP3A4, the allotropic phenomenon has been described for CYP1A2, CYP2E1 and CYP2D6 (15).

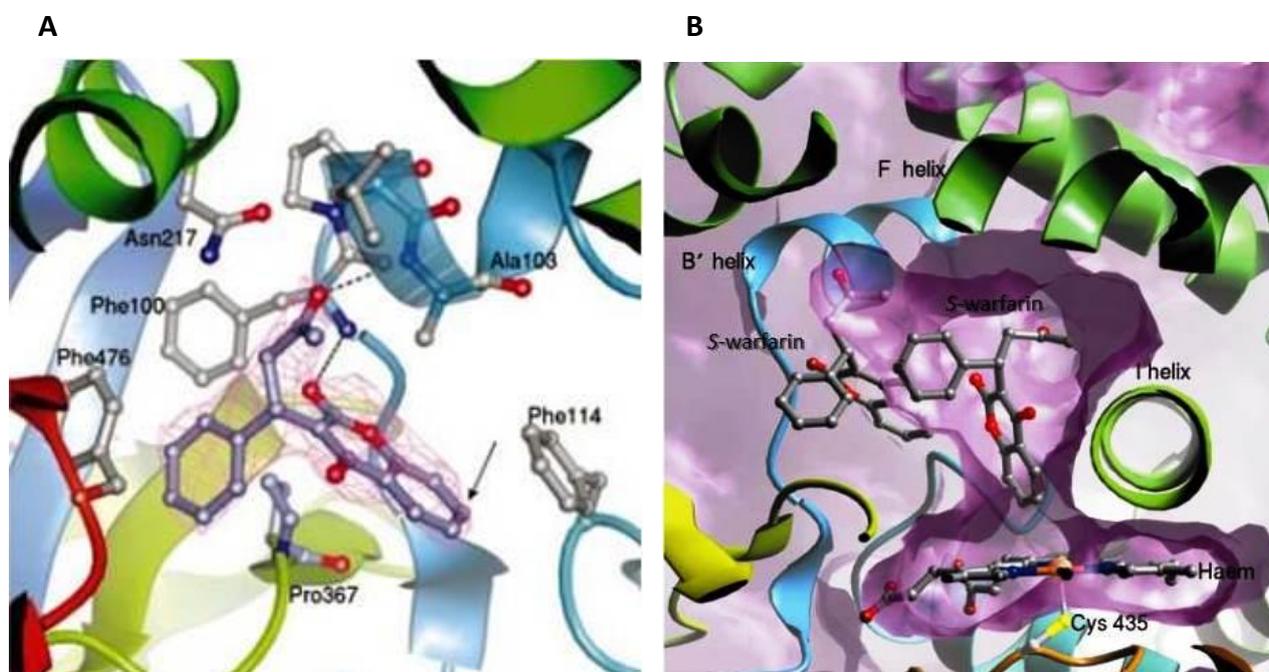
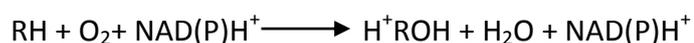


Figure 2. A. Crystal structure of human CYP2C9 that shows stereo view of the binding site of S-warfarin in CYP2C9. **B.** The surface of the active site of CYP2C9. The active site of CYP2C9 is large and could potentially accommodate another molecule of S-warfarin without substantial conformational movement. Part A is adapted from Ref. 17. Part B and C are reproduced with permission from ref. 17.

2.2. CYP enzyme; catalytic activity

Even though there exists a considerable diversity and multiplicity in the structure of CYPs, interestingly, all these enzymes essentially function in the same way (9). Generally, CYP enzymes have the capability to catalyze diverse reactions such as hydroxylation, dealkylation, reductive/oxidative dehalogenation, epoxide/oxide reduction and isomerization; however, their main function is monooxygenation i.e. to insert an oxygen molecule into a usually stable and hydrophobic compound. The CYP-catalyzed monooxygenation reaction has been thoroughly described and reviewed by Coleman (9). Briefly, the process consists of five main features including 1) substrate binding and

reduction; 2) oxygen binding; 3) oxygen scission; 4) insertion of oxygen into the substrate; and 5) release of product. As illustrated in Figure 3, the general CYP catalytic reaction is carried out as the following equation:



The substrate binds to the active site of CYP, in close proximity to the heme group and on the side opposite to the peptide chain and induces a change in the conformation of the active site as often displacing a water molecule from the distal axial coordination position of the heme iron (20), and/or changing the state of the heme iron from low-spin to high-spin (21). The change in the electronic state of the active site facilitates the transfer of an electron from nicotinamide adenine dinucleotide phosphate (NADPH)-CYP oxidoreductase (POR) (22), to the heme iron which is usually in ferric form (Fe^{+3}) to be reduced to its ferrous state (Fe^{2+}) (9). POR is a flavoprotein localized in the ER as the sole electron donor for all microsomal CYP enzymes (23,24). The POR protein contains 2 flavins: adenine dinucleotide (FAD) and mononucleotide (FMN). These flavins are located in 2 separate lobes of POR (25,26). Electrons pass from NADPH through FAD to FMN in the POR protein. At this point, a conformational change occurs in which the FMN-binding domain of the POR interacts with P450 enzymes and donates electrons to the CYP heme iron (Fe^{3+}) (25,27). Although reducing heme iron is indispensable, CYPs containing ferric heme iron can initiate catalysis by both directly binding to a substrate, and then reducing to ferrous state by POR, or first reducing to ferrous state by POR, and then binding to a substrate (28,29). After the first electron reduction, molecular oxygen binds covalently to the distal axial coordination position of the heme iron and then a second electron must be donated by POR reducing the dioxygen

adduct to a negatively charged peroxy group (O-O) (9,28). The peroxy group then is rapidly protonated twice by local transfer from water or from surrounding amino-acid side-chains, releasing one water molecule. The final step is the product formation and release. After the product has been released from the active site, the enzyme returns to its original state, with a water molecule returning to occupy the distal coordination position of the iron nucleus (9,28,29).

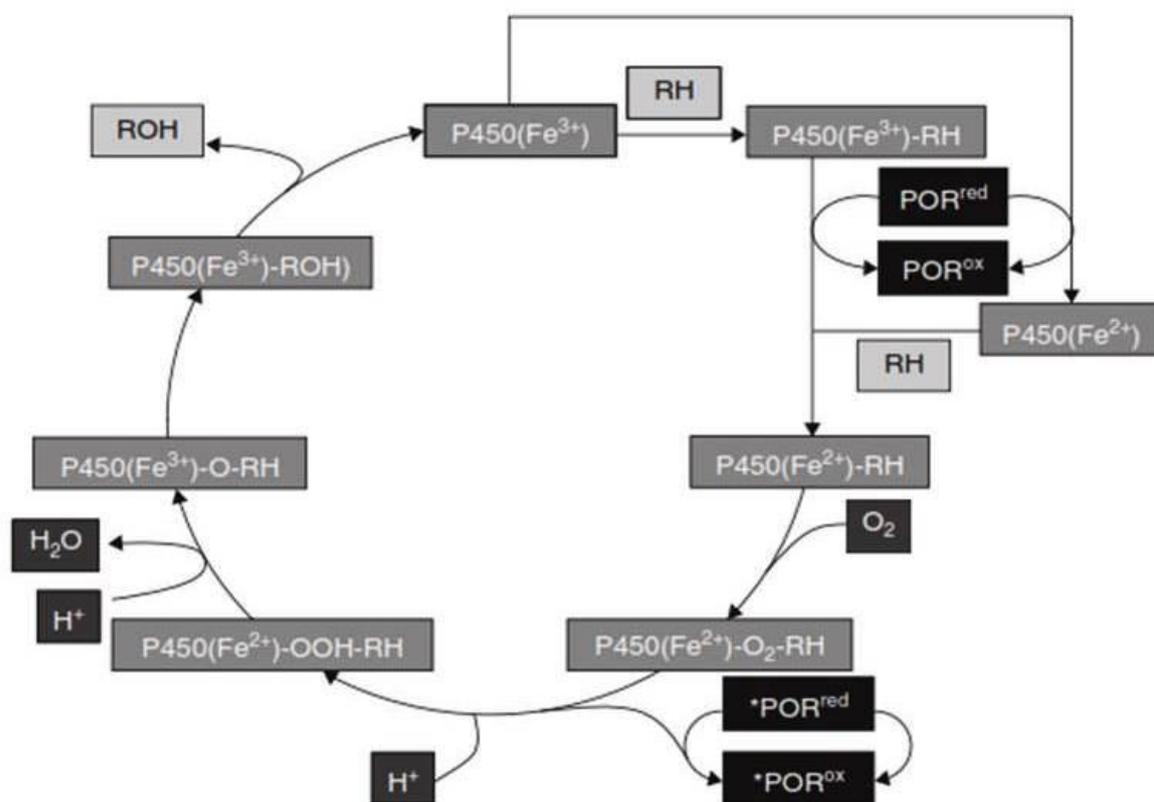


Figure 3. The CYP catalytic cycle. Figure is reproduced with permission from Ref. 29.

2.3. Human CYP epoxygenases; EET formation

CYP enzymes, particularly those in the CYP1, CYP2 and CYP3 genes families, catalyze the biotransformation of 75% of xenobiotics and pharmaceuticals in human (7,30). In addition, human CYPs play central roles in the overall metabolism and disposition of a wide range of

endogenous chemicals including steroid hormones (estrogen and testosterone), vitamin D, bilirubin, cholesterol and fatty acids (6).

AA is a 20-carbon, omega-6 polyunsaturated fatty acid (PUFA) that resides in the cell membrane phospholipids in the stereospecific numbering (*sn*)-2 position and is released upon stimulation by the cytosolic enzyme phospholipase A₂ (31). The free intracellular AA is converted to a series of biological active metabolites referred to as “eicosanoids” (32). Cyclooxygenases catalyze the biosynthesis of prostaglandins/thromboxanes and lipoxygenases mediate the production of leukotrienes (33,34). Referred to the “third eicosanoid enzymatic pathway”, AA is also metabolized to EETs and hydroxyeicosatetraenoic acids (HETEs) by CYP epoxygenases and CYP hydroxylases, respectively (35,36).

CYP epoxygenase enzymes belong to a complex superfamily of genes with a common evolutionary origin, a conserved peptide that provides them with a cysteine heme ligand, and the capacity to take an active form of atomic oxygen to ground state carbons (32). Indeed, CYP epoxygenases catalyze the epoxidation of four olefin bonds of the intracellular AA that gives rise to four corresponding regioisomers: 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET (37) (Figure 4). Several CYP subfamilies including CYP1A, CYP2B, CYP2C, CYP2E and CYP2J have been classified as CYP epoxygenases (36,38), and potentially contribute to the conversion of AA to EETs; however, human epoxygenases are predominantly members of CYP2C and CYP2J classes (34,36,38,39,40). In particular, CYP2C8, CYP2C9, CYP2C19 and CYP2J2 isoforms have been identified as the main CYPs involved in the biotransformation of AA to EETs in human (41-43). Each epoxygenase is, supposedly, capable to convert AA to all four EETs; however, 11,12- and 14,15-EETs are, in real, the main products in many cases (36,44,45). Furthermore, there is mounting evidence that human CYP epoxygenases are relatively regioselective for EET formation. For instance CYP2C8 catalyze the biosynthesis of

14,15-EET and 11,12-EET in a ratio of 1.3:1.0 whereas its capability for the formation of 8,9-EET and 5,6-EET is negligible. The CYP2C9 also generates 14,15-EET and 11,12-EET in a ratio of 2.3:1.0; this epoxygenase, however, does not form significant amounts of 8,9-EET (46,47).

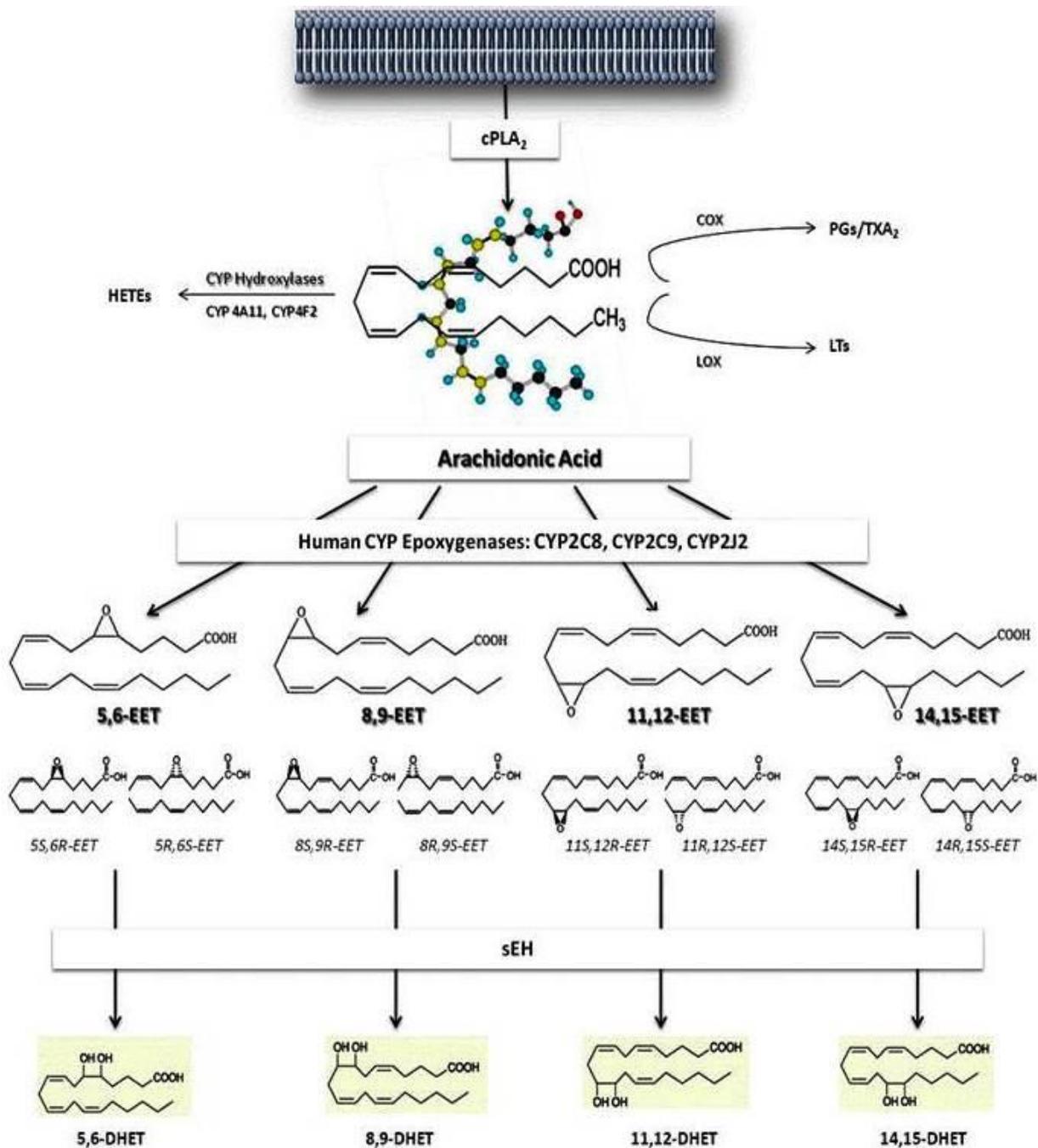


Figure 4. Biosynthetic pathways of arachidonic acid. CYP: cytochrome P450; COX: cyclooxygenase; EET: epoxyeicosatrienoic acid; HETE: hydroxyeicosatetraenoic acids; PG: prostaglandin; PLA: phospholipase A; LOX: Lipoxygenases; LT: leukotriene; sEH: soluble epoxyhydrolase; TX: thromboxane

2.4. *Physiological roles of EETs in vascular biology*

There is a large volume of evidence that EETs possess a wide range of potent cardiovascular protective effects including regulation of vascular tone (41,48-50), and homeostasis (31,32,37,41,42). Figure 5 is a schematic illustration of the protective effects and the postulated mechanism of action of EETs on the myocardium and vasculature. It long has been known that EETs act as endothelium-derived hyperpolarizing factors (EDHFs) and exhibit vasodilatory effects in various arterial territories including coronary, renal, mesenteric and cerebral vasculatures (31,32,41,42). In addition, EETs stimulate EC proliferation and angiogenesis, protect ECs from apoptosis (31,51), have anti-migratory effects on smooth muscle cells (SMCs) (31,37,52) and also possess potent anti-inflammatory properties (5,31,37,53). With a particular focus on their anti-inflammatory effects, we have previously reviewed various biological functions of EETs in the cardiovascular system(54).

A number of original studies suggested that EETs exert their effects, principally, in an autocrine and paracrine manner. However, further investigations demonstrated that blood cells, mainly red blood cells (RBCs) and platelets are importance sources of EETs (32,56-60), showing that on a wider spectrum of autocrine and paracrine agents, EETs could be potentially considered as “endocrine hormones”.

Interestingly, it has been indicated that the threshold concentration of exogenously applied EETs for exerting a specific biological response, differs across their vascular effects; while vasodilation and inhibition of vascular cell adhesion molecule 1 (VCAM-1) expression occur at low physiological concentrations (10^{-9} M and $<10^{-8}$ M, respectively), at least 10^{-7} M of EETs are required to exert fibrinolytic, anti-platelet and pro-angiogenic effects and SMC anti-migratory and anti-apoptotic properties of EETs occur at their greater concentrations i.e. at 10^{-6} M and 10^{-5} M, respectively (41).

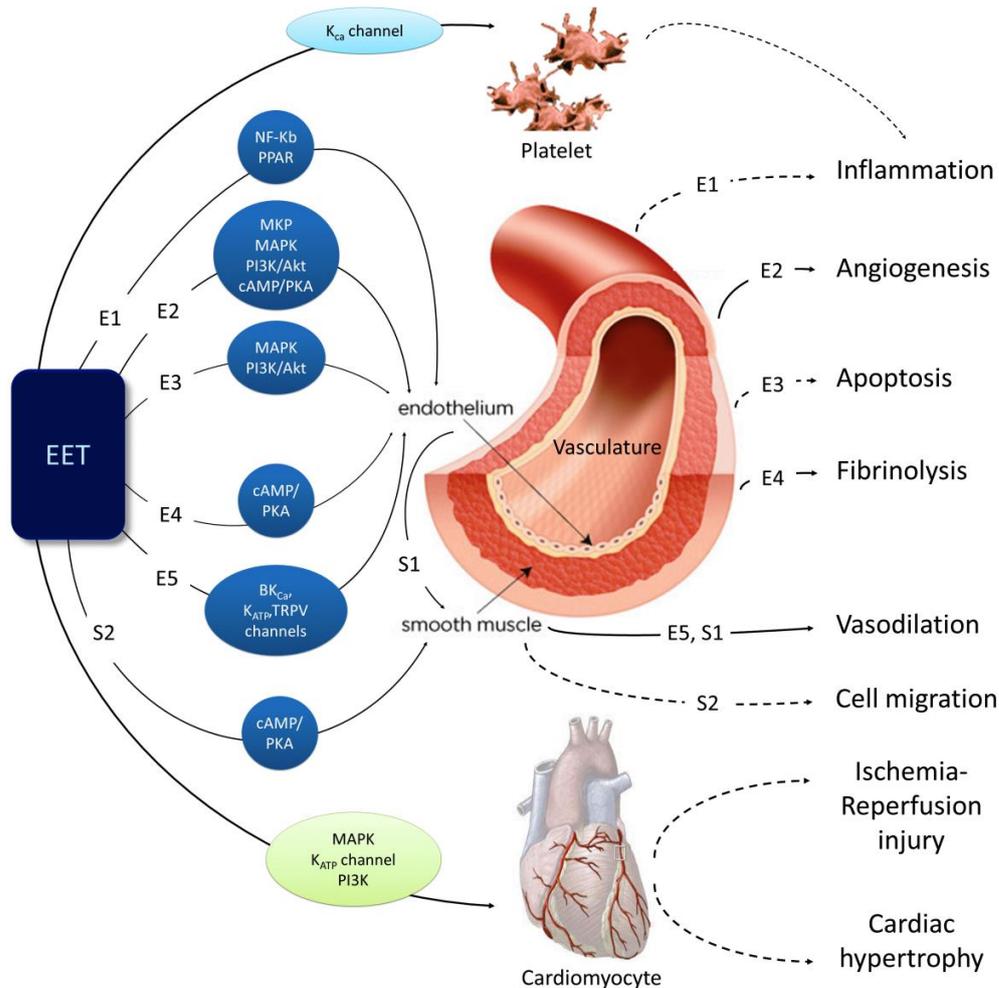


Figure 5. The main biological functions of EETs on the cardiovascular system and the main corresponding cellular signaling pathways. Solid line represents stimulatory influence and dash line represents inhibitory influence. «E» represents the ECs pathway and «S» represents the SMCs pathways. Akt (Akt is not an abbreviation): protein kinase B; ATP: adenosine triphosphate; cAMP-response-element binding protein; BK_{Ca}: Ca²⁺-activated K⁺ channel; EET: epoxyeicosatrienoic acid; K_{ATP} TRPV: ATP-sensitive K⁺ channel vanilloid transient receptor potential; MAPK: mitogen-activated protein kinase; MKP-1: MAPK phosphatase-1; NF-κB: nuclear transcription factor-kappa B; PI3K: phosphatidylinositol 3'-kinase; PPAR: peroxisome proliferator-activated receptor

2.5. Nuclear receptor-mediated transcriptional regulation of CYP epoxygenase genes

Accumulating lines of evidence suggests that the constitutive expression as well as xenobiotic-induced transcriptional activation of most CYP genes is regulated, chiefly, via a complex network of several ligand-dependent nuclear receptors (NRs), transcriptional

factors (TFs) and nuclear coactivators and corepressors (61,62). The NRs are a class of highly conserved proteins that transform extracellular and intracellular signals into cellular responses by triggering the transcription of target genes involved in cell growth, differentiation, lipid homeostasis, inflammation and immunity (63-65). To date, 49 members of the NR superfamily have been identified in the human genome (66). Members of NR family share a relatively common structure containing a DNA binding domain (DBD), which binds the receptors to specific promoter regions of the target genes, and a ligand binding domain (LBD) that permits the NRs to directly interact with the ligands (67,68). Moreover, NRs harbor two regions of ligand-dependent activation function (AF), AF-1 and AF-2, located in the N-terminal and C-terminal (within the LBD region of NR), respectively (69). The NR ligands, which are either endogenously synthesized small molecules (such as bile acids, phospholipids, steroid hormones, thyroid hormone, retinoids and vitamin D), or a variety of xenobiotics (including drugs, carcinogens, food additives, pesticides and environmental chemicals and pollutants), provoke a conformational change in the AF-2 upon binding to the LBD. This conformational change alters the cellular location of the NRs and/or their interaction with cofactors, which ultimately results in up-regulation or down-regulation of gene expression (69). However, for half of the NRs found in man, which are referred as “*orphan receptors*”, no relevant activators or ligands have yet been identified (70).

Several NRs such as Pregnant X receptor (PXR, NR1I2), Constitutive Androstane receptor (CAR, NR1I3), liver X receptor (LXR, NR1H3), Farnesoid X receptor (FXR, NR1H4), small heterodimer partner (SHP, NR0B2), VitaminD receptor (VDR, NR1I1), chicken ovalbumin upstream promoter transcription factor (COUPTF, NR2F1) and glucocorticoid receptor (GR, NR3C1), in interaction with Hepatocyte Nuclear Factor 4 α (HNF4 α , NR2A1), regulate, in a complex manner, the xenobiotic-induced expression of CYP gene families 2 and 3 in the

human liver (62, 70-79). In general, HNF4 α and other members of liver-enriched transcription factors (LETFs), including HNF1 α , HNF2 α (CCAAT/enhancer-binding protein α (C/EBP α)), HNF3 γ (FOXA) and HNF6, have been shown to regulate the constitutive expression of CYP2C genes (61), and the retinoid acid-related orphan receptors (RORs) have also been found as receptors which regulate CYP2C8, but not CYP2C9 and CYP2C19 (80). Unlike CYP2 and CYP3 families, xenobiotic-mediated induction of the expression of CYP1 family is regulated by Aryl hydrocarbon receptor (AhR) (76, 81). The ligand-mediated activation of NRs is mostly carried out in cytoplasm where unliganded inactive NRs reside. However, in some cases, like LXR, NR is localized and activated in nucleus (62).

Given the importance of CYP2C and CYP2J2 enzymes in the biosynthesis of EETs, it is essential to understand the molecular regulatory mechanisms of the constitutive and inducible expression of these enzymes to better understand how the regulatory mechanisms could provide a fundamental basis for the inter-tissue, intra-, and inter-individual variability in the expression of human epoxygenases and EET formation. Accordingly, the transcriptional modulation of CYP2C and CYP2J2 is briefly reviewed here.

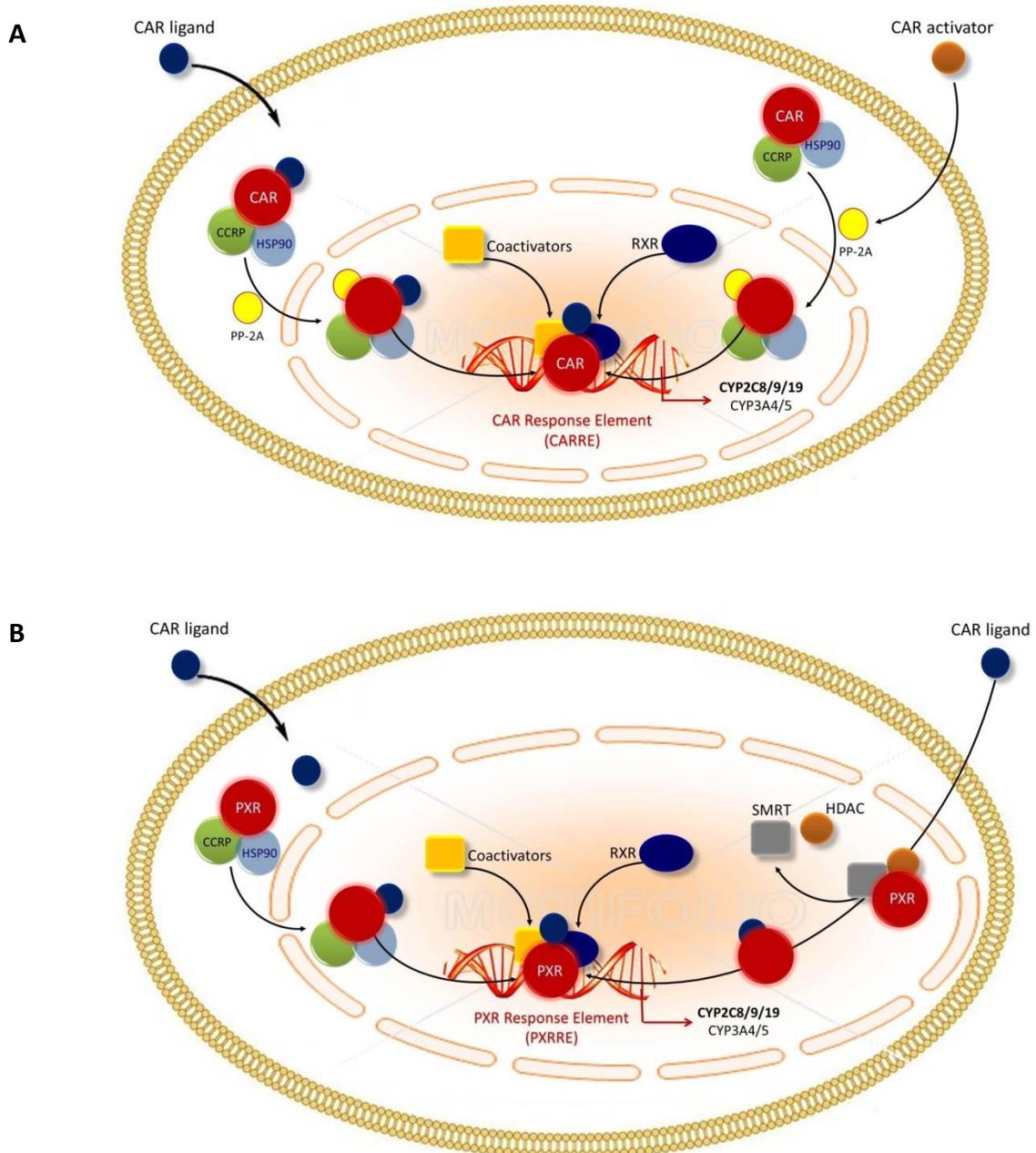
Information from *in vitro* studies in isolated human primary hepatocytes reveals that hepatic CYP2C genes are highly inducible (2-8 fold) at the levels of mRNA, protein and activity, with CYP2C8 being the most inducible gene followed by CYP2C9 and CYP2C19 (62, 82-84). CYP2C genes have been reported to be induced by a wide range of xenobiotic and endobiotic compounds. While all three CYP genes are induced by some molecules like rifampicin, phenobarbital, dexamethasone and hyperofin (an active ingredient of St. John's wort) (62), CYP2C genes are generally induced in an inducer-specific manner, as discussed later.

Many NRs have been identified as the xenobiotic-induced mediators of the transcriptional activation of human CYP2C genes. In particular, the ligand-activated NRs CAR, PXR and GR

are the main responsible for the transcriptional upregulation of CYP2C8, CYP2C9 and CYP2C19 (61,62,85,86); CAR agonists include drugs such as phenobarbital, phenytoin and artemisinin as well as the chemical CITCO {6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl oxime)}; rifampicin, artemisinin and hyperforin act as ligands for PXR; and dexamethasone can activate GR (61). In addition, the ligand-activated NRs Estrogen receptor α (ER α , NR3A1) (87), the transcription factors HNF3 γ (FOXA3) (88) and GATA-4 (89), have been shown to mediate induction of CYP2C19 gene. Also, VDR has been reported to contribute in the induction of CYP2C8 and CYP2C9 genes in the human hepatocytes by lithocholic acid and 1 α , 25-dihydroxyvitamin D₃, respectively (90,91). Detailed information on the structure and NR-specificity of >250 endobiotics and xenobiotics acting as NR ligands has been previously provided and is accessible in the Nuclear Receptor Signaling Atlas (<http://www.nursa.org/>) (63).

The mechanism of ligand-mediated CAR, PXR and GR activation and that of the subsequent transactivation of CYP2C genes have been schematically illustrated in Figure 6A, 6B and 6C, respectively. In contrast to PXR and GR, where activation depends exclusively on ligand-binding, CAR can be activated either directly via ligand binding or through a ligand-independent pathway. In fact, CAR mostly is constitutively active without ligand and many xenobiotics mediate its nuclear translocation instead of acting as real ligands (92-94). CAR is located in the cytosol in a complex with chaperones such as heat shock protein 90 (HSP90), cytoplasmic CAR retention protein (CCRP) and PPP1R16A (62). Xenobiotic CAR activators, like phenobarbital, trigger indirectly, cytoplasmic-nuclear translocation of unliganded CAR by recruiting protein phosphatase 2A (PP-2A) to the CAR/CCRP/HSP90/PPP1R16A complex. Seemingly, PP-2A is also responsible for ligand-triggered CAR nuclear translocation. In the nucleus, CAR dissociates from the complex and binds to retinoid X receptor (RXR) to form

CAR/RXR heterodimer and recruit coactivators. Coactivators are a class of protein factors which do not bind to DNA directly but interact with DNA binding TFs and recruit histone acetyltransferases and methyltransferases to the promoter region where NRs bind, termed response element (RE), allowing access of general transcriptional machinery to the promoter of the target genes (CYP2C8/9, CYP2C19 and CYP3A4/5). Binding the ligand/CAR-RXR heterodimer/coactivators complex to CARRE promotes the transcriptional expression of target gene by activation of RNA polymerase II (62,95).



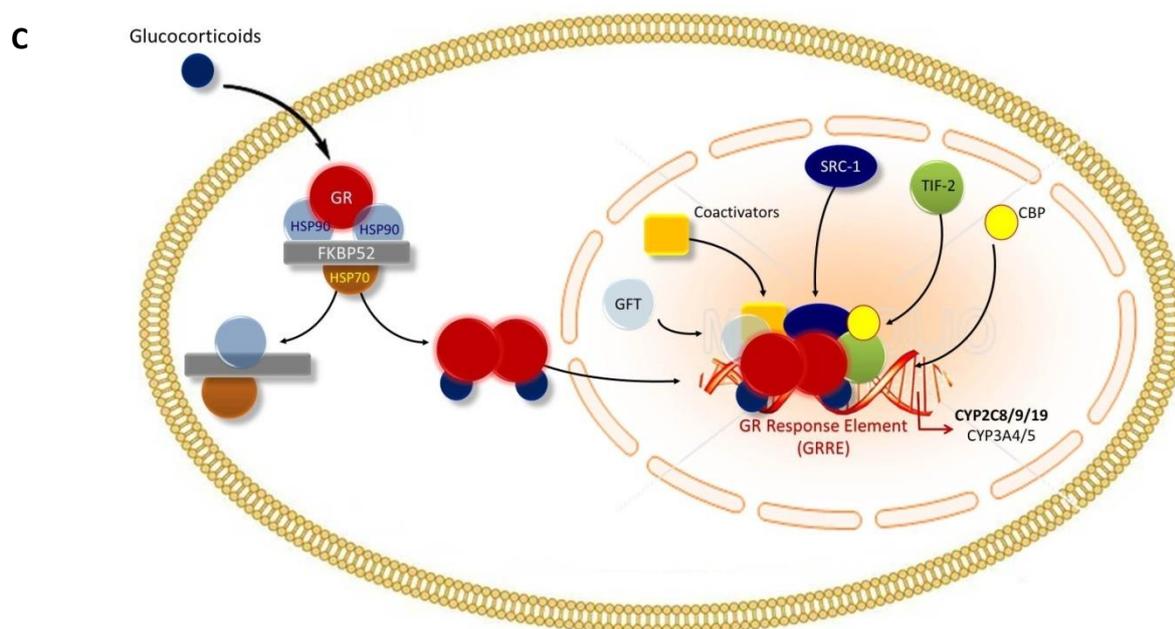


Figure 6. Schematic of CAR (A), PXR (B) and GR (C) nuclear receptor transactivation pathways. CAR: constitutive androstane receptor; CCRP: cytoplasmic CAR retention protein; GR: glucocorticoid receptor; GTF: general transcription factors; HDAC: histone deacetylases; HSP: heat shock protein; PXR: pregnant X receptor; RE: receptor element; PP-2A: protein phosphatase 2A; SMRT: Nuclear receptor corepressor 2 (NCOR2), silencing mediator for retinoid and thyroid hormone receptors; SRC-1: steroid receptor activator-1; TIF-2: transcriptional intermediary factor 2

In the NR superfamily tree, PXR is the closest relative to CAR (64); this might be the reason that PXR and CAR share similar mechanisms of xenobiotic-mediated gene activation and also coordinately regulate a largely overlapping set of CYP genes. Akin to CAR, PXR is found either in the cytoplasm as PXR/CCRP/HSP90 complex or in the nucleus as PXR/SMRT (NCOR2) corepressor/histone deacetylases (HDACs) complex. Ligand binding to PXR triggers translocation to the nucleus where it dissociates from the corepressor/HDACs and binds to RXR (forming heterodimer) and coactivators. The ligand/PXR/RXR/coactivators complex binds to PXRRE which eventually leads to a RNA polymerase II-mediated increase in the transcriptional expression of target genes (i.e. CYP2C8/9/19 and CYP3A4/5) (62,96,97). It is of note that there exists a feedback regulatory mechanism for PXR-mediated transcriptional

activation of CYP3A4 gene. In the absence of xenobiotic-induced PXR activation (unliganded PXR), SHR inhibits PXR-mediated transactivation of CYP3A4. Ligand-activated PXR, however, reduces the expression of SHR and consequently the inhibitory effect of SHR is abolished and CYP3A4 transcription is increased (62). Unlike CAR, PXR and VDR that form heterodimers with RXR, GR forms homodimers which are recognized GRREs within the CYP2C promoters. In the absence of ligand, GR resides in the cytoplasm as a complex of GR/HSP90/HSP70/FK506 binding proteins. Ligand binding to GR triggers dissociation of chaperone proteins, dimerization and translocation to the nucleus, where GR selectively recruits several coactivators such as steroid receptor activator-1 (SRC-1), transcriptional intermediary factor 2 (TIF-2), p300/CBP and general transcription factors (GTFs). The ligand/GR/SRC-1/TIF-2/P300/CBP/GTF complex activated RNA polymerase II and also acylates histone which eventually leads to the upregulation of DNA expression (62).

The REs of the CYP genes exhibit similar but distinct features; Figure 7 depicts the known REs within the CYP2C8, CYP2C9 and CYP2C19 upstream promoter regions that are binding sites for the known NRs which have been shown to be involved in the transcriptional activation of these genes (61,85,91,98-101). Both CYP2C9 and CYP2C19 promoters possess a similar proximal CAR/PXR RE located at -1839/-1824 and -1892/-1877 from the translation start point of CYP2C9 and CYP2C19, respectively (85). CYP2C9 also contains a second CAR/PXR RE in the more upstream region at -2897/-2881 (99). Interestingly, although both CAR/PXR REs contribute to activation of the CYP2C9 promoter, it appears that the former (at -1839) is more important since mutation at the distal site (-2897) alone has been shown to only decrease the activated PXR-mediated activation of CYP2C9 by 30%, while mutation at -1839 alone nearly abolishes the PXR-regulated transactivation of CYP2C9 (85). It also holds true for mutation of the CAR/PXR RE at -1892/-1877 of the CYP2C19 promoter which completely

abolishes activation of the CYP2C19 by activated CAR/PXR (61). Similar to CYP2C9, there are two CAR/PXR REs on the CYP2C8 promoter at -2796/-2780 and -8805/-8790 with the former being situated in a similar location of the latter CAR/PXR RE of the CYP2C9 promoter. Likewise, mutation of the CAR/PXR binding site at -2796bp does not affect significantly the CAR/PXR-mediated activation of the CYP2C8 promoter but mutation of CYP2C8 promoter in the far upstream (at -8805) halts activation of the CYP2C8 by the ligand-activated PXR and CAR in human hepatocytes (100). It is of great interest that there exists a symmetrical cross-talk between CAR and PXR in transcriptional activation of human CYP2C genes, i.e., the CAR/PXR REs in the promoters of all three human CYP2C genes are activated by either CAR or PXR (61). Likewise, a similar cross-talk exists between VDR and CAR/PXR for the expression of CYP2C9 (61).

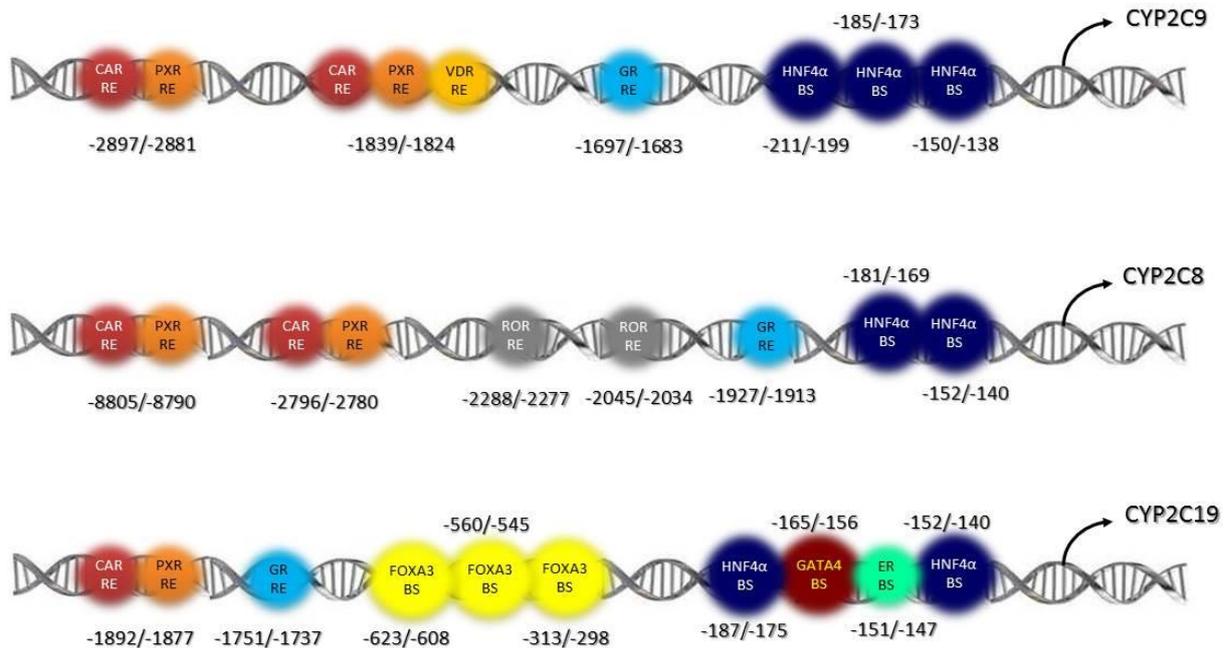


Figure 7. The known Res for NRs regulating CYP2C8, Cyp2C9 and CYP2C19 genes. Figure adapted from Ref. 61.

The three CYP2C genes, CYP2C8/9 and CYP2C19, have also been found to be the target genes for GR. The GRRE at -1697/-1682, -1927/-1913, -1751/-1737, respectively, in the promoter region of CYP2C9, CYP2C8 and CYP2C19 have been demonstrated to be functionally active and mutations of GRREs within CYP2C promoters, abolish the dexamethasone-GR mediated induction of these genes (98,100,101).

As alluded earlier, in addition to CAR, PXR and GR, ligand activated nuclear receptor ER has been recently demonstrated to contribute, specifically, to the regulation of the transcription of CYP2C19 (86). The ERRE is located in the CYP2C19 promoter at -151/-147, and estrogen derivatives, including 17- β estradiol and 17- α ethinylestradiol, can downregulate CYP2C19 expression via ER-mediated interaction with the ERRE in the CYP2C19 promoter site. This effect decreases, but does not abolish, with mutation of this element (87).

In general, the constitutive expression of CYP2Cs is mainly under the control of LETFs (61). Particularly, the significant contribution of C/EBPs and FOXA3 in the maintenance of expression of CYP2C genes and that of RORs in the transcriptional activation of CYP2C8 has been reported, previously (61,88,102). C/EBPs are basic leucine zipper (bZIP) TFs with a DNA binding basic region and a leucine zipper dimerization domain (61). It has been demonstrated that all CYP2C genes have a C/EBP-specific RE in the 5' flanking region of their promoters (103), and that the deletion of this element is associated with a significant decrease in the transcriptional activity of the CYP2C9 promoters (104). Likewise, FOXA3, that is a member of the forkhead box/winged helix family of TFs (61,88), has an important role in regulating the constitutive expression of CYP2Cs so that the promoter studies revealed that FOXA3 can activate the promoter activity of CYP2C8, CYP2C9 and CYP2C19 by 25-fold, 4-fold and 4-fold, respectively (88). Several FOXA3 REs have been identified in the 5' flanking region of all three CYP2C promoters. For instance, it has been shown that the CYP2C19 promoter

harbors three FOXA-3 functional sites at -313/-298, -560/-545 and -623/-608 (88). Whether the mutations in the FOXA3 REs affect the expression of CYP2Cs remains to be elucidated.

Additionally, GATA-4, that is a member of the zinc-finger TF family, has been found to be important specifically in the maintenance of transcription of CYP2C19. Two REs of GATA-4 have been detected in the CYP2C19 promoter at -165/-162 and -159/-156 (89); deletion mutation in these REs results in completely abolished transcription (89). In addition to all mentioned NRs which have been found to be significantly contribute specifically to the expression of CYP2C19, following TFs and REs have been discovered recently within the promoter of CYP2C19 which may be important in the transcription of this gene: - ATF-2 (-806 to -786), CEBP- β (-1505/-1491 and -1443/-1429), CDP repressor protein (-105/-87), GATA-1 (-103/-91) and an additional GRE (-828/-810); however, further studies are needed to elucidate the functionality of these factors (105).

There is mounting evidence that, beyond their direct and discrete regulatory effects on the transcription of target genes, NRs often cooperate with each other and with other factors such as TFs, coactivators and corepressors, within a highly integrated and intricate network, to achieve maximum induction of the target genes (61,106). Notably, it has been shown that NR HNF4 α is at the center of this network (107,108). Known as a master regulator of liver-specific gene expression (106), HNF4 α has been identified to play a pivotal regulatory role in the constitutive and xenobiotic-induced expression of CYP2C8, CYP2C9 and, to a lesser extent, of CYP2C19 (61,109-111). In this way, several NRs interact with HNF4 α to regulate the expression of CYP2C genes in a complex manner. Specifically, HNF4 α has been found to be involved in crosstalk with CAR and PXR signalling pathways on the upregulation of CYP2C8 (100), and CYP2C9 (112), and also to synergize with CAR/PXR in the induction of CYP2C promoters.

To date, three HNF4 α binding sites have been identified in the CYP2C9 promoter (at -150/-138, -185/-173 and -211/-199) (61,111,112). Mutation of these binding elements is clearly associated with the abolition of the drug-induced transactivation of CYP2C9 mediated by CAR and PXR, indicating that HNF4 α is required for the drug responsiveness of the CYP2C9 promoter (61). Because of the distance between the drug responsive elements (e.g. CARRE, PXRE, GRRE) and HNF4 α binding sites in the CYP2C9 promoter, there is apparently an indirect crosstalk between the receptors, probably through a coactivators or other TFs, which modulate the synergistic activation of the CYP2C9 gene by HNF4 α and CAR/PXR (61). Specifically, it has been shown that NCoA6 provides a bridge between and interacts with both the HNF4 α binding sites and CARREs in the CYP2C9 promoter (113) (Figure 8). Of note is that knockdown of NCoA6, disrupts this bridge and reduces the additive effects of HNF4 α and CAR on the induction of the expression of CYP2C9 gene (61).

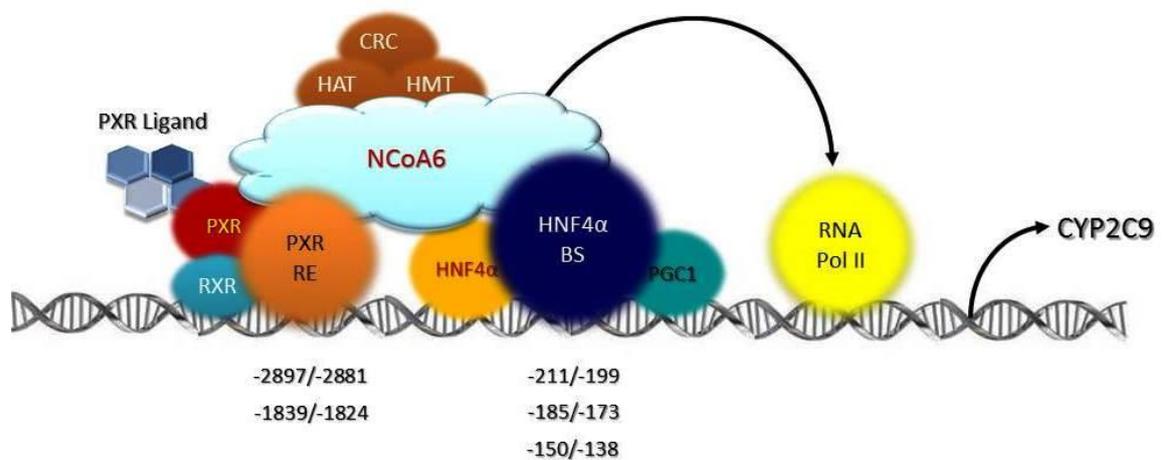


Figure 8. Interaction between NRs, coactivators and REs precisely regulates the transcription of CYP2C9 gene.

In addition to CYP2C9, both CYP2C8 and CYP2C19 have been demonstrated thus far to harbor two identical HNF4 α binding elements in their promoters (at -152/-140 and -181/-169 for CYP2C8; at -152/-140 and -187/-175 for CYP2C19) (61,100,111).

It is of great interest that NRs not only co-regulate the transcription of the target genes (e.g. CYP2Cs), but also regulate the expression of each other (61,106). For instance, glucocorticoid-activated GR has been shown to induce the expression of CAR, PXR and RXR through GRREs within the promoter region of these NRs, resulting in an indirect enhancement in the expression of CAR/PXR target genes including CYP2Cs (114-116). Likewise, HNF4 α has been shown to have regulatory effects on the expression of several NRs such as CAR, PXR, FXR and SHP (117-121). In sum, it could be concluded that in addition to a protein-protein interaction, there is a protein-promoter interaction between HNF4 α and other NRs (106) (Figure 9).

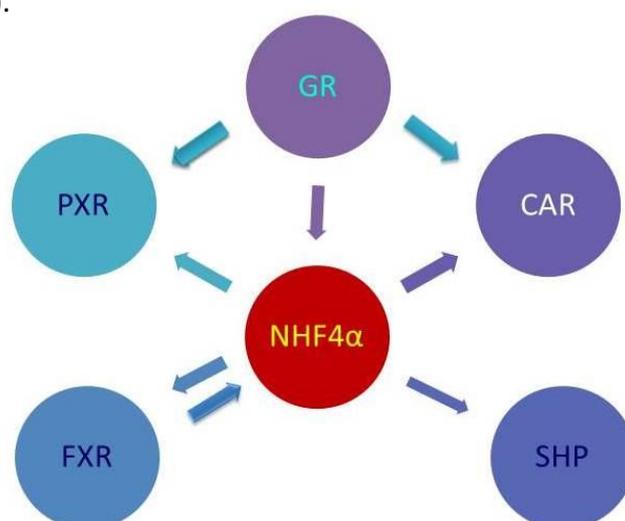


Figure 9. Interactive network of NRs; HNF4 α is at the center of the network. Figure adapted from Ref. 63 and 114-16.

Collectively, considering the role of CYP2C isoforms in the production of EETs, it would be logical to speculate that the slightest changes in the function and and/or activity of each part of the interactive network of NRs, including functional mutations both in the genes encoding the proteins involved in the network and in their responsive/binding elements in the CYP promoter regions, would potentially have a marked effect on the NR-mediated transcriptional modulation of the constitutive and inducible expression of CYP2C genes and

subsequently on the EET formation. As such, a functional mutation in gene encoding NHF4 α protein, may not only directly alter the expression and activity of NR NHF4 α (and consequently the NHF4 α -mediated transcription of CYP2C genes), but also can indirectly modify the NHF4 α -mediated expression of other NRs (such as CAR and PXR) resulting in the dysregulation of CYP2C isoforms and the EET synthesis.

As mentioned earlier the constitutive as well as xenobiotic-induced expression levels of most CYPs is regulated via the above-mentioned NR-mediated mechanism; however, for a number of CYPs, the basal and induced level of transcription is mediated through a different molecular pathway. For instance, the constitutive expression of CYP2J2, a main human epoxygenase, has been shown to be maintained by bZIP protein c-Jun which is an important component of the activator protein-1 (AP-1) transcription complex (122). The CYP2J2 promoter harbors two c-Jun RE in its proximal region, at -56/-63 and -105/-95 bp, which contribute to CYP2J2 c-Jun dependent transactivation (122,123). Interestingly, deletion of upstream between -152 and -50 bp relative to the start codon is associated with complete loss of c-Jun-mediated transcription and mutation of both the -56/-63 and -105/-95 regions bp results to partial loss of gene activation (122). Of note, in hypoxia, the upregulation of the AP-1 subunit c-fos leads to CYP2J2 downregulation indicating that hypoxia can influence on the CYP2J2 expression (122).

2.6. *MicroRNA-mediated transcriptional and post-transcriptional regulation of CYP genes*

MicroRNAs (miRNAs) are a family of small non-coding RNAs of 20-25 nucleotides that have been shown to govern transcriptional and post-transcriptional expression of target genes in many pathways and in a multitude of different organisms (124,125). At present, release 17 of the miRNA database miRBase (<http://www.mirbase.org/>) shows more than 1000 human

miRNAs (126), but the number keeps increasing,, so that it has been estimated that miRNAs are involved in the regulation of expression of 70% of protein-coding genes in human (127). miRNAs are encoded by specific genes. Fifty two per cent of human miRNA-encoding loci are located in intergenic regions and 48% of miRNAs are encoded from intragenic (intronic: 43%; exonic: 5%) regions (128,129). It is, therefore, not surprising that like other parts of the genome, the transcription of miRNA genes is itself under the control of a complex network of several NRs, coregulators and polymerase II (124,128).

In general, miRNAs bind a partially complementary segment within the 3' untranslated region (3'UTR) of target mRNA to reduce its stability and translation (124). Accordingly, miRNAs could potentially reduce the gene expression, either transcriptionally or post-transcriptionally. Indeed, miRNAs can suppress the transcription of target gene through direct repression of NRs involved in the transcriptional regulation of that gene, leading to an indirect downregulation of target gene transcription. Alternatively, miRNAs can contribute to post-transcriptional gene silencing via direct targeting the 3'UTR transcript of target gene leading eventually to a reduced expression of the target gene. Specifically speaking, miRNAs have been found to act as important transcriptional and post-transcriptional regulators of several CYP genes (130-134). The regulatory effects of miRNA on the expression of CYP genes could be exemplified by the miRNA-mediated transcriptional/post-transcriptional modulation of human CYP epoxygenases. In fact, several NRs that are implicated in the constitutive as well as xenobiotic-induced transcriptional upregulation of CYP2C genes, including PXR, VDR, RXR, HNF4 α , ER α and GR, have been found to be regulated post-transcriptionally by various miRNAs (126). The miRNA-mediated post-transcriptional degradation of NR transcripts could be associated with alteration in the NR-mediated transcriptional regulation of CYP2C genes. For instance, miRNA-24 and miRNA-34a

negatively regulate the translation of NR HNF4 α (135) and, thereby, could indirectly downregulate the HNF4 α -mediated transcription of CYP2Cs. Likewise, PXR is regulated by miRNA-148a (136); VDR is regulated by miRNA-125b (137); ER α is regulated by miRNAs 206, 221/222 and 22 (138-140); and GR is regulated by miRNAs-18 and 124a (141). The matter is further complicated by reminding the fact that NRs interact with each other for achieving a precise regulation of CYP2Cs and also have regulatory influence on the transcriptional expression of each other.

Additionally, the contribution of miRNAs to the post-transcriptional regulation of human CYP epoxygenases has been the focus of some important recent researches (130-134, 142,143). Notably, CYP2Cs have been recently demonstrated to be downregulated post-transcriptionally by miRNA-103 and miRNA-107, with CYP2C8 being the main target and CYP2C9 and CYP2C19 being affected to a lesser degree than CYP2C8 (142). Likewise, the results of a very recent study revealed that miRNA let-7b reduces CYP2J2 expression so that the decreased expression of this let-7b could lead to the high expression of CYP2J2 protein (143).

Taking together, there exists a complex interaction network involving NRs and miRNAs; miRNAs inhibit NR signalling and NRs induce miRNA gene transcription. This complex set of interactions leads to an extensive network of feedback and feedforward regulatory loops. The NR-miRNA bidirectional interplay in addition to the direct transcriptional regulatory effects of miRNAs, develop an intriguing system to precisely regulate the expression of human epoxygenases in both transcriptionally and post-transcriptionally manners.

2.7. *Epigenetic-mediated transcriptional and post-transcriptional regulation of CYP genes*

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene expression that do not involve changes in DNA sequences (144). A broader definition of epigenetic phenomena is structural modifications to chromatin, the nucleoprotein complex of approximately 146 bp of DNA and an octamer of the histone subunits H2A, H2B, H3 and H4 (145), that mark and signal transient and frequently reversible (stochastic) alterations in transcriptional states (146). Both heritable and transient epigenetic events contribute to the gene expression, generally through two main mechanisms that structurally change chromatin architecture:

1. Histone modification (HM): HM is post-translational modifications of the amino acids that make up histone proteins. These modifications including phosphorylation, acetylation, methylation and ubiquitination which occur on the N-terminal of the histones, can result in changes in the shape of the histone sphere and consequently in regulation of gene expression (147).

As discussed earlier, NRs regulate HM through interacting with coactivators including CBP/p300, corepressor proteins including HDACs as well as chromatin remodeling complexes to regulate gene expression through their enzymatic activity by epigenetically altering chromatin structure (144,149,150). Indeed, the highly compact structure of chromatin hinders accessibility of the underlying DNA. Having access to chromatin at regulatory elements is required for regulatory factors. Through the recruitment and interaction with coactivators and chromatin remodeling complexes, NRs modify local chromatin structure resulting in chromatin exposure to regulatory factors and eventually gene expression (144). Detailed description of the NR-mediated mechanisms of HM is out of the scope of this paper and has been comprehensively reviewed by Biddie (144), and Kato et al (150).

2. DNA methylation (DMet): DMet is the addition of methyl groups to the DNA by the DNA methyltransferase enzyme, predominantly at CpG sites of the mammalian genome (151,152). Of the four bases, DMet occurs only in cytosine at C5 position of the pyrimidine ring, converting cytosine to 5-methylcytosine (153). Three to four percent of all cytosines are methylated and the 5-methylcytosines compose up to 1% of all nucleotide bases in the DNA of normal human tissues (154). It has been shown that some areas of the genome are methylated more frequently than others, and highly methylated areas tend to be less transcriptionally active through two general mechanisms (155): First, DMet of gene promoters may prevent the physical binding of some TFs to their DNA binding sites (155-157). Second, the transcriptional silencing capability of DMet may occur via indirect mechanisms involving changes in chromatin conformation (155,158-160).

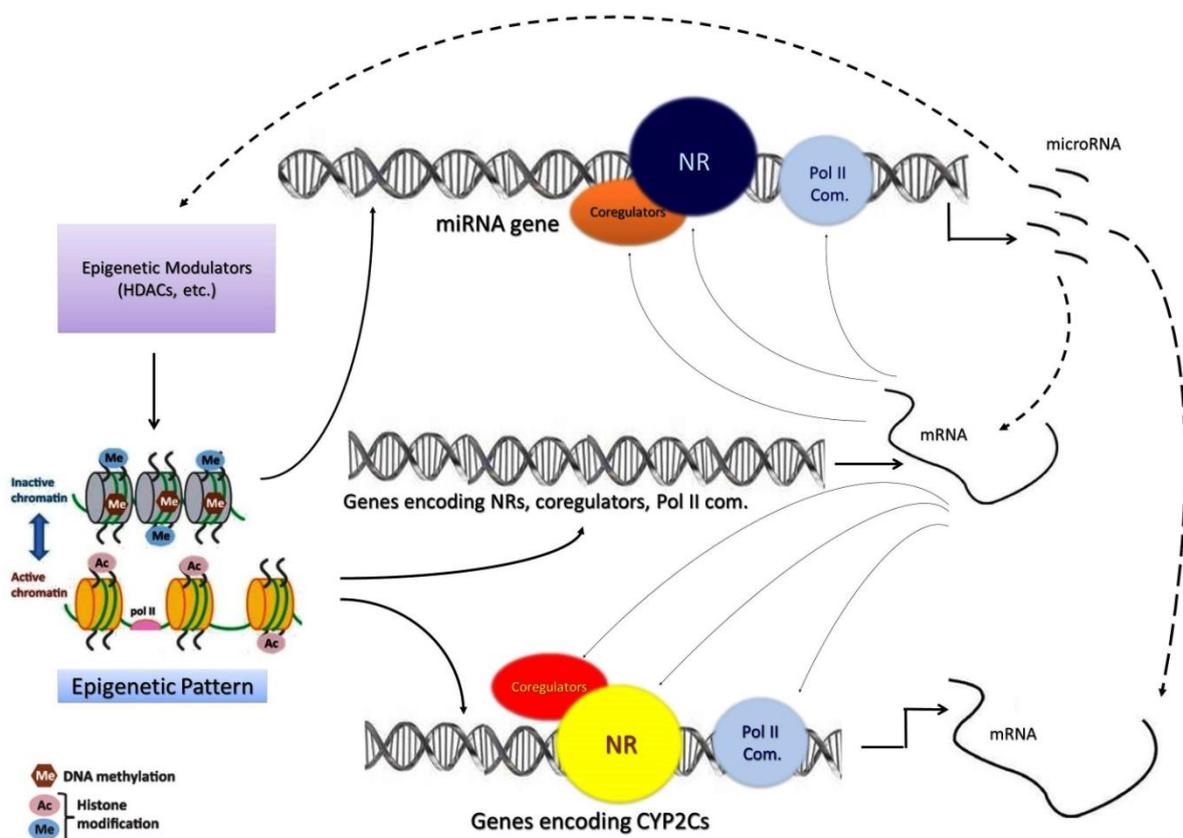


Figure 10. NR-Epigenetic-miRNA network of CYP regulation

To date, transcription of several CYPs has been proven to be influenced by epigenetic regulation, particularly DMet. Specifically, expression of CYP1A1, CYP1A2, CYP3A, CYP1B1, CYP2D6, CYP2E1, CYP2R1 and CYP2S1 has been found to be affected by DMet. In addition, of the main human CYP epoxygenases, the effect of epigenetic factors on the expression levels of CYP2C19 and CYP2J2 has been documented, so far (155,161-163). The DMet-mediated alteration in the expression of CYP enzymes could have significant consequences on drugs and endogen compounds metabolism (155).

Beyond their direct roles as transcriptional regulators, epigenetic mechanisms have also profound indirect transcriptional and post-transcriptional effects on controlling gene expression via regulation of miRNAs. Recently, it has been shown that epigenetic changes not only regulate the expression of protein-encoding genes, but also miRNAs, such as let-7a, miRNA-9, miRNA-34a, miRNA-124, miRNA-137, miRNA-148 and miRNA-203 (164-166). As an example, DMet negatively regulates transcription of CYP2J2 (155). Furthermore, epigenetic mechanisms could theoretically affect expression of CYP2C8 transcriptionally and post-transcriptionally through the regulation miRNA-34a (that is involved in the expression of NR HNF4 α and subsequently in the expression of CYP2C8) (135), and miRNA-103/107 (143), respectively. On the other hand and to complicate matter further, another subset of miRNAs has been shown to control conversely the expression of important epigenetic regulators, including DNA methyltransferases and histone deacetylases genes (164).

The complicated network of feedback between miRNAs and epigenetic pathways appears to form an epigenetics-miRNA regulatory circuit that could potentially organize the whole gene expression profile including those genes involved in the xenobiotic-, endobiotic metabolism such as CYP epoxygenases. Not surprisingly, a disruption in this regulatory circuit may cause

significant changes in the expression and activity of human epoxygenases and consequently in the EET formation.

2.8. NR-epigenetic-miRNA network-mediated regulation of CYP epoxygenases

Taking together, in theory, transcription of CYP epoxygenases and consequently EET biosynthesis could be orchestrated by an extremely complex network of NR, miRNAs and epigenetic factors. In this network, CYP expression is primarily induced by NRs, coregulators and Pol II complex. In addition, miRNAs regulate the expression of CYP genes, at post-transcriptional level, by inducing the degradation of CYP epoxygenase mRNAs. Likewise, miRNAs suppress CYP expression, transcriptionally, by repressing the translation of NR/coregulators/Pol II complex. The expression level of all the three main components of this network, i.e. NRs, miRNAs and CYP epoxygenases, is regulated by genetic/epigenetic factors (Figure 10).

2.9. Genes counter-interaction

As illustrated in Figure 11, genes counterinteract with each other in a complicated way. Indeed, the expression of a given gene could be regulated by other genes. For instance, NR genes contribute to upregulation of CYP epoxygenases. In addition, CYP2C8 and CYP2C9 are coexpressed. Therefore, apparently, all of variables that contribute to the regulation of the expression of CYP2C8 can simultaneously affect the expression of CYP2C9.

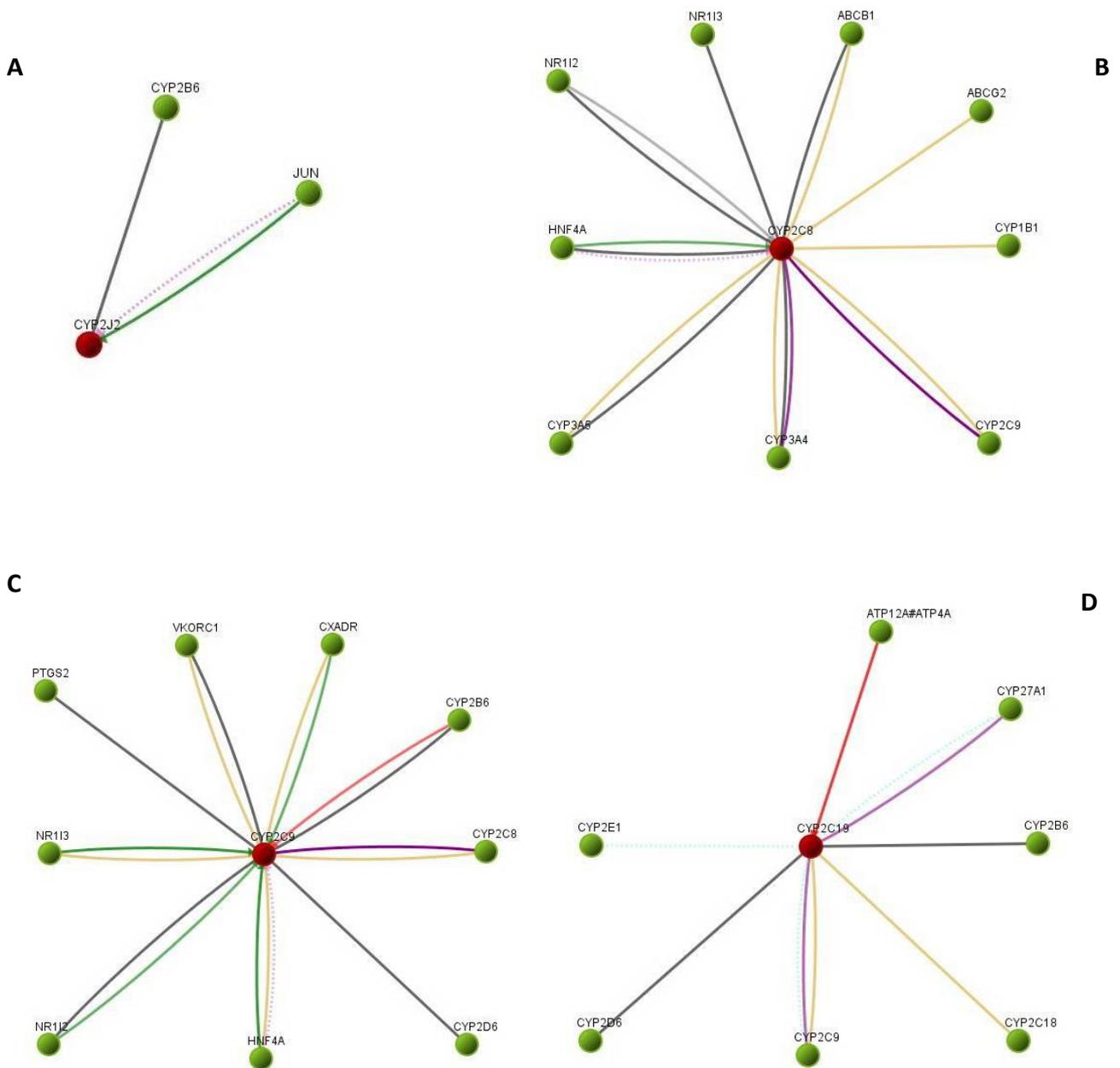


Figure 11. Genes which are in counteraction with main human epoxygenases. Figures reproduced from images available at: <http://www.genecards.org/>

3. Inter- and intra-individual variability in CYP expression

Multiple studies now have demonstrated that there exists a wide inter-individual variability in the expression of CYP mRNA and protein (167-171). There is >50-fold inter-individual

variation in the liver enzyme content for CYP2A6, CYP2B6 and CYP2D6; 20-fold for CYP1A2; 12-fold for CYP2E1; and 5-fold for CYP2C and CYP3A4 (167). In addition, highly variable catalytic activities of CYP enzymes have been detected among individuals (167). Likewise, in a given individual, the basal CYP expression level could vary under different physiological and pathological conditions. Herein, we provide a comprehensive overview of the variables that have been identified so far as the potential sources of inter- and intra-individual variability in the CYP expression as well as CYP activity.

3.1. *Age*

Based on the accumulating evidence, it is now certain that the CYP expression and activity changes markedly throughout the course of physical and sexual maturation with greatest variability noted during gestation, infancy, childhood and adolescence (172-175). Such alteration, also known as CYP ontogeny, explains, at least in part, the fact that the developmental changes profoundly influence the response to medications and is indispensably considered as integral part of the pediatric clinical pharmacology (172). Taking into the account that CYP3A subfamily is the responsible for the biotransformation of almost one half of the drugs prescribed for children (176), the ontogeny of CYP3A isoforms has been more extensively investigated.

Altogether, transcriptomic and proteomic analyses revealed that CYP3A7 is the predominant CYP expressed in fetal liver (177). CYP3A7 remains the dominant CYP3A enzyme in children up to one year after birth; however, substituting with CYP3A4, hepatic CYP3A7 protein level decreases continuously and dramatically during the childhood and adolescence so that CYP3A4 represents the major component of adult hepatic CYP content comprising 10 to 50% of the total CYP (Figure 12 A) (173). In addition, although limited in number, the studies on

the developmental expression of CYP3A5 have suggested that CYP3A5 protein level is highly variable, but generally independent of age. For more detailed review on CYP3A ontogeny, we refer to the review by Hines (175).

As typified by CYP3A isoforms, the developmental expression patterns of other key CYP enzymes, including human epoxygenases, have also been investigated (174,175). Specifically, studies on the ontogeny of the CYP2C subfamily (Figure 12 B) have indicated that CYP2C9 is detectable at 1% (0.3 ± 0.2 pmol/mg) of reported adult values between 8 and 24 weeks of gestational age (170,174,175,178). CYP2C9 levels increases significantly between 25 and 40 weeks of gestation to levels that are approximately 10% (5.0 ± 4.0 pmol/mg) of adult values (174,175). Increases in CYP2C9 continue postnatally; in infants 0 to 5 months old, the hepatic CYP2C9 content is about 25% (11.8 ± 7.0 pmol/mg) of that is found in adults and the mean CYP2C9 levels in children between 5 months and 18 years old reached to 50% of adult values (18.0 ± 6.1 pmol/mg) (174,175). Compared to CYP2C9, a distinct developmental expression pattern has been observed for CYP2C19. At levels that are approximately 10% to 20% of those reported in adults (3.4 ± 2.2 pmol/mg microsomal protein), the hepatic CYP2C19 is detectable at 24 weeks of gestational age and, unlike CYP2C9, expressed at relatively constant levels throughout gestation and at the birth. Rather, a postnatal linear increase in CYP2C19 occurs to enzyme levels 50% to 75% of observed adult values (11.7 ± 7.9 pmol/mg microsomal protein) over the first five months after birth and enzyme levels indistinguishable from adult levels (14.9 ± 4.1 pmol/mg microsomal protein) are observed in post-puberty adolescents (174,175). To date, very little is known about the ontogeny of CYP2C8 and nothing is known about the ontogeny of any of the CYP2C enzymes in extrahepatic tissues. In addition, the expression and activity of CYP2J2 has been characterized in 58 prenatal and 18 postnatal liver samples. As a result, CYP2J2 mRNA has

been found to be expressed in human fetal liver as early as 11 weeks gestational age and the CYP2J2 expression level remains unchanged postnatally from 4 days to 18 years of age (179). Despite ample animal studies showing significant age-dependent changes in Cyp expression and activity and although abundant literature exists regarding the developmental expression of CYPs in human during gestation and throughout the first two decades of life, the influence of ageing on CYP enzyme expression and activity in human during adulthood and old age has not been a persistent finding in all investigations and the results of different studies in this field are sometimes contradictory (180-185). For instance, while the results of a study on 54 liver samples showed that the hepatic content and activities of CYP enzymes are preserved in normal aging (186), another investigation undertaken on 226 subjects with hepatic histopathologic changes found significant decreases by 32% and 29%, respectively, in the total hepatic content and *in vivo* clearance of antipyrine, as an indicator of CYP enzymes activity, in subjects more than 70 years as compared with young adults (187). Moreover, it has been suggested that, in general, hepatic CYP content declines at a rate of 0.07 nmol/g of liver after 40 years and that there is approximately 20% reduction in the antipyrine clearance in subjects aged 25-75 years (187). Specifically, an age-related reduction by roughly 25% has been reported in CYP2C9 and CYP2C19 catalytic activities (184). On the other hand, neither CYP2C8/9 activity (again using antipyrine as a probe), nor CYP2C8 mRNA or protein expression levels were associated with subjects' age in two distinct studies on 37 and 60 hepatic samples of healthy donors, respectively (181,183). Likewise, the results of other study on 150 samples of human liver microsomes and 64 samples of cryopreserved human hepatocytes showed that there is not a statistically significant difference in CYP2C8, CYP2C9 and CYP2C19 activity as a function of age (185). The influence of aging on the expression of CYP2J2 after adolescence has been less investigated.

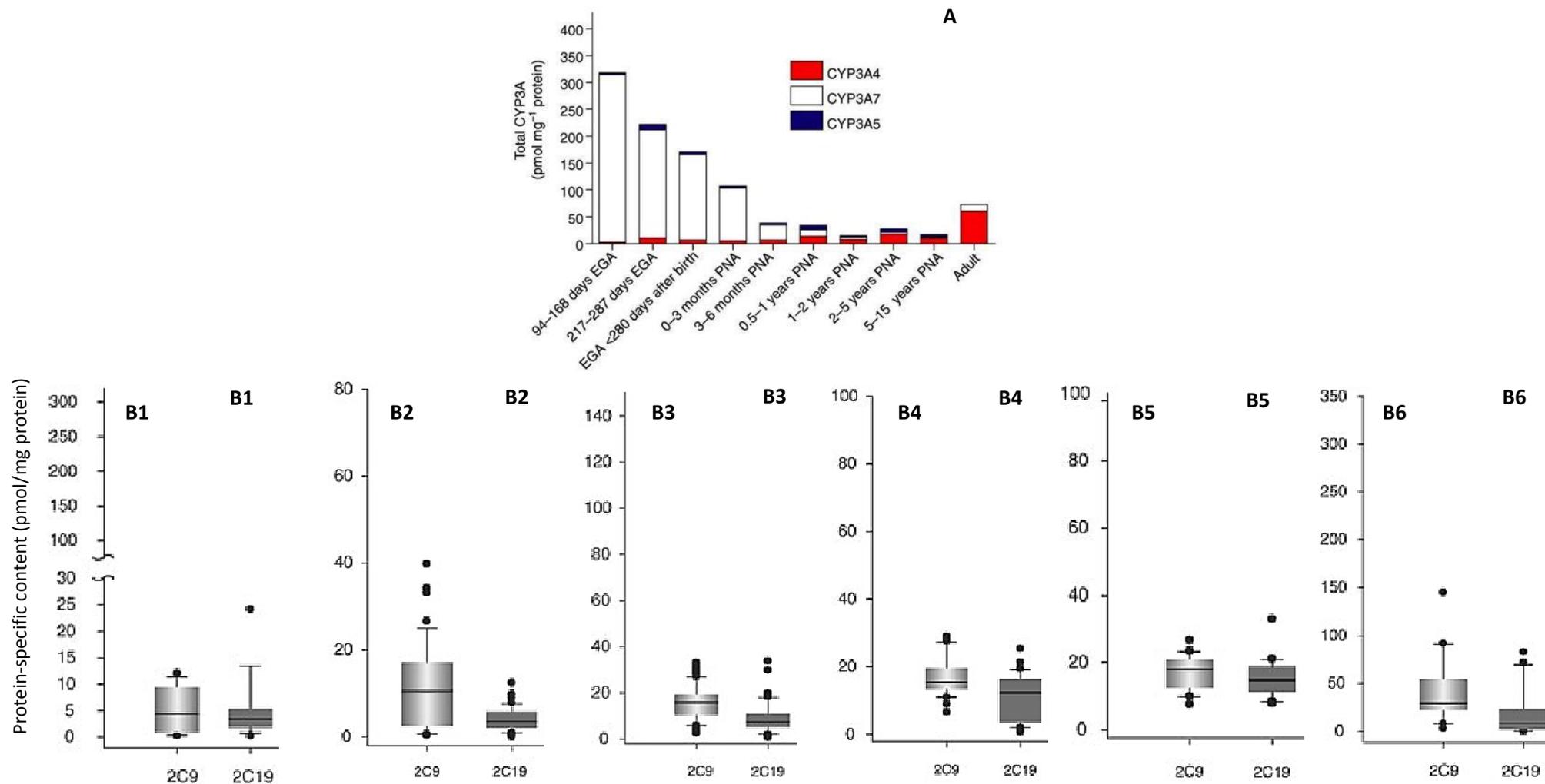


Figure 12. A. Levels of CYP 3A4 (red bars), CYP3A5 (blue bars) and CYP3A7 (open bars) protein as a function of age. EGA: estimated gestational age; PNA: postnatal age. **B.** Expression of hepatic CYP2C9 and CYP2C19 enzymes in fetus (**B1**), neonate (**B2**), infant (>30 days after birth but less than 1 year) (**B3**), prepubertal (>1 year but <10 years) (**B4**), adolescent (>10 years but <18 years) (**B5**) and adult (**B6**). The boxes represent the 25th to 75th percentiles and the horizontal bar the median of the data. The vertical bars represent 10th to 90th percentiles. Outliers (circles) were defined as having specific contents outside 1.5 times the 25th or 75th percentiles. Part A and B are reproduced with permission from Ref. 173 and Ref. 174, respectively.

It is noteworthy to mention that, due to the following reasons, the relationship of hepatic CYP expression and activity with aging has been challenging and difficult to assess in human. Firstly, as it mentioned later, there are host of factors, such as demographic; genetic and environmental variables, which contribute to inter-subject variability in CYP expression and activity. Secondly, obtaining liver samples from healthy subjects is not readily feasible, ethically; this is the reason why most of the available *in vitro* investigations have been performed on the liver biopsies of patients with hepatic pathology. Thirdly, it has been recently shown that human gene expression becomes, qualitatively and quantitatively, heterogeneous with age. The age-correlated heterogeneity of expression is a pervasive characteristic of human genome including CYP enzyme. Lastly, most of the existing studies have been carried out on relatively small number of subjects and also have used antipyrine as a non-specific substrate for the assessment of CYP activity (182). However, a recent large investigation on 466 human liver samples applied a systems biology approach, via integration of genetics, gene expression and enzyme activity measurements, to systematically assess the full spectrum of functionality of individual CYPs (188). In this study, the activity of each CYP enzyme was measured using a single specific probe and the effect of age and gender on CYP activities were assessed. Interestingly, the primary analysis on all subjects, including both young and adult subjects, showed that CYP activities increase as age advances. Specifically, age had a significant positive impact on the activities of CYP2C8, CYP2C9, CYP2A6 and CYP3A4 in both genders; CYP1A2 in females only; and CYP2B6 in males only. After correction for 30 tests and performing additional analyses on adults only, the effect of age remained significant only on CYP2C9, suggesting weaker age effect in adults. Interestingly, when both age and gender were considered simultaneously, gender

demonstrated different effects at different age bands; at younger ages (e.g., <10 yr), females appeared to have lower activity than males, whereas, at older ages females tended to have higher activities than males. However, the authors concluded that, although not very strong, both age and gender influence on CYP activities (188).

Collectively, considering the fact that gene expression has been suggested to become heterogeneous with aging (189), and that CYP enzymes play a central role in drug and endobiotic metabolism, further studies are warranted to investigate the real effect of age on CYP expression and activity. Notably, more ambitious efforts are needed to investigate the age-dependent variation in the expression of human CYP epoxygenases and its consequence on the EET production.

3.2. *Gender*

Multiple lines of evidence from various species, including human, support the notion that there exists a sex-specific gene expression pattern both in the reproductive and somatic tissues including the liver (190-199). In a recent genome-wide association study, the sex-differences in human hepatocyte gene expression have been characterized (200). Accordingly, 1,249 sex-biased genes were identified with females showing 70% higher expression rates; the chromosomal distribution of male- and female-based genes has been demonstrated in Figure 13 (200). Interestingly, many of the genes differentially expressed in the human liver are involved in drug and steroid metabolism (190); therefore, sex differences in drug metabolism, drug safety and drug efficacy, which have long been recognized and widely studied in epidemiological and clinical reports (201-207), are attributed, at least partly, to the sex-

differences in the expression of drug metabolizing genes, including CYP enzymes, in the human liver (201-204,207,208). Specifically, the sex-related difference in the expression and catalytic activities of fundamental human CYPs involved in drug metabolism, namely CYP3A; CYP1A2; CYP2A6; CYP2B6; CYP2D6; CYP2C8; CYP2C9; CYP2C19 and CYP2E1, have been more explored in several previous *in vivo* and *in vitro* studies in which, the pharmacokinetic parameters, mainly the rate of clearance of specific CYP substrates, have been used to compare the activity of individual human CYPs in males and females (7,185,188,194,202,203,207,208). Similar to the studies investigating the age-related changes in human CYP expression/activities, existing studies aimed at identifying sexual dimorphic expression/activity of human CYPs have yielded conflicting results.

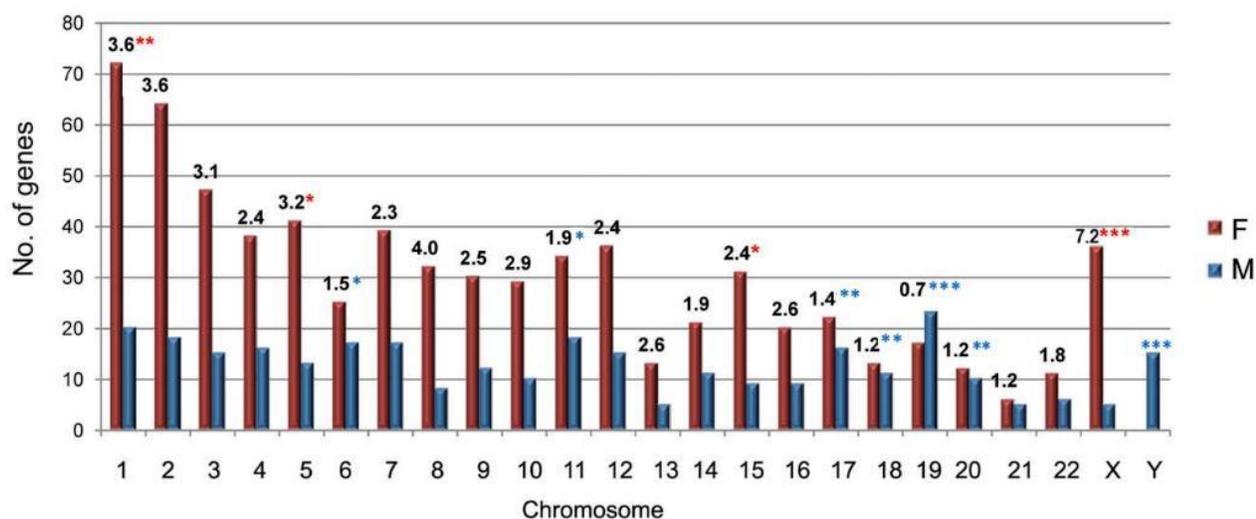


Figure 13. Chromosomal distribution of male-biased and female-biased genes identified in human liver. Number of male- and female-biased genes on each chromosome, based on the criteria $|\text{fold change}| \geq 1.15$ and composite array score ≥ 14 . Numbers at the top of each bar indicate the ratio of the number of female-biased genes to male-biased genes on each chromosome. Asterisks indicate the significance of the sex ratio based on Chi-square tests (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; red asterisks indicate significant enrichment of female-biased genes and blue asterisks indicate significant enrichment of male-biased genes). Figure is reproduced with permission from Ref. 200.

Specifically, for human CYP epoxygenases the issue of sex-based expression has not been well elucidated and the currently available data are inconsistent and, in some cases, contradictory. For instance, the gender-mediated difference in human CYP2C9 activity was investigated in 640 and 629 patients following administration of piroxicam and naproxen, respectively (209). The plasma levels of these two drugs were significantly varied between males and females indicating greater CYP2C9 activity in males. Using phenytoin as the substrate marker, other investigations have yielded contradictory results, suggesting that CYP2C9 has higher activity in females compares to males (210,211). Contrary to these studies, other researches with phenytoin and other substrates reported lack of gender-specific in human CYP2C9 activity (185,212,213). Similar to CYP2C9, the results of few studies performed on the human CYP2C19 activity are inconsistent (185,214-217). Specifically, the gender-related variation in CYP2C19 activity was explored in 641 healthy Swedish subjects who received (*S*)-mephenytoin (214). No significant differences were observed between males and females. Likewise, other investigation with (*S*)-mephenytoin in 611 Caucasians revealed no significant gender-dependent differences in CYP2C19 activity (215). In contrast, investigating the CYP2C19 activity in 4301 Dutch men and women following administration of (*S*)-mephenytoin showed that men have significantly higher CYP2C19 activity than women (216). Surprisingly, another study undertaken in Chinese population using (*S*)-mephenytoin as substrate, demonstrated greater CYP2C19 activity in women than those in men (217). Little is known about the sex-specific difference in the expression and activity of CYP2C8 and CYP2J2. In a study on 150 samples of human liver microsomes, no significant effect of gender on CYP2C8 activity has been observed (185). In addition, a recent study showed that CYP2J2 is expressed 1.19 fold higher in females than males

(202). In addition to CYP2J2, this study, which has systematically explored the sex differences in the expression of 374 drug-metabolizing enzyme and transporter (DMET) genes in the human liver samples of 234 males and 193 females (202), detected 76 additional DMET genes which were found to be transcribed sexually dimorphic in the human liver. Of them, 16 genes were of CYP subfamilies: CYP7A1 (2.1 fold higher in females); CYP3A7 (1.83 fold higher in females); CYP3A4 (1.73 fold higher in females); CYP2A6 (1.49 fold higher in females); CYP2A7 (1.46 fold higher in females); CYP2A13 (1.43 fold higher in females); CYP2B6 (1.33 fold higher in females); CYP2B7P1 (1.33 fold higher in females); CYP3A43 (1.3 fold higher in females); CYP39A1 (1.29 fold higher in females); CYP3A5 (1.27 fold higher in females); CYP51A1 (1.2 fold higher in females); CYP2J2 (1.19 fold higher in females); CYP27A1 (1.16 fold higher in females); CYP4Z1 (1.16 fold higher in females); and CYP1B1 (1.13 fold higher in males) (202).

Collectively, at this time and according to the available literature, it is difficult to ascertain if gender has an influence over the activity of human epoxygenases. Therefore, more well-conducted studies are required to be carried out in order to make a solid conclusion regarding the effect of gender on the activity of these CYP isoenzymes and consequently on the EET production.

The biological basis of sex differences in CYP expression and activity are believed to be mainly related to the fundamental role of gender-related hormonal regulation in gene expression; the effects of growth hormone and sex hormones have been documented (202,218). For instance, CYP3A4 has been documented to be expressed at higher level in women than in men and its expression has been shown to be increased by continuous treatment with growth hormone in men and to be decreased by pulsatile treatment with growth hormone in women

(203,207,219,220). Interestingly, although in human have not yet been demonstrated, in rodents, the sex differences in the expression of CYP enzymes is attributed to NRs including HNF4 α and PXR (208,220,221).

3.3. *Molecular genetics*

It has been demonstrated that the polymorphic nature of the CYP genes influences individual CYP-mediated reactions including xenobiotic- and endobiotic metabolism. This variation includes copy number variation (CNV), miRNAs, variability of POR, polymorphisms of NRs that regulate the expression of CYP genes and genetic/epigenetic variations of CYPs (155).

3.3.1. *Variation in POR expression*

As discussed before, as the exclusive electron donor for all microsomal human CYP monooxygenases, POR has a unique role in the CYP-mediated xenobiotic and endobiotic metabolisms. Similar to most microsomal CYP enzymes, but in a lesser extent, POR is expressed differently among individuals (7), and potentially in a given individual under various conditions. The variability in the POR expression is attributed, at least in part, to the inducibility of POR genes by NRs CAR and PXR (222), hormonal influence on POR expression (223), and POR gene mutations (7,29,109,224-230).

In human POR gene is located on chromosome 7q11.2 (7). Several functional polymorphisms have been identified so far in the human POR gene (29). Unsurprisingly, POR polymorphisms have been shown to be associated with significant modification of the enzymatic activity of some CYPs such as CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4

(7,29,224-230). Specifically in human CYP epoxygenases, POR mutations A287P and R457H have been discovered to result in almost complete loss and significantly reduced activities of CYP2C19 and CYP2C9, respectively. In contrary, polymorphisms A503V and Q153R showed increased activity of CYP2C19 to 113% and 284% of wild type, respectively; these polymorphisms have also been found to increase slightly the activity of CYP2C9 (7,225,229). The influence of POR genetic variations on the expression of CYP2C8 and CYP2J2 has been less investigated. However, since POR has been shown to be directly involved in CYP activity and CYP-mediated biological pathways, it is logical to speculate that POR polymorphisms potentially could also be associates with alerted CYP2C8/CYP2J2 activities.

Collectively, it could be assumed that the NR- and hormonal-mediated regulation of POR expression may result in a marked inter- and intra-individual variability in the expression and activity of this enzyme. In addition, the functional POR gene polymorphisms are important sources of inter-individual variation of POR expression. Given the important contribution of POR to the activity level of human CYP epoxygenases, the inter- and intra-individual variability in the POR expression/activity could be considered potentially significant cause of the inter- and intra-individual variability in the epoxygenase activity and consequently in the EET production.

3.3.2. Polymorphisms of genes encoding NRs and NR binding sites

The crucial role of NRs and also the importance of NR-RE interaction in the regulation of constitutional and xenobiotic-induced expression of human CYP epoxygenases were discussed thoroughly in section 2.5. Accumulating evidence indicates that mutations in NR binding sites in CYP epoxygenase gene promoters could be associated with dysregulation of these genes

(section 2.5). Also, several SNPs have been identified, so far, in genes encoding NRs (106, 231-241). The genetic variations in NR coding regions have been shown to result in altered level of NR proteins (106) and, by this means, can contribute to a variety of local and systemic disorders (242-251), and also to inter-individual differences in drug response through changes in the expression of genes involved in drug metabolism, including CYPs (252-256). For instance, a single nucleotide polymorphism (SNP) in the HNF4- α gene (253), and a genetic variant of the CAR (239), have been identified to be associated with alerted expression of CYP2B6 and several SNPs in the PXR gene may result in CYP3A4 expression and metabolic activity (321,239). Although little is known about the potential influences of NR polymorphisms on the expression and activity of human CYP epoxygenases, considering the importance of NRs in the regulation of these enzymes, it would be logical and makes sense to expect that the genetic variations in genes encoding NRs may potentially be a significant cause of inter-individual variability in the expression of CYP epoxygenases and eventually in the EET formation.

3.3.3. *MicroRNAs*

As described before, miRNAs regulate the transcription of a wide range of genes such as the xenobiotic-, endobiotic-metabolizing enzymes including CYPs. A growing body of reports has shown that the expressions of miRNAs are readily influenced by xenobiotics, chemicals, carcinogens, drugs, hormones, stress and diseases including cancer, Alzheimer's disease, CVDs and schizophrenia (126,256-258). This condition-related dysregulation of miRNAs and subsequently the miRNA-related polymorphisms could potentially lead to a considerable inter- and intra-variability in the expression of CYP genes and consequently to changes in the drug

and endobiotic metabolism and effects. The miRNA-CYP-mediated variation in drug response would provide useful information in pharmacogenomics and personalized medicine.

Additionally, as discussed, miRNAs themselves are controlled by NR proteins which are, in turn, under control of polymorphic genes (section 3.3.2). The instability in the miRNA expression as the result of NR polymorphisms would provide a potential source of inter-individual variability in the transcription of CYP genes. To complicate matter further, SNPs of genes encoding miRNA precursors, their target sites and silencing machinery were found to alter miRNA function and they are likely to affect phenotypic variation, including disease susceptibility and potentially xenobiotic-, endobiotic metabolism (259,260). In fact, SNPs of miRNA genes may alter their sequences and therefore enhance, diminish or even generate or cancel out their ability to bind to target sites (261-263). Therefore, genetic variations of genes encoding miRNAs could provide an additional source of inter-subject variability in the expression and activity of CYP epoxygenases and in the EET formation.

3.3.4. Epigenetic factors

It has been suggested that there exists a significant inter-individual variability in the epigenetic modification of genes and that the difference in the epigenetic-related regulation of genes could explain a much greater fraction of inter-individual phenotypic variation than differences in genotype, alone (264-268). There is an ever growing body of evidence that environmental factors, including chemical pollutants; dietary components; smoking; temperature changes and other external stressors, can affect the epigenetic landscape of an individual (264-268). Considering the fact that most phenotypic traits are determined by interplay between

individual's gene and environment, the "environmental epigenetics" as a mechanistic link between genes and environment has attracted considerable interest (264). The molecular mechanisms that mediate environment-epigenetic-induced modification of gene expression have been thoroughly reviewed by Feil and Fraga (264).

Additionally, since environmental conditions are dynamic and constantly changing, the environmental epigenetics is being considered a major source of inter- and intra-individual variability in the expression of the genome including genes involved in the metabolism of drugs and endogenous compounds such as CYPs (269-273). The study of epigenetic-mediated inter- and inter-personal differences in the expression of drug-metabolizing enzymes is a rapidly emerging discipline called "pharmacoepigenetics"; it is expected that pharmacoepigenetics, complementary to pharmacogenetics, will play a crucial role in future pharmacology and personalized medicine (269-273).

Similarly, the regulatory impact of epigenetic variations and the environmental epigenetics on human CYP epoxygenase genes, specifically CYP2C19 and CYP2J2 (section 2.7), may result in a significant inter- and intra- variability in the expression of epoxygenases and eventually in the biosynthesis and biological function of EETs.

3.3.5. *CYP gene polymorphism*

The human CYP genes are highly polymorphic (7,34,40,155,274). The polymorphisms can modify the function of CYPs through several mechanisms: loss-of-function polymorphisms mostly affect splicing and expression, but surprisingly not transcription or protein structure (7,275); gain-of-function polymorphisms influence enzyme expression and activity via CNV,

promoter variation (e.g. CYP2B6 and CYP2C19) (7), and amino acid variations that resulting in increased substance turnover (e.g. CYP2B6 and CYP2C8) (276). Unexpectedly, from all the discovered polymorphisms, there are few that influence on substrate selectivity and xenobiotic-mediated inducibility of CYP enzymes (7).

CYPs represent the most important phase I drug metabolizing enzymes and genetic polymorphisms within CYPs remarkably affect the metabolism of drugs that are substrates for those particular enzymes which eventually leading to differences in drug response and risk of adverse drug reactions. Very recently, the characteristics, functional effects and clinical implications of pharmacogenetically important CYP polymorphisms have been comprehensively by Zanger and Schwab (7).

Additionally, since EETs, as epoxygenase-mediated AA metabolites, possess potent protective effects on the cardiovascular system, genetic variations in the CYP epoxygenase pathway have attracted considerable interest. The main human CYP epoxygenases including CYP2C8, CYP2C9, CYP2C19 and CYP2J2 exhibit genetic polymorphisms. The most common epoxygenase gene polymorphisms (Table 1) have been demonstrated to be associated with a significant decrease in their epoxidation activity and consequently in the EET biosynthesis (34). For instance, *CYP2C8*3* has shown to be defective in the metabolism of AA to 11,12-EET and 14,15-EET having a turnover of 35-40% of wild type CYP2C8 (*CYP2C8*1*) (34). Likewise, *CYP2C9*2* displays half of CYP2C9 (*CYP2C9*1*) wild type epoxygenase activity (34). Moreover, compared to other CYP2C8/CYP2C9 haplotypes, samples genotyped as *CYP2C8*3/*3/CYP2C9*2/*2* exhibited 34% decreased EET biosynthesis (34). Similarly, *CYP2J2*7* (G-50T) polymorphism, the most frequent and the most studied functional variant of CYP2J2, results in a reduced transcription of the

CYP2J2 gene and also lower EETs plasma concentration (34). Considering the fact that CYP epoxygenase polymorphisms cause lower activity of these enzymes and that EETs play important roles in cardiovascular physiology, it is logical to assume that genetic variations in the epoxygenase pathway could be generally associated with an increased risk of several CVDs including HTN and CAD and, thereby, could be considered one of the determinants of individual susceptibility to these disorders (34,40).

As demonstrated in Table 1, the frequencies of CYP epoxygenases polymorphisms are highly dependent on the ethnic background (7,34). While the frequency of *CYP2C8*2* has been reported to be up to 18% in African-Americans (rare in Caucasians), *CYP2C8*3* is primarily occurred in Caucasians (up to 19.8%) and neither is occurred in Asians. Furthermore, *CYP2C9*2* and *CYP2C9*3* are considerably frequent in Caucasians (up to 19% and 16.2%, respectively), while these polymorphisms are less frequent in African and are rare in African-American and east Asians. Likewise, the frequency of *CYP2C19*2* has been reported to be 45.5%, 7.8% and 13% in Chinese, Bolivian and Caucasians, respectively. There is also a large inter-ethnic difference in the prevalence of *CYP2J2*7* polymorphism, seemingly being most frequent in Africans (7,34).

It is also important to be mentioned that, beyond their effects on the expression of CYP enzymes, polymorphisms of CYP genes are also associated with alteration in inducibility of these enzymes by xeno- and endobiotics. For instance, the induction of hepatic CYP2C19 protein and mRNA exhibits marked inter-individual variability which is attributed, at least in part, to the polymorphisms of this gene (61).

Table 1. Most common polymorphisms of human CYP epoxygenases

CYP allele designation	Genomic position (rs)	Protein effect	Allele frequencies (%)	Functional activity
CYP2C8				
CYP2C8*2	11054A>T (rs11572103)	I269F	Global MAF: 3.9 AA, Af: 10 - 22 Ca: 0 - 1.2 As: 0	Dec. activity
CYP2C8*3	30411A>G (rs10509681)	K399R	Global MAF: 6.5 AA, Af: 0 - 3.3 As: 0 Ca, Hs: 6.5 – 19.8	Dec. activity
CYP2C8*4	11041C>G (rs1058930)	I264M	Global MAF: 2.6 AA, Af, As: 0 - 1 Ca, Hs: 0 – 7.5	Dec. activity
CYP2C9				
CYP2C9*2	3608C>T (rs1799853)	R144C	Global MAF: 6.9 AA: 0 - 1 Af: 0 - 12 As, Pc: 0 - 2 Ca, Hs: 10.6 - 19	Dec. activity
CYP2C9*3	42614A>C (rs1057910)	I359L	Global MAF: 4.3 AA, Af: 0.5 - 6.0 As: 2 - 6 Ca, Hs: 5 - 16.2	Dec. activity
CYP2C19				
CYP2C19*2	19154G>A (rs4244285)	-	Global MAF: 19.9 AA, Af: 10 - 25 As, Pc: 15 – 45.5 Ca: 6 – 15.9 Hs: 15	Null allele
CYP2C19*3	17948G>A (rs4986893)	W212X	Global MAF: 1.4 Af, Ca, Hs: 0 - 1 As, Pc: 0 - 14.3	Null allele
CYP2C19*17	-806C>T (rs12248560)	Promoter	Global MAF: 15 AA, Af: 15 - 27 As: 0 - 2 Ca: 21 - 25	Inc. exp. & activity
CYP2J2				

MAF, minor allele frequency as reported in the 1000Genome phase 1 genotype data (released May 2011). Selected frequencies of individual ethnicities (AA, African American; Af African; As Asian; Ar, Arab; Ca Caucasian; Hs, Hispanic; In, Indian; Pc, Pacific; SA, South American); Data adapted with permission from Ref. 7 and Ref. 34.

Altogether, genetic polymorphisms account for considerable inter-ethnic and inter-individual variability in the expression and activity of CYP epoxygenases in human. This variability can result in a reduced biotransformation of AA to EETs and eventually in an increased CVD risk at the population level.

3.3.5.1. Copy number variation

As a form of structural variation, CNV is the alteration of the DNA of a genome at 1 kb or larger, resulting in the cell having variable number of copies compared with a reference genome (276-278). CNVs can potentially alter gene dosage and expression, disrupt coding sequences and perturb long-range gene regulation resulting in dramatic phenotypic consequences (276-281); however, some CNVs affecting numerous genes and consisting of millions of bases have been found in the general population with no observable consequences (277). For long, it has been thought that SNPs were the most prevalent and the main sources of genetic variation. However, it is now well established that CNVs encompass at least three times the total nucleotide content of SNPs and are the most important source of genetic and phenotypic variation (277). To date, approximately 2000 CNVs have been identified in the human genome, covering more than 12% of the genome (278). Undoubtedly, further CNVs will be discovered as technologies improve and more DNA samples from different population and ethnicities are explored (282).

Like SNPs, CNVs can contribute to disease susceptibility and also to inter-individual differences in drug response by affecting the expression level of drug-metabolizing enzymes (283). CNVs have been described for several xenobiotic-metabolizing enzymes including GSTT1, GSTM1,

SULT1A1, UGT2B17 and CYPs (283-288). In particular, CYP2A6 (277,283,288,290), CYP2B6 (283), CYP2C19 (277,291), CYP2D6 (277,288,292,293), and CYP2E1 (283) exhibit deletion, duplication and even multiplication. The results of a recent study CNVs were described for CYP2A6, CYP2B6 and CYP2E1 with multi-ethnic frequencies ranging from 0 to 8.3% and 0 to 4.1% for deletion and duplication, respectively (283).

CNVs can influence CYP activity through altering gene expression. For instance, CYP2A6, CYP2B6, CYP2E1, CYP2C19 and CYP2D6 CNVs have been shown to be associated with a significant alteration in response to those medications which are metabolized with these enzymes (155, 277, 283-293). In 2004, the United States Food and Drug Administration (FDA) approved the first DNA microarray molecular diagnostic test, the Ampli Chip CYP (Roche Molecular Diagnostics, Alameda, CA, USA), for the analysis of SNPs and CNVs of CYP2D6 and CYP2C19, genes which are involved in the metabolism of many drugs prescribed widely for treatment of depression, gastroesophageal reflux, and cardiovascular diseases (294). There is also a large inter-ethnic variation in the CNV of metabolizing genes (155,277,283). For example, CYP2A6 deletion (*CYP2A6*^{*4}) has a higher frequency in Asians (7–24%) than in Caucasians and Africans (about 1%), CYP2D6 deletion (*CYP2D6*^{*5}) has similar frequency (about 4%) in the different populations, but CYP2D6 duplication/multiduplication is more frequent in Africans than in Asians and north Caucasians (277,295).

To date, little is known about the CNVs of human CYP epoxygenase genes and their potential effects on the metabolism of AA and the formation of EETs; however, the biological significance of this aspect of molecular genetics in causing inter-individual variation in human epoxygenase expression and activity will, definitely, be the focus of research efforts.

3.4. *Xenobiotics*

Most CYPs are subject to induction and inhibition. There is mounting evidence that there are a great number of xenobiotics such as chemical agents and drugs, including herbal medications, influencing the expression and/or activity of the CYP enzymes including human epoxygenases (7,167,296-306)

Enzyme induction is defined simply as an increase in the amount of CYP present and consequently in the oxidation and clearance of drug (167,307,308). As discussed earlier, the xenobiotic-mediated induction of CYP genes occurs, mainly, via ligand-activated NRs and TFs (section 2.5). On the other hand, CYP inhibition is reduced enzyme activity due to direct interaction with a drug (308). The xenobiotic-associated inhibition of CYPs can be categorized generally as reversible (competitive vs. non-competitive) and non-reversible (mechanism-based inactivation) (308-314).

Reversible CYP inhibition occurs immediately when the inhibitor is added to the CYP enzyme and the inhibition stops when the inhibitor is removed (167). As mentioned in section 2.1, the allotropism phenomenon makes CYPs able to accommodate multiple ligands and substances, simultaneously. It is believed that when bound to the active site, the second molecule should have enough space to move from the primary SRS toward the heme to facilitate the catalysis (17,21). It is also possible that some ligands that bind in the active site are unable to move closer to the heme and could behave as competitive inhibitors by occupying this binding site (17). Indeed, in the competitive inhibition, the inhibitor directly binds to CYPs and competes with the substrate at the active site of the enzyme. The reversible non-competitive inhibition, however, models a system where the inhibitor (allostetrics) binds to site other than active site

(allosteric site); therefore, the inhibitor and the substrate may both be bound to the enzyme, at the same time. The enzyme-substrate-inhibitor complex cannot form product and can only be converted back to the enzyme-substrate complex or the enzyme-inhibitor complex (315).

As another type of xenobiotic-mediated CYP inactivation, the irreversible mechanism-based CYP inactivation (also known as time-dependent and catalysis-dependent CYP inhibition), involves CYP-catalyzed bioactivation of the xenobiotic to a reactive intermediate, which covalently modifies an active site amino acid residue and/or coordinates to the heme prosthetic group of the same CYP enzyme. Indeed, the mechanism-based inactivator is any compound that is catalytically transformed by CYP to give a reactive intermediate that inactivates the enzyme without leaving the CYP (167,308-314).

Table 2 lists the drugs and herbal supplements which have been identified so far to induce or inhibit the main human CYP epoxygenases. Unsurprisingly, the xenobiotic-mediated regulation of CYP expression could be another important source of intra- and inter-individual variability in the CYP-dependent AA metabolism and the EET formation. Interestingly, it is also noteworthy to note that in addition to the variability in the CYP expression induced by drugs, there is a large inter-individual variability in response to enzyme inhibition and induction (167). The sources of inter-individual variability in xenobiotic-mediated induction and inhibition of CYP enzymes have been described thoroughly in an excellent review by Lin and Lu (167).

Table 2. Cytochrome P450 inducers and inhibitors

CYP isoform	Inhibitors	Inducers
CYP2C8	Gemfibrozil	
	Montelukast	
	Zafirlukast	
	Trimethoprim	
	Thiazolidinediones	
	Quercetin	
	Deferasirox	
	Lapatinib	
	Candesartan cilexetil	
	Irbesartan	
	Ketoconazole	
	Clotrimazole	
	Felodipine	
	Nifedipine	
	Mometasone furoate	
	Salmeterol	
	Raloxifene	
	Fenofibrate	
	Ritonavir	
	Levothyroxine	
	Tamoxifen	
	Loratadine	
	Oxybutynin,	
	Medroxyprogesterone	
	Ethinyl estradiol	
	Lovastatin	
	Simvastatin	
	Cerivastatin	
	Fluvastatin	
	Spironolactone	
	Repaglinide	
	Star fruit juice (H)	
White Grapefruit (H)		
Naringin (H)		
Peppermint oil (H)		
Dimethylsulfoxide (H)		
Feverfew (H)		
Kava (H)		
Quercetin (H)		
		Cyclophosphamide
		Dexamethasone
		Gemfibrozil)
		Hyperforin
		Imatinib
		Lithocholic acid
		Nelfinavir
		Paclitaxel
		Phenobarbital
		Phenytoin
		Rifampicin
		Ritonavir
		Atorvastatin
CYP2C9	Fluconazole	Aprepitant
	Naringenin	Atorvastatin
	Sulphaphenazole	Avasimibe
	Tienylic acid	Barbiturates

Voriconazole	Bosentan
Amiodarone	Carbamazepine
Fenofibrate	Cyclophosphamide
Fluvastatin	Dexamethasone
Fluvoxamine	Glutethimide
Isoniazid	Hyperforin
Lovastatin	Nelfinavir
Phenylbutazone	Nifedipine
Probenicid	Norethindrone
Sertraline	Phenobarbital
Sulfamethoxazole	Prednisone
Sulfaphenazole	Rifampicin
Teniposide	Ritonavir
Voriconazole	Secobarbital
Zafirlukast	St. John's Wort (H)
5-hydroxyeugenol (H)	Glycyrrhizin (H)
Apigenin (H)	Hyperforin (H)
Black pepper (contains piperine) (H)	Licorice (H)
Capsaicin (found in cayenne pepper, habanero peppers) (H)	Limonene (H)
Cayenne pepper (H)	Orange EO (H)
Cinnamon (but not cinnamon oil) (H)	Valerian root (H)
Echinacea (H)	Artemisia scoparia (H)*
Ginger (H)	Artemisinin (H)*
Ginkgo biloba (H)	Atractylodes macrocephala (H)*
Kava (H)	Berberine hydrochloride (H)*
Pummelo (H)	Cafestol (H)*
Quercetin (H)	Chinese angelica (H)*
Saint John's Wort (High doses) (H)	Coptis chinensis (H)*
Turmeric (H)	Curcumin (H)*
Eugenol (H)	Epimedium brevicornum (H)*
Glabridin (in licorice) (H)	Eugenol (H)*
Licorice (contains Glabridin) (H)	Gardenia fruit (H)*
Star fruit juice (H)	Ginkgo biloba (H)*
Bergamottin (H)	Guggulsterone (H)*
Garlic (H)	Ligusticum chuanxiong (H)*
Luteolin (H)	Ligustilide (H)*
Milk Thistle (H)	Luteolin (H)*
Silymarin (H)	Lycium chinense (H)*
	Milk Thistle (H)*
	Ophiopogon japonicas (H)*
	Paeonia lactiflora (H)*
	Polygonum multiflorum (H)*
	Rehmannia root (H)*
	Resveratrol (H)*
	Schisandra chinensis (H)*
	Schisantherin A (H)*
	Trichosanthes kirilowii (H)*
	Turmeric (H)*

CYP2C19	Chloramphenicol	
	Cimetidine	
	Clopidogrel	
	Fluoxetine	
	Fluvoxamine	
	Felbamate	
	Indomethacin	
	Ketoconazole	
	Lansoprazole	
	Modafinil	Acetylsalicylic acid
	(-)-N-3-benzyl-phenobarbital	Antipyrine
	Naringenin	Artemisinin-type antimalarials
	(+)-N-3-benzyl-nirvanol	Baicalin
	Omeprazole	Barbiturates
	Oxcarbazepine	Carbamazepine
	Pantoprazole	Dexamethasone
	Probenicid	Dihydroepiandrosterone
	Rabeprazole	Efavirenz
	Ticlopidine	Hyperforin
	Topiramate	Nelfinavir
	Voriconazole	Norethindrone
	Capsaicin (H)	Prednisone
	Curcumin (H)	Rifampicin
	Nootkatone (H)	Ritonavir
	Resveratrol (H)	St. John's Wort (H)
	Saint John's Wort (H)	Ginkgo biloba (H)
	Turmeric (H)	Sage (H)
	Bergamottin (H)	Valerian Root (H)
	Carvone (H)	
	Dimethylsulfoxide (H)	
	Feverfew (H)	
	Glycyrrhizin (H)	
	Kava (H)	
Licorice (H)		
Peppermint EO (H)		
Pomegranate juice (H)		
CYP2J2	Arachidonic acid	
	α -naphthoflavone	
	Danazol	
	Hydroxyebastine	Not known
	Ketoconazole	
	Terfenadone	
	Tranlycypromine	
Troglitazone		

H: Herbal preparation; *Induce CYP2C9 through PXR activation

3.5. *Environmental factors*

Over the years, considerable efforts have been put into finding the possible influences of environmental factors, including diet, smoking and alcohol, on the expression and activity of CYP genes (308,316-322).

The CYP enzymes have been shown to be highly responsive to nutrient and non-nutrient factors presented in the diet (320). The non-nutrient chemicals, such as phytochemicals, food additives, trace contaminants and other xenobiotics that found in foodstuffs, have significant effects on the CYP expression and activity (320); similar to the other xenobiotics, the non-nutrients diet components can regulate CYP expression via a NR/TF-mediated mechanism, as described before. More importantly, alterations in the dietary content of macronutrients, such as protein and lipid, and micronutrients, including vitamins and minerals, are associated with considerable changes in the rate of CYP-dependent biotransformation of xenobiotics and endobiotics (320). Although the detailed mechanistic information of the diet-induced regulation of CYP expression has yet to be discovered, a number of recent reports have provided important evidence suggesting that growth hormone, NRs, TFs and cellular signalling pathways contribute to the dietary control of the expression of some, but not all, CYP genes (320). For more detailed review on the cellular mechanism of the regulatory effects of diet on the CYP expression and function, we refer to the review by Murray (320). The effect of diet on the human epoxygenase as well as CYP-dependent EET synthesis may be best exemplified by the influence of dietary fatty acid composition on the CYP-dependent branch of eicosanoid biosynthesis and the EET formation (319,322). It has been shown that virtually all human CYP isoforms involved in AA metabolism accept a wide variety of other PUFAs, including

eicosapentaenoic (EPA) (an omega-3 PUFA) and docosahexaenoic acids (DHA) (an omega-3 PUFA), as efficient alternative substrates, converting them to their corresponding metabolites, i.e. epoxyeicosatetraenoic acid (EEQ) and epoxydocosapentaenoic acid (EDP), respectively (319,322). These fatty acids are produced endogenously in a very limited and competitive manner and the demand for them is covered usually through dietary supplementation (319,323). EPA and DHA are abundantly found in fish, fish oil and other sea foods (319). Therefore, dietary n-3 PUFA supplementation may result in an increase accessibility of AA metabolizing CYPs to EPA and DHA and consequently decrease in the AA metabolism and EET formation (319,322). Interestingly, it has been recently demonstrated that in rats given dietary EPA/DHA supplementation, the endogenous EET:EEQ:EDP ratio was significantly shifted in different tissues. For instance, in the heart, this ratio shifted from 87:0:13 to 27:18:55 and in the kidney from 95:1:4 to 38:56:6 (322).

Additionally, the impacts of other environmental factors on the expression and activity of certain CYPs have been reported so far. The ethanol-mediated induction of CYP2E1 has long been known (324-326). In addition, the alcohol-induced liver damage has been demonstrated to be associated with a generalized induction of CYP2A6, CYP2E1 and CYP3A4 (326). Moreover, CYP1A2 and CYP1B1 expression have been found to be, respectively, 1.55 and 16.43 times higher in smokers than that of non-smokers (327-327). Moreover, exposure to polycyclic aromatic hydrocarbons, such as those found in char-grilled foods, induces expression of CYP1A1 and CYP1A2 (308). Also, compared with non-smokers, the expression of human CYP epoxygenase CYP2C9 was induced in the bronchial biopsies of smokers, whereas CYP2J2 was

repressed and CYP2C8 and CYP2C19 showed no changes (330). Likewise, the results of a recent study found no effect of smoking on CYP2C19 activity (331).

Collectively, the environmental-dependent regulation of the CYP expression causes significant inter- and intra-individual variability in the protein content and activity of these enzymes and consequently in the EET synthesis.

3.6. Sex steroid-dependent states

Accumulating evidence indicates that sex steroid hormones including endogenous androgens (e.g. testosterone and DHEA), estrogens and progesterones are important in regulating expression and activity of CYPs (203,207,208,332-337). In general, androgenic steroids cause marked variation in human CYP enzymes, estrogens decrease CYP expression and the reported effects of progesterones on CYP activity are variable and inconsistent with some investigations reporting an inhibitory effect and others reporting an inductive effect (332-337). More specifically, it has been shown that, with some exceptions, the physiological, pathological and pharmacological alterations in female sex hormones are associated with significant changes in the CYP activities and CYP-dependent drug clearance (332). For instance, while the activities of CYP isoforms remain generally unchanged during the menstrual cycles (338), menopause has been suggested, by some pharmacokinetic studies, to be associated with significant alteration in CYP3A, CYP1A2 and CYP2B6 activities (339-344); however, other pharmacologic studies failed to confirm the influence of the menopausal period on the activity of CYP3A isoenzymes (341,345,346). Furthermore, use of oral contraceptives (OCPs) is associated with remarkable CYP isoform-specific alteration in drug clearance (308). Pharmacological phenotyping studies

using specific probe substrates revealed that, in particular, the activities of CYP1A2, CYP2C19 and CYP2B6 decrease during combined OCP therapy (203,214-216,344). Additionally, results from clinical pharmacokinetic studies suggest that fluctuations in female sex steroid levels may lead to alterations in CYP activity throughout pregnancy (308). Particularly, the metabolism of CYP2A6, CYP3A4, CYP2C9, and CYP2D6 substrates is increased by approximately 50, 100, 20 and 50%, respectively, in the third trimester as compared with non-pregnant controls, whereas that of CYP2C19 and CYP1A2 substrates is decreased by 40–50% (337,349-353). It has been suggested that the pregnancy-mediated regulation of CYP enzymes occurs through the activation of corresponding NRs (308).

Collectively, given the regulatory effects of sex steroid hormones on the activity of human CYP enzymes, including epoxygenases, sex hormone change in the physiological, pathological and pharmacological conditions can be considered an important cause of inter- and intra-variability in the CYP activities and eventually in the EET formation.

3.7. Immunological response - local/systemic disorders

The phenomenon of inflammation/infection-induced down-regulation of hepatic and extrahepatic CYPs, particularly CYP2C and CYP2J epoxygenases, and its consequent effect on xenobiotics and AA metabolism has been reported in several human and animal studies (5,354-360). Infection with several viruses, like Herpes simplex, adenovirus and human immunodeficiency virus has been identified to depress CYP metabolism and reduce drug clearance (360). In addition, acute hepatitis A (361), and chronic hepatitis C (362), have been demonstrated to decrease CYP2A6 activity and CYP2D6/CYP3A4 activities, respectively.

Likewise, bacterial infections can impair CYP activity (360). Also, stimulation of the immune system through influenza immunization (363,364), and bacillus Calmette–Guerin (BCG) vaccination (365), is associated, in some cases, with decrease in CYP-mediated theophylline and aminopyrine clearance in healthy subjects.

Depending on the specific CYP gene, type of inflammatory stimuli and the time point in the response, multiple transcription-related mechanisms are implicated in the inflammation-induced downregulation of CYP activity (354-360). For most of CYPs studied, however, it has been found that inflammation suppresses, pre-transcriptionally, the expression of CYPs via cytokine-dependent activation of nuclear TF-kappa B (NF-κB) (356). NF-κB refers to a mammalian ubiquitously expressed pleiotropic nuclear TF that controls the expression of genes involved in a wide variety of pathophysiological processes such as inflammatory responses (367,368). NF-κB can be activated by a number of stimuli including inflammatory cytokines as well as exposure of cells to lipopolysaccharide (LPS), pathogens, toxins, drugs and oxidants (368). In regard to the influence of inflammation on the CYP expression, cytokine-induced activated NF-κB interacts with NRs which are involved in the CYP upregulation (356). For instance, suppression by inflammatory stimuli of induced transcription of CYP1A1 and CYP1A2 is due to mutually antagonistic interaction of NR AhR with NF-κB; NF-κB binds to AhR and these two transcription factors repress each other's function (369). The interaction of NF-κB with PXR and CAR are other mechanisms proposed to be involved in the downregulation of inducible CYP in response to inflammation (355,356,370,371).

The cytokines which are involved in this process are, in general, interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interferon-α (INF-α) and INF-γ

(354,360); however, cytokines have enzyme-specific effects on CYP expression so that the subsets of CYPs regulated by individual cytokines are different (354-357,360). For example, while CYP3A4 and CYP2C8 are down-regulated by all cytokines, CYP2C9 and CYP2C19 were found being down-regulated by IL-6 and TGF- β but not significantly affected by LPS, TNF, IFN, or IL-1. Moreover, CYP2B6 responds only to IL-6 and IFN (354,360).

Even though pre-transcriptional downregulation is the main mechanism in the inflammation-induced suppression of many CYPs, there are also several post-transcriptional mechanisms that are implicated in this effect. For instance, it has been shown that inflammation induces nitric oxide (NO) synthesis through the induction of NOS (NO synthase) resulting in the downregulation of CYP2B1 and CYP2B6 (372,373).

The regulatory effect of inflammation on CYP expression may provide, at least in part, a fundamental mechanistic explanation for the impacts of other pathological conditions like CVDs, rheumatoid arthritis, diabetes mellitus and cancers in which inflammation has been known to occur (316,360). However, the influence of diseases on the level and pattern of CYP expression is not limited to the inflammation-associated disorders. As an example, it has recently been demonstrated that inflammation has not an important contribution to the pathogenesis of varicose veins (374). We have previously shown that compared to normal veins, CYPs are differently expressed in human saphenous varicose veins (375). We demonstrated that CYP1B1, CYP2C, CYP2E1, CYP2J2, CYP3A5 and CYP4A11 are expressed in the human saphenous veins and that the expression levels of most of these CYPs is higher in varicose veins when compared to the normal controls (375). The available data on the effects of

different diseases on the expression and activity of an individual CYP, including human epoxygenases, has been tabulated in a review by Rendic and Guengerich (316).

These findings collectively contain an important message; the downregulation of CYPs seems to be a common feature of a variety of diseases, including those involving an immune response. The depression of CYP activity in response to an increased immunological response and also to diseases could potentially lead to a significant inter- and intra-individual variability in not only the expression of xenobiotics-metabolizing CYPs, but also the expression and activity of human CYP epoxygenases and the EET formation.

3.8. Nervous system and stress mediated CYP regulation

It has been demonstrated that both the central nervous system (CNS) and peripheral nervous system (PNS) are important contributors to the regulation of liver CYPs, mainly of CYP3A, CYP2C and CYP2D subfamilies (376-380). The CNS-mediated regulation of hepatic CYP expression occurs mainly via the tuberoinfundibular pathway and the dopaminergic D2 receptors of the pituitary, as well as the mesolimbic pathway engaging the D2 receptors of the nucleus accumbens. These two dopaminergic pathways stimulate the secretion of pituitary hormones, which directly (growth hormone) or indirectly (adrenocorticotrophic hormone and thyroid stimulating hormone) activate hepatic NRs controlling CYP genes. In addition to dopaminergic pathways, recent studies suggest also involvement of the brain noradrenergic and serotonergic systems in the regulation of liver CYP (376-380). Moreover, it has been demonstrated that the PNS can be involved in the physiological regulation of hepatic CYP activity through application of several neurotransmitters (acetylcholine, noradrenaline, adrenaline, dopamine and

serotonin) (376). It is also of interest to note that the autonomic sympathetic and parasympathetic nervous systems are also implicated in the regulation of CYP expression in the liver and the hypothalamus-pituitary-adrenal axis has a crucial role in this regulatory effect (376). Also, there is an increasing body of evidence supporting the involvement of stress in the regulation of hepatic CYPs so that stress has been suggested as a critical regulator of CYP3A, CYP2C and CYP2D expression (381). The hypothalamus-pituitary-adrenal axis and consequently catecholamines and the adrenergic-dependent systems mediate the stress-mediated regulation of CYP genes (381).

Taking together, physiological and/or pathological changes in the functioning of central, peripheral and autonomic nervous systems affect liver CYP expression provide potential sources of inter- and intra-individual variability in the expression of CYP enzymes, including epoxygenases, and the EET formation. Moreover, stress may regulate CYPs in the liver and is potentially a major cause of inter- and intra-individual difference in the metabolism of endogenous substances (e.g. EETs) and clinically important drugs.

3.9. *Radiation*

It has been suggested that both ionizing radiation (γ -rays, X-rays, high energy UV and fast neutron), caused by environmental pollution or clinical treatments, and non-ionizing radiation (low energy UV), contribute to the expression and activity of drug-metabolizing enzymes including CYPs (382). Accordingly, the inter-individual differences in sun exposure behaviors might cause a significant inter-individual variability in the CYP expression and consequently in the xenobiotic and endobiotic metabolism. More interestingly, it is not bizarre expect that even

in a given individual, the CYP enzymes are more active in a sunny wethaer than under cloudy sky. This will also to lead to a marked inter-individual variation in the metabolic activity of CYPs, including epoxygenases, and consequently in the production of EETs.

4. Inter-tissue variability in CYP epoxygenase expression

Even though expressed ubiquitously in hepatic and extrahepatic tissues, CYP genes exhibit a remarkable degree of tissue-specific expression so that there exists a considerable difference in the distribution and activity of CYPs across human tissues (62,167,171,383-389). The inter-tissue variability in CYP genes expression has been attributed, at least mainly, to a NR-dependent mechanism (62). Supposedly, human CYP expression is independently regulated in different tissues and the level of expression of individual CYPs in different organs is mediated by different NRs. For more detailed review on the contribution of NRs to the inter-tissue variability in the CYP expression, we refer to the review by Pavek and Dvorak (62).

Several lines of evidence indicate that, with few exceptions (i.e. CYP2F1 and CYP2S1) (62), CYPs are predominantly expressed in the liver and therefore, this organ plays the dominant role in the determination of the systemic level of drug and also endogenous metabolites. However, the CYPs are also expressed noticeably, but inconsistently, in extrahepatic organs such as gastrointestinal (GI) and respiratory tracts, central nervous system (CNS), genitourinary (GU) system, skin and cardiocirculatory system (62,167,171,383-389). The extrahepatic CYPs contribute to the tissue burden of xenobiotics and also, although at a lower extent than the liver, to the biosynthesis and bioavailability of endogenously-produced AA metabolites, EETs

(385). In general, each tissue has an exclusive profile of CYP enzymes that determines, to a large extent, the contribution of that organ to a given xenobiotic and/or endobiotic substrate and, therefore, leads to a tissue-specific response to chemical exposure (32,41).

Taking into account their significant contributions to the metabolism of endo- and xenobiotics, considerable research efforts have been directed in recent decades toward the identification of the expression rates of CYPs in the human extrahepatic tissues. With focus on human CYP epoxygenases, the expression and activity of CYPs in the extrahepatic tissues has been compared with those of the liver and are briefly reviewed below. In addition, the expression level of the main human CYP epoxygenases has been illustrated in Figure 14 and Table 3.

It is also noteworthy to mention that in addition to an inter-tissue variability in the CYP expression in a given individual, there is also considerable inter-individual inter-tissue variability in the expression of CYP enzymes. Likewise, the expression pattern of CYP in a given tissue varies between individuals. For instance, the expression of human CYP epoxygenases has been determined qualitatively and quantitatively in human cardiovascular tissues (390). As depicted in Figure 15A, a significant inter-individual variation can be observed among the cardiac, coronary artery and aortic expression level of CYP epoxygenases. Similarly, as depicted in Figure 15B, the expression level of CYP2E1 differs significantly in 18 human liver samples (391). This similarity has also been shown for other hepatic CYPs (171,392,393).

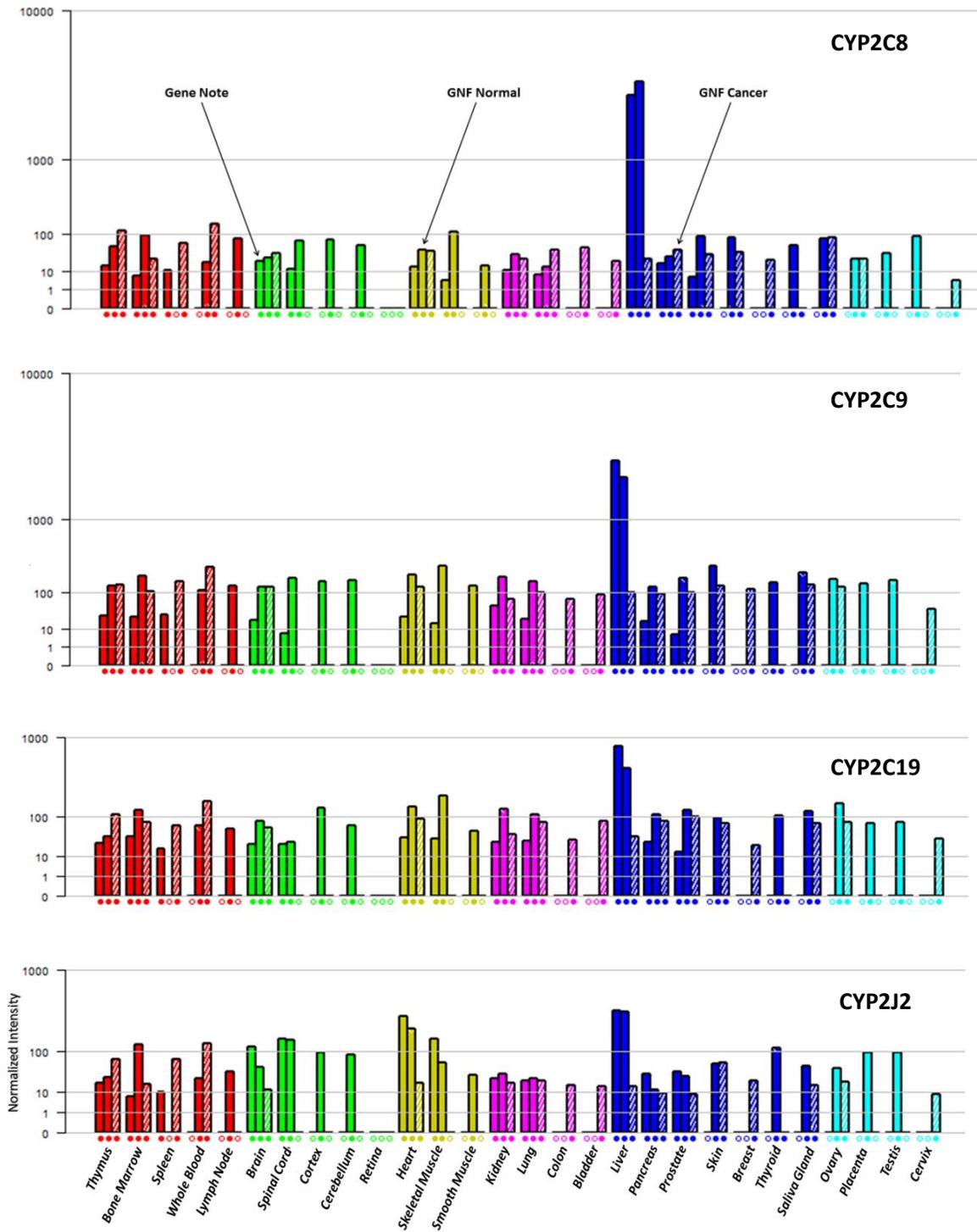


Figure 14. Gene Note/ GNF Normal and GNF Cancer expression array image showing the relative expression rates of human epoxygenases in different hepatic and extrahepatic tissues based on transcriptomic studies. Figures adapted from images available at: <http://www.genecards.org>

4.1. *CYP expression in liver*

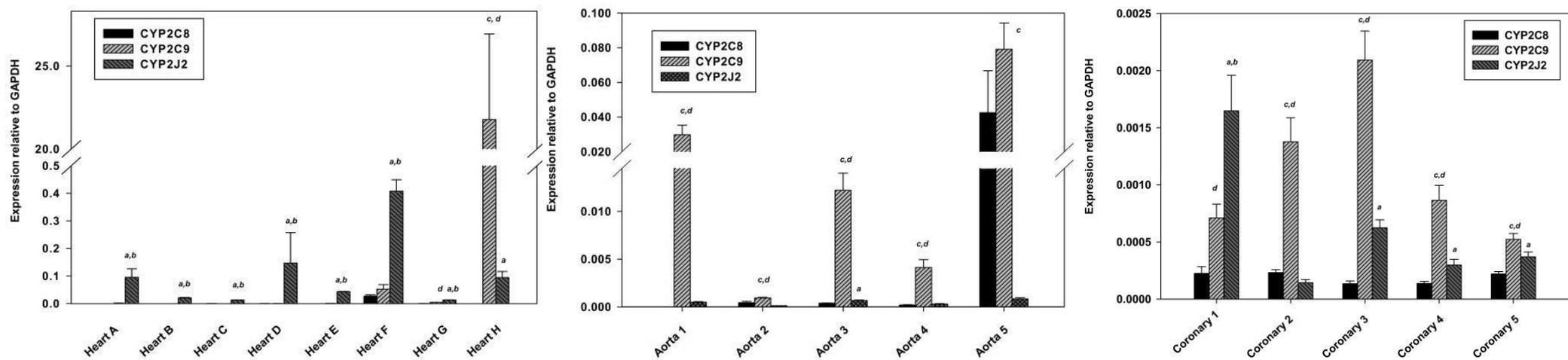
B2**B3**

Liver constitutes approximately 2.5% of human adult body weight (167). In general, CYP3A (CYP3A4, CYP3A5, CYP3A7 and CYP3A43), CYP2C (CYP2C8, CYP2C9, CYP2C18 and CYP2C19) and CYP1A (CYP1A1 and CYP1A2) are the major CYP subfamilies expressed in the human liver and account for, roughly, 40%, 25% and 18% of total hepatic immunoquantified CYPs, respectively. Although expressed at lower levels, the isoforms of CYP1B, CYP2A, CYP2B, CYP2D, CYP2E, CYP2R and CYP2J constitute the rest pieces of the human hepatic CYP pie (170,388) (Figure 16A).

4.2. *CYP expression in gastrointestinal tract*

Anatomically, the gastrointestinal tract is defined as comprising the esophagus, stomach, small intestine (subdivided into the duodenum, jejunum and ileum) and large intestine (385). As the portal-of-entry organs, GI tract is the first site of CYP-catalyzed metabolism of orally digested xenobiotics. Along the human GI tract as well as other extrahepatic tissues, intestinal CYP enzymes are probably the most important contributors to the extrahepatic metabolism of drugs and theoretically endogen compounds (62,385,388,394,395). However, the expression and activity of intestinal CYPs are still much lower than those of the liver (167). For example, the levels of CYP3A4 were estimated to be 350 pmol/mg of microsomal protein in the liver and 160, 120, and 70 pmol/mg of microsomal protein in the duodenum, jejunum and ileum, respectively (389). Furthermore, the CYP3CA4 catalytic activity, measured by erythromycin metabolism, was calculated to be 2.8, 1.6, 1.1 and 0.15 nmol/min/mg of protein in the liver, duodenum, jejunum and ileum, respectively (389). Furthermore, the estimated total mass of CYP2C and CYP2D6 in the whole intestine is 100- to 200-fold lower than that in the whole liver (389).

A



B

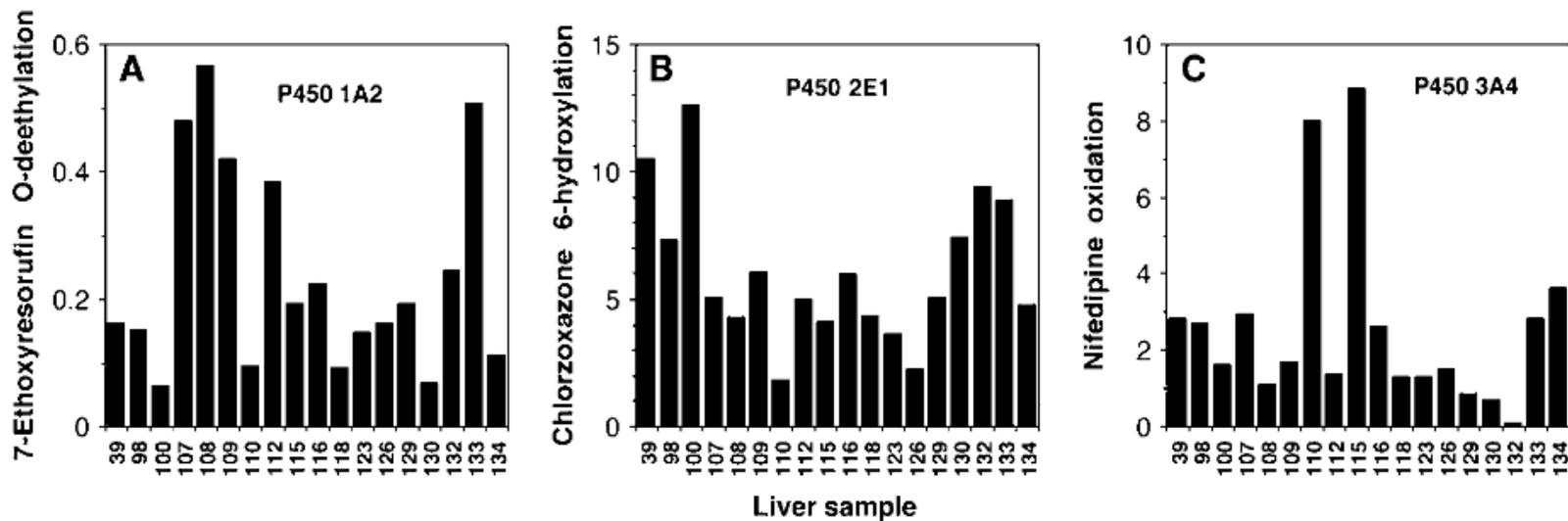


Figure 15. A. Variability in the expression of CYP epoxygenases across the organs of cardiovascular system. B. The expression of CYP1A2, CYP2E1 and CYP3A4 varied between the liver samples obtained from different individuals. Part A is reproduced with permission from Ref. 390 and Part B from Ref. 391.

Interestingly, total CYP content increased faintly in proceeding from the duodenum to the jejunum and then decreased abruptly toward the ileum (385,395).

As in the liver, CYP3A and CYP2C represent the major parts of the human intestinal CYP pie, accounting for, on average, 82% and 16% to total immunoquantified CYPs, respectively (Figure 16B) (388); however, except for CYP3A4, CYP2C9 and CYP2C19 for which there exists a relative consistency in the existing data about their expression in the intestine, there is an obvious discrepancy in the findings of studies that have explored the CYP profile of the human intestine. For instance, while the results of a transcriptomic analysis, on 27 patients who were referred for a routine gastro-colonoscopy, revealed that CYP2E1 has the highest mRNA expression among the studied CYPs in all parts of the GI tract including the duodenum (396), the CYP profile of the human intestine determined on the microsomes from duodenal/jejunal samples of 31 donors did not identify CYP2E1 among the detected CYPs (388). Moreover, there is also variability in the expression of CYP1A1 and CYP3A5 in the intestine between the previous reports. This inconsistency gets even more noticeable when different methods, including quantitative reverse transcription-polymerase chain reaction assays (RT-PCR), immunohistochemistry or immunoblotting, are employed for the characterization of CYP expression in various studies. In an investigation on enterocytes from 10 small intestines, RT-PCR of enterocytes revealed the expression of CYP1A1, CYP1B1, CYP2C, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 mRNAs; CYP1A2, CYP2A6, CYP2A7, CYP2B6, CYP2F1, CYP3A7, and CYP4B1 were not detected (397). However, when probed by immunoblots, only CYP3A4, CYP1A1, and CYP2C proteins were detectable, and CYP1B1, CYP2E1, CYP2D6, and CYP3A5 proteins were not detectable. In a larger study on 15 patients, the rank order for CYP2C mRNA expression in the

intestine, determined by RT-PCR, was CYP2C9 = CYP2C18 > CYP2C8 > CYP2C19, whereas the rank order for expression of CYP2C protein, using Western immunoblotting, was CYP2C9 > CYP2C19 > CYP2C8 (content below limit of quantification) > CYP2C18 (not detected) (386). This inter-study variation might be explained by different study designs, different methods, different populations under study (healthy vs. patient) and, most importantly, by marked inter-individual, even more general, inter-ethnic variability in the CYP expression pattern in the intestine.

In addition to the intestine, human CYP genes expressed in other parts of GI tract have been investigated in several studies (385). The CYP profile in the human esophagus consists of CYP1A1, CYP1A2, CYP2A, CYP2E1, CYP2J2 and CYP3A5; CYP1A1, CYP1A2, CYP2C, CYP2J2, CYP2S1 and CYP3A4 were found to be expressed in the stomach and the main CYP expressed in the colon are CYP1A1, CYP1A2, CYP1B1, CYP2J2, CYP3A4 and CYP3A5 (385).

Together, the main AA-metabolizing CYP epoxygenases are found ubiquitously in the GI tract. The expression pattern and level of the human epoxygenases varies inter-individually between 33% and 87% for CYP2C9, 4% and 38% for CYP2C19 and 0.5% and 7% for CYP2J2 (398). Accordingly, the intestine and other organs in the GI system could contribute to the biosynthesis of eicosanoids and might be potentially important sources of EET formation. Notably, the relative abundance of EET in the intestine has been reported to be 40 ng/g of intestine of which 14,15-, 11,12- and 8,9-EET contribute 31%, 36% and 33%, respectively (399).

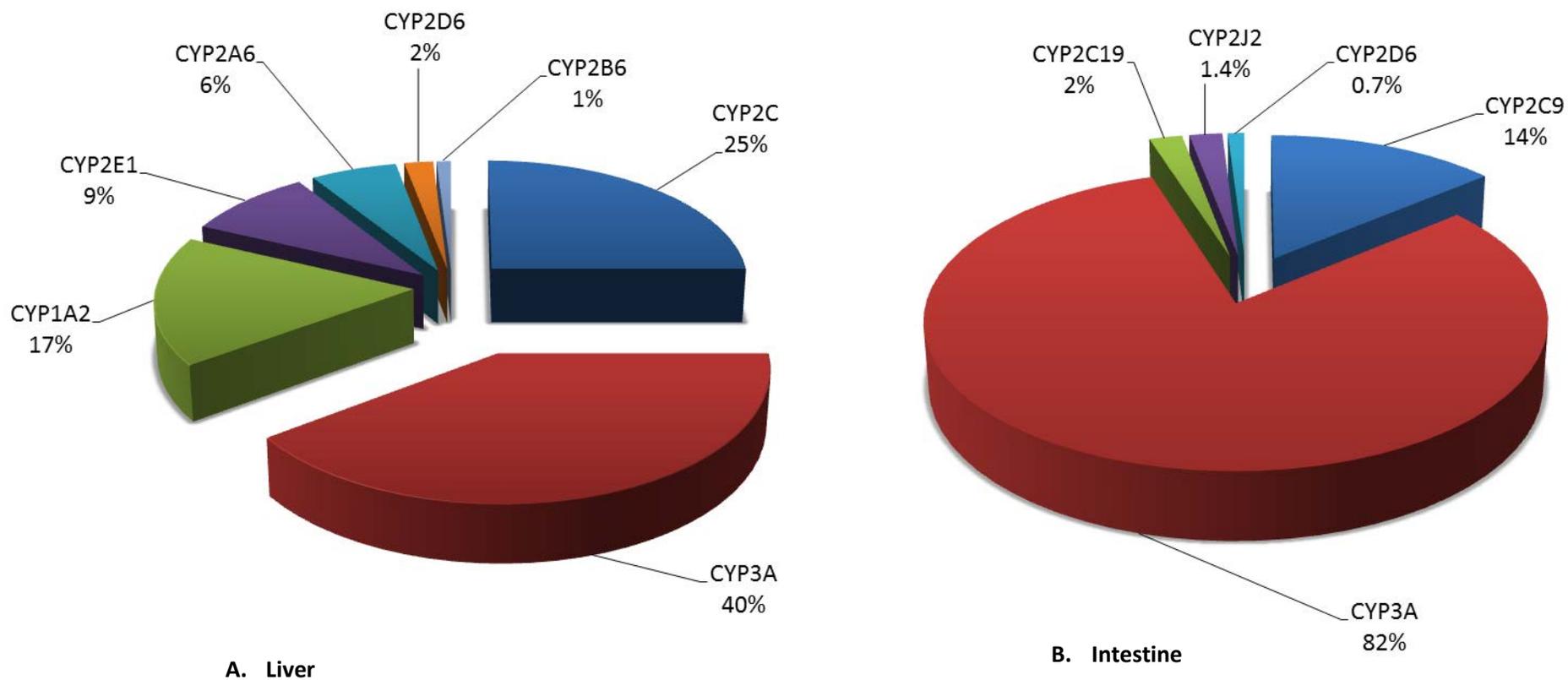


Figure 16. The relative abundance of CYPs in human liver and small intestine. Figure adapted with permission from Ref. 388.

4.3. *CYP expression in respiratory tract*

The respiratory system, consisting mainly of the nasal cavity, trachea, larynx and the lungs, is considered as the portal-of-entry for the inhaled xenobiotics and, therefore, the tissues of the respiratory tract are frequently exposed to elevated concentrations of exogenous inhaled compounds and are important targets for environmental toxicity (385). However, the tissues are continuously in contact both with the inhaled and blood-borne xenobiotics, the CYPs expressed in the respiratory tract would contribute to the biotransformation of wide ranges of exogenous chemicals including drugs (385).

The CYP profile of the human lung has been characterized in several studies. Many microsomal CYPs including CYP1A1, CYP1A2, CYP2B6, CYP2E1, CYP2F1, CYP3A4, CYP3A5, CYP4B1, CYP1B1, CYP2A6, CYP2A13, CYP2C, CYP2D6, CYP2J2, and CYP2S1 have been found to be expressed in bronchial and bronchiolar epithelium, Clara cells, type II pneumocytes and alveolar macrophages (62,385,400); however, Individual CYP isoforms have different patterns of localization within pulmonary tissue. Specifically, the CYP3A isoforms (CYP3A4 and CYP3A5) represent significant parts of total pulmonary CYP content (62). Both CYP3A4 and CYP3A5 are expressed in the lung (401); however, the lung cells show a significantly higher expression rate of CYP3A5 than that of CYP3A4 (CYP3A5 is found 4 times more than CYP3A4); therefore, CYP3A5 is regarded as the dominant CYP3A isoform in the respiratory tract (62). Even though most of the xenobiotic-metabolizing CYP enzymes are expressed in the lung at remarkably lower levels than in the liver, several CYPs including CYP2A13, CYP2F1, CYP2S1, CYP3A5 and CYP4B1 were demonstrated to be preferentially expressed in the lung. Furthermore, in the human larynx, mRNAs for the CYP1A1, CYP2A6, CYP2B6, CYP2C, CYP2D6, CYP2E1 and CYP3A5

were detected and predominantly expressed CYPs in the trachea are CYP2A6, CYP2A13, CYP2B6 and CYP2S1 (62,385,400). In the nasal mucosa, the CYP expression pattern was, interestingly, found to be slightly different in adult vs. fetal nasal mucosa. While CYP2A6, CYP2A13, CYP2C and CYP3A have been detected in adult nasal mucosa, CYP2A6, CYP2A13, CYP2B6 and CYP2J2 were found in fetal nasal mucosa (385).

Collectively, the fact that several CYPs which are involved in the metabolism of xenobiotic compounds are expressed ubiquitously along the respiratory tract and that certain xenobiotic-metabolizing CYPs, such as CYP2F1 and CYP2S1, are selectively expressed in the tracheopulmonary system, have made the lungs, and to a lesser extent other tissues of respiratory tract, to be pharmacologically active organs which contribute, particularly, to the metabolism of and response to the specific drugs. Furthermore, since the main human epoxygenases are universally expressed in the respiratory system, the lungs and other organs in this system are significant sources of EET formation. Specifically, it has been demonstrated that the overall concentration of EETs in the lung is approximately 110 ng/g lung tissue of which 14,15-EET, as the most abundant EET, contribute 43% and 11,12-EET and 8,9-EET contribute 26% and 31%, respectively (402).

4.4. *CYP expression in genitourinary system*

The CYPs expressed in the GU system have been demonstrated to play significant role in the metabolism of certain drugs as well as endogenous compounds (62). In general, CYP1B1, CYP2B6 and CYP3A5 are the predominant renal CYPs; however, CYP1A1, CYP2A13, CYP2C8,

CYP2C9, CYP2C19, CYP2J2, CYP2D6, CYP2E1, CYP2R1, CYP2S1, CYP3A4, CYP3A7 and CYP3A43 have also been reported to be expressed at lower levels in the GU system (62).

Specifically, the expression of human epoxygenases is extensively studied in the kidney (398). It has been shown that CYP2C8, 2C9 and 2J2 are all highly expressed in proximal and distal tubules, and collecting duct of the kidney (43). Therefore, it is not surprising the kidney cortex have a high relative abundance of EETs (i.e. 250 ng/g of the kidney tissue) (403). EET content of the human kidney cortex is composed predominantly of 14,15-EET (44%), followed by 11,12-EET (33%) and 8,9-EET (23%) (403).

4.5. *CYP expression in central nervous system*

The CNS composed of a set of highly specialized components of extremely heterogeneous cytoarchitectonic organization and functions consisting of an outstandingly complex meshwork of highly ordered interconnections among hundreds of billions of individual neurons (404). Since the brain is the target-tissue of a wide range of drugs, the contribution of the CYP profile to the biotransformation of xenobiotics as well as the metabolism of endobiotics in the CNS has been the focus of many researches (405-409). A substantial body of evidence indicates that, even though the total microsomal CYP content in the brain has been reported to reach up to 10% of the corresponding level in the liver microsomes (406,410), CYPs in the brain may have a role in the activation or inactivation of centrally acting drugs, in the metabolism of endogenous compounds and in the generation of damaging toxic metabolites and/or oxygen stress and that the CYP content and catalytic activity in the brain cells would be as important as, or even more

important than, that of the liver (405,408,409). Therefore, considerable effort has been expended toward identifying and quantifying the CYPs expressed in the human brain.

Among the human CYPs, CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C8, CYP2D6, CYP2J2, CYP2E1, CYP3A4, CYP3A5, CYP8A1, CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP19A1, CYP21A2, CYP26A1, CYP26B1, CYP27B1 and CYP46A1 have been found to be expressed in several sections of the human CNS; however, the characterization of CYP1A1, CYP1A2, CYP2B6, CYP2D6, CYP2E1 and CYP46A1 have been more extensively studied (405). The CYPs in human CNS are distributed largely, and extremely heterogeneously, in nearly all brain regions; however, along the human CNS, the highest and lowest CYP contents have been reported, respectively, from the brain stem/cerebellum and the striatum/hippocampus (405). For more detailed review on CYP expression in different parts of the CNS, we refer to the review by Dutheil, Beaune and Lorient (405).

In regard to human epoxygenases, the isoforms of CYP2C subfamily and also CYP2J2 have been detected in different parts of the CNS and accordingly, the relative abundance of EETs is evidently high in the cerebral tissues (398,405,411). EETs have been found to be produced in astrocytes, pituitary and hypothalamus (408), and have proven effects on the cerebral blood flow and cerebral function (408). The cerebral EETs also exhibit anti-inflammatory and cerebrovascular protective effects, and can also modulate the release of neurohormones that influence many physiological functions (408).

4.6. *CYP expression in cardiocirculatory system*

The tissues in the cardiovascular system are the main target organs of numerous widely-prescribed medications such as β -blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers and anticoagulant agents; the metabolic activity of cardiocirculatory CYPs involved in drug metabolism may have a significant effect on the effectiveness of pharmacotherapy (412-415). For example, the expression of CYPs in the right ventricle has been found to influence the metabolisms of β -blockers and verapamil and may explain, at least in part, lack of efficacy of these drugs in certain patients (412-414). Moreover, the cardiac CYPs have a potential role in the detoxification of noxious foreign compounds (412-414).

Additionally, strong evidence indicates that CYPs expressed in the cardiocirculatory tissues also contribute to the biotransformation of endogenous compounds such as AA (i.e. to EETs) (45,56-60,390,414-417). Considering the facts that EETs act, predominantly, in an autocrine and paracrine manner, and that EETs have potent cardiovascular protective properties, the cardiovascular CYPs may have a clinically important impact on the synthesis and biological activities of endobiotics, particularly EETs.

It has been demonstrated that the expression pattern of CYPs is highly variable among different human cardiovascular tissues. For instance, the results of an investigation in which the relative transcriptomic and proteomic expression of human epoxygenases, CYP2C8/9 and CYP2J2, has been compared between a limited number of samples from human cardiomyocytes, aorta and coronary artery revealed that CYP2J2 mRNA is approximately 10^3 times higher than CYP2C9 or CYP2C8 in human heart; in human aorta, mean CYP2C9 mRNA levels are ~ 50 times higher than

CYP2J2 and 5-fold higher than CYP2C8; in human coronary artery, mean values for CYP2C9 mRNA were ~2-fold higher than CYP2J2 mRNA and 6-fold higher than CYP2C8 mRNA. The proteomic analyses of the samples showed relatively high levels of CYP2J2 and CYP2C8 protein in human hearts; CYP2C9 was present at higher levels than CYP2J2 in aorta and coronary artery, while CYP2C8 protein was below the limits of detection (390).

To date, little is known about the human cardiac (i.e. cardiomyocyte) CYP profile and the findings of few available studies are somehow contradictory. While the original report of the expression of CYPs in human heart revealed the expression of CYP1A1, CYP2B6, CYP2C8, CYP2C19, CYP2D6 and CYP4B1 genes, predominantly in right ventricle (412,413), the results obtained in a larger and more recent study indicate that CYP2J2, CYP4A11, CYP2E1, CYP1A1 and CYP2C8 mRNA are the most abundant isoforms, at similar levels in the left and right ventricles, in human cardiomyocytes and that CYP2B6 and CYP2C9 mRNA were present at low levels in only certain samples (415). Specifically, in regard to CYP epoxygenases, CYP2J2 and, to a lower extent, CYP2C9 isoenzymes have been well demonstrated to be the primary epoxygenases producing EETs in the heart (36,39,45,390,398,414,415). Moreover, the overall abundance of EETs in the heart has been reported to be 60 ng/g of cardiac tissue; 14,15-, 8,9- and 11,12- comprise, respectively, 39%, 37% and 24% of the total cardiac EET content (45,47,420).

The vascular system is the largest organ in the human body and is continuously exposed to wide range of chemical compounds from both endogenous and exogenous sources including drugs. The blood vessel cells, comprising SMCs and ECs, may be the target organ and can potentially contribute to the biotransformation of circulating xenobiotics such as drugs and environmental contaminants. A very recent study has shown that CYP metabolizing enzymes expressed in

aortic SMCs and ECs can bioactivate clopidogrel; an antiplatelet drug which is commonly used in patients with acute coronary syndrome and those who undergo percutaneous coronary intervention (PCI) and stenting (418). Moreover, human vascular SMC and ECs are both the main target tissues of AA-derived eicosanoids. ECs are the main sources for EETs in the cardiovascular system (41,42). In fact, ECs are the sole source of EETs in the vascular wall; even though endothelium-derived EETs diffuse into and paracrinely act on the contiguous SMCs, none of the EET regioisomers are not produced by SMCs (41,390). Many CYP isoenzymes of CYP1A, CYP1B, CYP2B, CYP3A, CYP4A, CYP4F, CYP2E, CYP26B, CYP2C and CYP2J subfamilies have been identified in human blood vessels; however, considering the significant contribution of EETs in the vascular hemostasis, the expression and metabolic activity of human epoxygenases, CYP2C8/9 and CYP2J2, have been more extensively explored. Interestingly, there is a great heterogeneity in the expression pattern of CYP epoxygenases across the vascular beds and the blood vessels within different organs can express different epoxygenases (31,32,41,419). Accordingly, the relative abundance of EETs differs among vascular beds depending on which CYP isoforms and at what expression levels are expressed in that given vessel (31,32,41,419). Nevertheless, functional studies point that, in general, 11,12-EET followed by 14,15-EET are the main EETs released from ECs (32,37,41,42). Collectively, it is logical to speculate and makes sense to assume that, in function of their CYP profiles, various blood vessels across the human body contribute significantly, but meaningfully at different extent, to endogenous chemicals as well as drugs metabolism.

Further important components of the circulatory system are the blood cells and platelets. It has been suggested that neutrophils and monocytes can metabolize drugs to reactive metabolites

(421), and that the most relevant drug metabolizing CYPs (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) are differentially expressed in leukocytes, making them as potential extrahepatic sites of xenobiotic metabolism (417). In addition, the mRNA levels of CYPs in leukocytes have been found to strongly correlate with hepatic CYP expression, allowing a good estimation of the hepatic drug metabolizing activities and providing a useful and simple tool in personalized medicine.

In addition to ECs as the primary source of EETs, there is increasing evidence that circulating blood cells can contribute to the production, storage and release of EETs and that EETs produced in the blood cells and platelets could likely have functionally significant effects on cardiovascular hemostasis (56-60,416,417). For instance, CYP2J2, as a main human epoxygenase, has been identified to be expressed in human monocytes and alveolar macrophage, making them capable to generate EETs (416,417). Additionally, erythrocytes and platelets serve as important reservoirs for EETs and have the potential to regulate EET levels. These findings supports the hypothesis that, in addition to autocrine and paracrine effects, EETs may act as endocrine hormones on the cardiovascular tissues, such as ECs, which are far from the organs that they are produced in.

Table 3. Hepatic and extrahepatic expression of human CYP epoxygenases

CYP	Liver abundance (pmol/mg)	% of hepatic P450 pool	Ad.	Br.	Ht.	Kid.	Lu.	Ova.	Plc.	Prs.	Sk.	Sm. Int.	Tes.
2C8	29.3-30	~7.5	R	R	RPA	R	R	R	R	R		R	R
2C9	18-116	4.5-26	R	N.Q.	R	R	R	R	R	R	R	RPA	R
2C19	3.6-15	0.9-3.8	R	N.D.		R	R	R	R	R	R	RPA	R
2J2	1-2	<1	R	RP	RP	R	R	R	R	R		RP	R

Ad.: Adrenal; Br.: Brain; Ht.: heart; Kid.: Kidney; Lu.: lung; Ova.: ovary; Plc.: placenta; Prs.: prostate; Sk.: skin; Sm. Int.: small intestine; Tes.: testise; R, RNA detected by real-time PCR (N.D., not detectable; very low values reported as N.Q., not quantifiable); P, protein detected by Western blot, immunohistochemistry, or mass spectrometry (average hepatic P450 pool assumed as 0.4 nmol/mg microsomal protein); A, enzyme activity detected using appropriate probe drug. Data collected from reference 7.

5. Inter-tissue, inter- and intra-individual variability in CYP epoxygenases; clinical implication

Given the importance of inflammation in the development of a large number of illnesses, particularly of CVDs, the anti-inflammatory properties of EETs have gained great interest in recent decades among the researchers as well as pharmaceutical industries. The clinical implication of the inflammation-reducing effects of EETs and the application of epoxygenase pathway, including sEH enzyme, as potential therapeutic targets for the prevention and treatment of a variety of inflammation-related cardiovascular pathologies including hypertension, atherosclerosis, cardiac hypertrophy and ischemic injuries, have been the subject of several recent review papers (422-427). However, the potential influences of the inter-tissue, intra-individual- and inter-individual variability in the expression of main human CYP epoxygenases on the local and systemic EET production (Figure 16), and its possible pathophysiological consequences have been less discussed.

Although ECs are the main sources and target organs of EETs, theoretically all of the human tissues in which CYP epoxygenases are expressed can contribute to EET formation. However, there exists a significant inter-tissue variability in the expression of human epoxygenases and accordingly, the relative abundance of EETs varies across the vascular bed. It would be logical, therefore, to assume that the contribution of EETs to the regulation of vascular inflammation varies among arterial ECs of different organs. This hypothesis may be a reasonable explanation for the difference in the prevalence of an inflammation-associated phenomenon, e.g. atherosclerosis, among the blood vessels across the body. For instance, atherosclerosis is a result of local and systemic inflammation; however, it is detected more frequently in the

coronary circulation and brain arterial system. This theory would provide the impetus for developing EET-based medications that selectively induce the expression of epoxygenases and the formation of EETs in a specific arterial territory, coronary arterial tree, for example. On the other hand, by considering the body an integrated system, it is logical to speculate that, under physiological condition, nearly all human tissues, but certainly at different extent, cooperate in an extensive “EET-mediated anti-inflammatory network”. This network provides an excellent opportunity and may be an emerging target for further pharmacological therapy of disorders in which increased inflammatory responses are known to occur.

Additionally, the CYP expression is under control of a large number of biological variables (Figure 17). Therefore, it could be assumed that the relative as well as absolute abundance of EETs, and consequently their anti-inflammatory properties, is subject to change with respect to the presence or absence of the parameters which regulate the expression and activity of CYP epoxygenase. This may explain, at least in part, why some conditions predispose an individual to be affected by an inflammation-related disease such as CAD. For instance, it has been shown by several studies that the expression and activity of CYP epoxygenases decrease with age resulting in a decrease in the relative and absolute abundance of EETs in older ages. This might be a reason why the incidence of CAD and hypertension increases with age. Also, the significant inter-individual variability in the CYP expression results in an inter-subject variability of EET levels which might explain, at least in part, the heterogeneous pattern of the prevalence of the inflammation-associated CVDs, including HTN and atherosclerosis, among different individuals. For example, as discussed earlier, the expression rate of CYP2J2 and CYP2C9 has been demonstrated to be higher in females than in males. This would be the reason why the

incidence of CAD, as a prototype of inflammatory CVDs, is significantly higher in males than that of the females. Besides, the functional polymorphisms of genes encoding CYP epoxygenases, as well as the genetic variations of genes involved in the expression and activity of the epoxygenases, including NRs, POR and miRNAs, might potentially contribute to the inter-individual and, more generally, to the inter-ethnic variability in the prevalence of those diseases in which inflammation is known to occur. To make the matter more complicated, beside an inter-individual variability in the CYP epoxygenase expression and activity, there is an inter-tissue-, inter-individual variability in the expression of CYPs. For instance, not only the transcription rate of a given CYP epoxygenase, CYP2J2 for example, differ in the cardiac vs. intestinal vascular bed in a given individual, but also the CYP2J2 expression rate varies between the cardiac endothelium of two different subjects, as well. This fact also could provide a logical reason for the inter-subject difference in the prevalence of tissue-specific inflammation-related illness such as CAD.

6. Summary

The key points of this review can be summarized as follow:

1. The CYP system is a superfamily of enzymes that involved in approximately 80% of oxidative drug metabolism, as well as the metabolism of many endogenous chemicals like AA.
2. The main human CYP epoxygenase, CYP2C8/9/19 and CYP2J2, convert AA to four corresponding EET regioisomers. The epoxide metabolites of AA exert potent anti-inflammatory effects in the cardiovascular system.

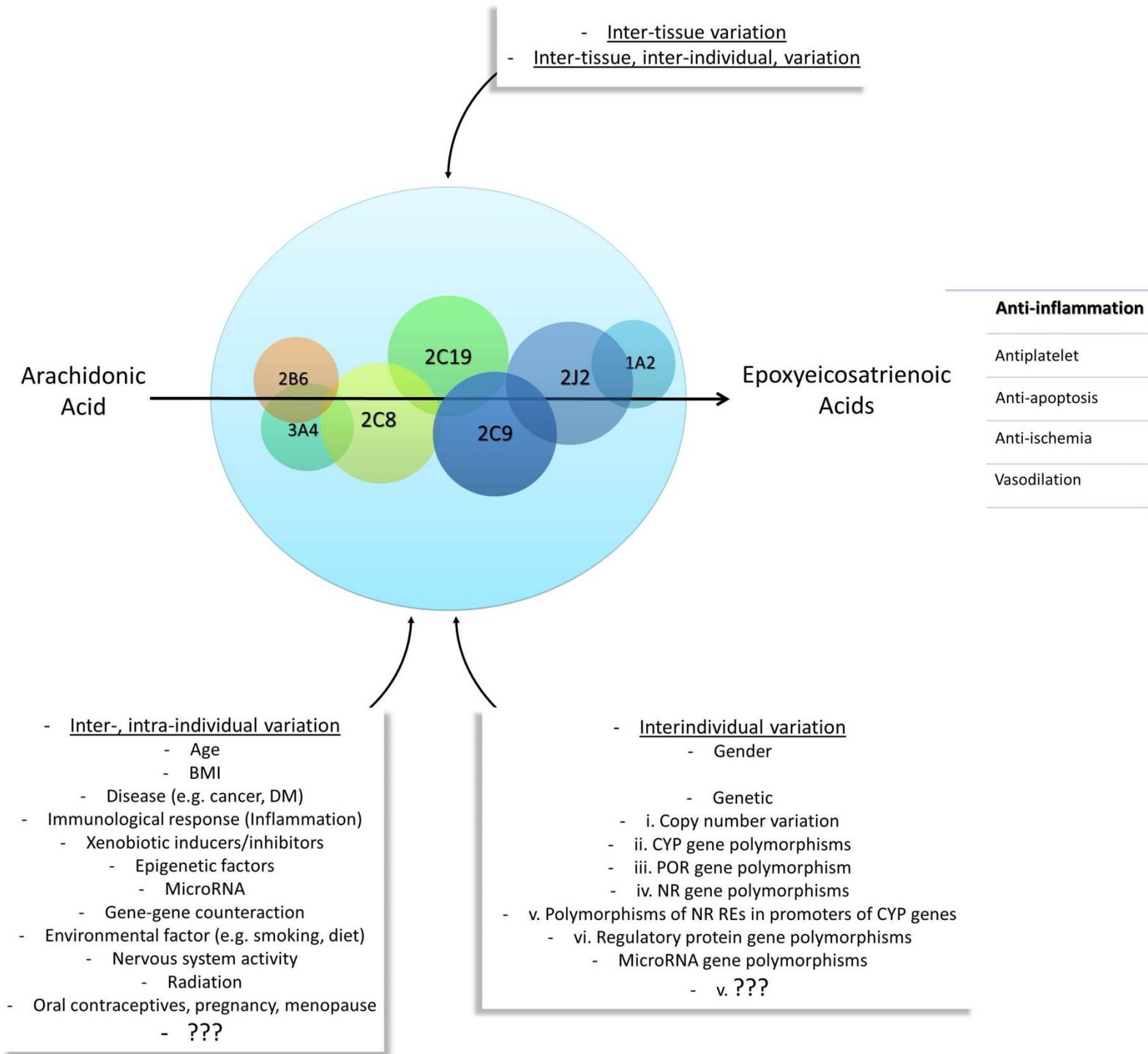


Figure 17. Summary of variables affecting the expression of CYP epoxygenases and resulting to inter-tissue, intra-individual and inter-individual variability in the expression of these enzymes.

3. The expression of CYP epoxygenases is regulated mainly through a highly intriguing network of NRs, ERs in the promoters of CYP genes, miRNAs and genetic/epigenetic factors. Accordingly, a large number of variables, including xenobiotics and environmental factors, can potentially modify the expression and activity of epoxygenase genes, leading to ten- to hundred-fold inter-individual and also a significant condition specific intra-individual variability in the CYP epoxygenase expression and activity.
4. The organ that expresses the highest levels of CYP epoxygenases is the liver. However, epoxygenase genes are expressed in nearly all extrahepatic tissues; mainly in the intestine, the lung, the brain, the kidney and the heart. However, there is a significant inter-tissue variability in the CYP expression rate and activity. On the other word, CYP2C and CY2J enzymes are expressed heterogeneously in different tissues and each tissue has a unique profile of CYP enzyme. Since CYP epoxygenases are relatively regioselective for EET formation which means that each CYP epoxygenase produces its own unique profile of EET regioisomers, the relative abundance of each EET regioisomer are organ specific.
5. The inter-tissue, intra-individual and inter-individual variability in the expression and activity of CYP epoxygenases and consequently in the EET formation may provide reasonable explanations for the differences in the prevalence of the inflammation-related diseases, such as CADs, between tissues of a given individual, in an individual under various conditions and between individuals of a given population and/or different ethnics, respectively.

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III. Influence of Inflammation on Cardiovascular Protective Effects of Cytochrome P450 Epoxygenase-Derived Epoxyeicosatrienoic Acids

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Key terms: Inflammation, cytochrome P450, epoxygenase, epoxyeicosatrienoic acids, nuclear transcription factor-kappa B, soluble epoxy hydrolase

Abstract

In addition to their role as xenobiotic metabolizing enzymes, cytochrome P450 (CYP) epoxygenases actively contribute to the metabolism of endogenous substances such as arachidonic acid. Epoxyeicosatrienoic acids (EETs) are epoxide derivative of arachidonic acid. CYP2C8/9 and CYP2J2 are the main epoxygenases expressed in human tissues including endothelial cells which are the chief sources of EET formation in human body. Once formed, EETs are primarily metabolized to their less biologically active metabolites, dihydroxyeicosatrienoic acids, by soluble epoxy hydrolase (sEH) enzyme. EETs possess a wide range of established protective effects on human cardiovascular system of which vasodilatory, angiogenic and anti-inflammatory actions have been more extensively described. On the other hand, inflammation has shown to decrease the expression and activity of CYP enzyme, including epoxygenases. Given the fact that CYP epoxygenase-derive EETs exhibit potent cardiovascular protective effects, including anti-inflammation, and that inflammation suppress CYP activation and EET formation, it would make sense to speculate that under inflammatory conditions there exists an inflammation-epoxygenase-EET-inflammation vicious cycle in which the inflammation-induced downregulation of CYP epoxygenases causes a decrease in the EET production. Insufficient EET synthesis would, in turn, lead to an ineffective EET-mediated anti-inflammatory effect, leading to an augmentation of systemic and regional inflammatory responses and further downregulation of CYP epoxygenase activity/EET production. This cycle, if any, might help to better understanding of pathophysiology of chronic cardiovascular diseases and also could be an emerging target for further pharmacological therapy of disorders in which increased inflammatory responses are known to occur.

III. Influence de l'inflammation sur les effets protecteurs cardiovasculaires des acides époxy-eicosatriénoïques dérivés des époxygénases du cytochrome P450

Outre leur rôle d'enzymes métabolisant les xénobiotiques, les époxygénases du cytochrome P450 (CYP) contribuent de façon active au métabolisme de substances endogènes telles que l'acide arachidonique. Les acides époxy-eicosatriénoïques (EET) sont des dérivés époxydiques de l'acide arachidonique. CYP2C8/9 et CYP2J2 sont les principales époxygénases qu'expriment les tissus humains, dont les cellules épithéliales qui sont les principales sources de formation d'EET dans l'organisme humain. Une fois formés, les EET sont principalement métabolisés par l'enzyme époxy-hydrolase soluble (EHs) en leurs métabolites biologiquement moins actifs, les acides dihydroxy-eicosatriénoïques. Les EET possèdent un large éventail d'effets protecteurs établis sur le système cardiovasculaire humain, parmi lesquels ont été décrites de façon plus étendue leurs actions vasodilatatrices, angiogènes et anti-inflammatoires. Il a été démontré que des concentrations physiologiques d'EET, une surexpression d'époxygénases du CYP et la prise d'analogues des EET et d'anti-EHs freinaient puissamment l'inflammation vasculaire en inhibant l'activation des cellules endothéliales et l'adhésion endothéliale des leucocytes qui représentent, l'une comme l'autre, des événements essentiels dans la réponse inflammatoire vasculaire. Plusieurs mécanismes et voies de signalisation sont impliqués dans les effets anti-inflammatoires à médiation endothéliale induits par les EET, le point clé étant l'inhibition de l'activité du facteur de transcription nucléaire kappa B. De plus, l'inhibition de l'agrégation et de l'activité plaquettaires constitue également un autre mécanisme proposé de réduction de l'inflammation par les EET. D'autre part, il est établi que l'inflammation diminue l'expression et l'activité des enzymes du CYP, y compris les époxygénases. On a attribué la régulation à la baisse inflammatoire des enzymes du CYP aux effets des cytokines inflammatoires, surtout le facteur de nécrose tumorale alpha, les interférons et l'interleukine-6. Etant donné le fait que les EET dérivés des époxygénases du CYP présentent de puissants effets anti-inflammatoires et que l'inflammation freine l'activation du CYP ainsi que la formation d'EET, il serait logique de supposer que dans des conditions inflammatoires existe un cercle vicieux n inflammation-époxygénase-EET-inflammation dans lequel la régulation à la baisse des époxygénases du CYP induite par l'inflammation a pour conséquence une moindre production d'EET. La synthèse insuffisante d'EET se traduirait à son tour par une inefficacité de l'effet anti-inflammatoire à médiation par les EET, entraînant une augmentation des réponses inflammatoires systémique et régionale et un surcroît de régulation à la baisse de l'activité des époxygénases du CYP/de la production d'EET. Ce cycle, s'il se vérifie, pourrait aider à une meilleure compréhension de la physiopathologie des maladies cardiovasculaires chroniques et serait également susceptible de constituer une nouvelle cible émergente pour la poursuite du traitement pharmacologique des troubles où l'on sait que se produisent des réponses inflammatoires accrues.

Mots clés : Inflammation, cytochrome P450, époxygénase, acides époxy-eicosatriénoïques, facteur de transcription nucléaire kappa B, époxy-hydrolase soluble

1. Vascular arachidonic acid metabolism

Cytochrome P450 (CYP) enzymes, the ubiquitous membrane-bound hemoproteins, are involved in the metabolism of 75% of xenobiotics and pharmaceuticals.^{1,2} Even though for many years they were believed to be biologically important only for the exogenous substances, several lines of evidence now clearly show that CYP enzymes play an integral part in the metabolism of endogenous compounds like cholesterol, fatty acids and arachidonic acid.³

Arachidonic acid is a 20-carbon polyunsaturated omega-6 fatty acid that resides in the cell membrane phospholipids in the stereospecific numbering (*sn*)-2 position and is released upon stimulation by the cytosolic enzyme phospholipase A₂ (cPLA₂).⁴ The free intracellular arachidonic acid is converted to a series of biological active metabolites referred to as “eicosanoids”.⁵ Cyclooxygenases (COX) catalyzes the biosynthesis of prostaglandins (PGs)/thromboxanes (TXs) and lipoxygenases (LOX) mediates the production of leukotrienes (LTs).^{6,7} Referred to the “third pathway” of arachidonic acid metabolism, CYP enzymes also catalyze the conversion of arachidonic acid to two other eicosanoids; the epoxyeicosatrienoic acids (EETs), produced by CYP epoxygenases, and the hydroxyeicosatetraenoic acids (HETEs), produced by CYP ω -oxidases.^{8,9} A number of early studies suggested that EETs possess potent cardiovascular protective effects including regulation of vascular tone,¹⁰⁻¹² stimulation of angiogenesis,¹³ prevention of arterial smooth muscle migration,¹⁴ and suppression of inflammation.¹⁵ Subsequently, a surprising number of further preclinical investigations as well as experimental studies have clearly documented the contribution of EETs to vascular and cardiac physiology; these findings have been the focus of a plethora of recent reviews.^{3,5,6-21} In

this review, we first provide an overview of CYP epoxygenase pathway of arachidonic acid and EET metabolism. The review then presents in detail the biological functions of EETs in the cardiovascular system, and the molecular mechanisms by which EETs exert their protective effects. Next, we discuss how the potential interaction between epoxygenase-derived EETs and inflammation may affect the beneficial effects of EETs in the human cardiovascular system.

2. EETs synthesis and metabolism; overview

EETs are epoxide derivatives of arachidonic acid.²² Several CYP subfamilies including CYP1A, CYP2B, CYP2C, CYP2E and CYP2J have been categorized as CYP epoxygenases and potentially contribute to the conversion of arachidonic acid to EETs;^{9,23} however, the CYP epoxygenases that synthesize EETs are predominantly members of the CYP2C and CYP2J classes.^{9,16,24,25} In particular, the CYP2C8, CYP2C9 and CYP2J2 isoforms have been identified as the main human epoxygenases involved in the biotransformation of arachidonic acid to EETs.^{16,18,26} It is worth mentioning that the catalytic activity of the CYP2J for the production of EETs is markedly lower than 2C enzymes.^{5,16,27} The CYP epoxygenases, which are situated in the endoplasmic reticulum,²⁸ catalyze the epoxidation of four olefin bonds of the intracellular arachidonic acid that gives rise to four *cis*-regioisomers: 5,6-EET, 8,9-EET, 11,12-EET and 14,15 EET (Figure 1).¹⁶ Interestingly, both *cis*-EETs and *trans*-EETs are found in plasma;^{29,30} however, the biochemical pathway responsible for *trans*-EET formation has not yet identified. Since the epoxide groups can react with the double bonds of arachidonic acid in two different configurations, each EET regioisomer is biosynthesized either as *R*, *S*- and/or *S*, *R*- enantiomers (Figure 1).¹⁶

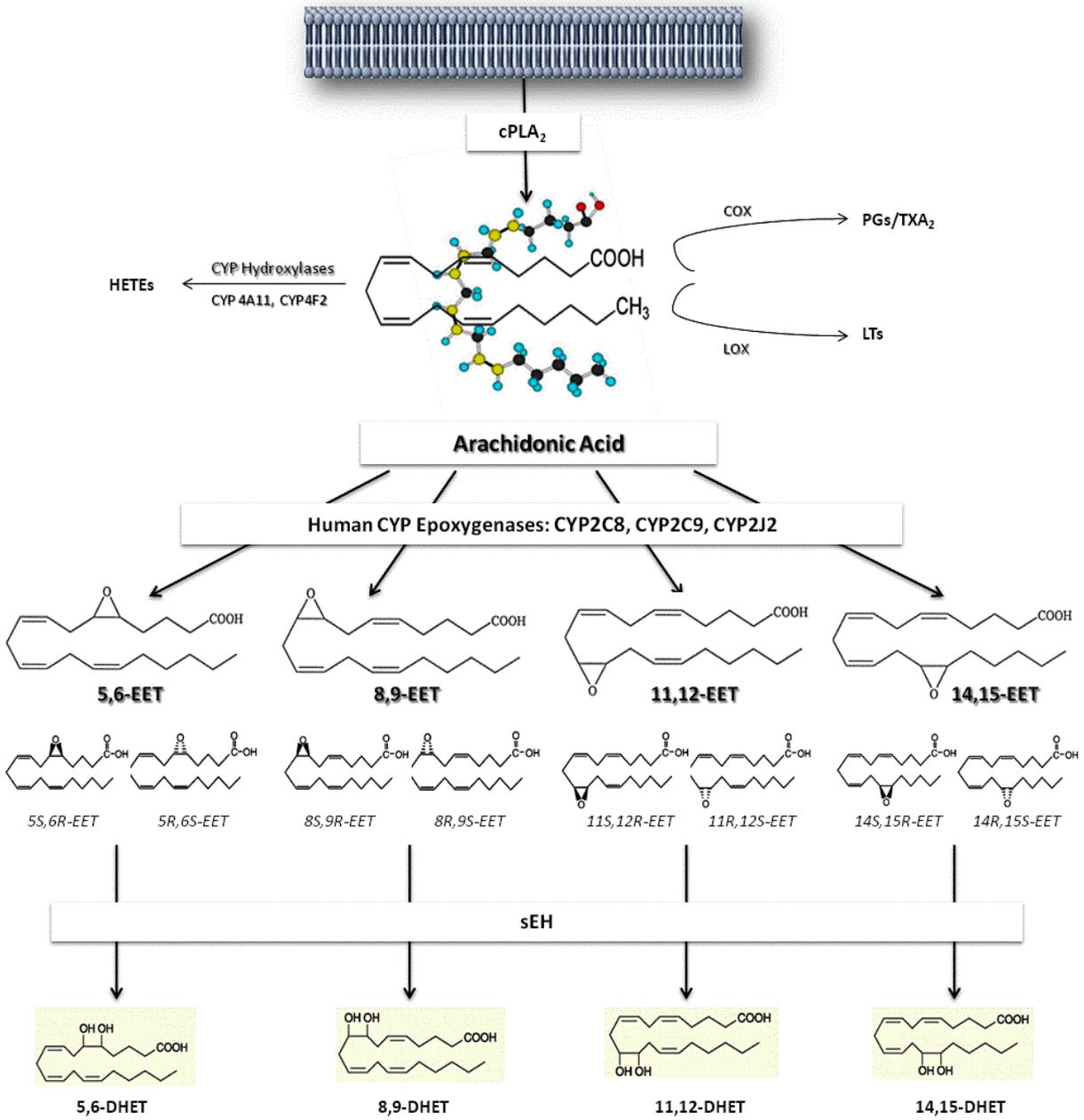


Figure 1. Biosynthetic pathways of arachidonic acid

Each epoxygenase is, supposedly, capable to convert arachidonic acid to all four EETs; however, 11,12- and 14,15-EETs are, in real, the main products in many cases.^{9,31,32} Furthermore, there is mounting evidence that human CYP epoxygenases are relatively regioselective and stereoselective for EET formation. For instance CYP2C8, the main human epoxidase,^{5,33} catalyze the biosynthesis of 14,15-EET and 11,12-EET in a ratio of 1.3:1.0 whereas its capability for the formation of 8,9-EET and 5,6-EET is negligible. The CYP2C9 also generates 14,15-EET and 11,12-EET in a ratio of 2.3:1.0; this epoxygenase, however, does not form significant amounts of 8,9-EET.^{34,35} In addition, 82% of the 11,12-EET produced by CYP2C8 and 76% of the 14,15-EET produced by CYP2C9 are the *R, S* enantiomer. In contrast, the 14,15-EET synthesized by CYP2C9 is 69% *S, R*.^{34,35}

Once formed, EETs could function in an autocrine and paracrine manner (discussed in next section) and/or could be further metabolized through the three main pathways as follows (Figure 2A):

1) *EET Recycling*: All EET regioisomers can be esterified, almost exclusively in the *sn*-2 position,^{36,37} in the cellular membrane phospholipids through a coenzyme A (CoA)/ adenosine triphosphate (ATP)-mediated acyltransferase reaction.^{38,39} Even though representing only 0.04% of the total fatty acyl chains in the membrane phospholipids,³⁸ the esterified incorporated EETs have shown to be potentially capable to modulate the cell membrane channel activity.^{5,40} Incorporation into the phospholipids could also serves as a source for EETs until stimulated to be released into the cytosol through a PLA₂-catalyzed hydrolysis reaction or

into the plasma and extracellular tissue.⁴¹ More than 90% of EETs in the plasma are present in the phospholipids and most of the phospholipids that carry EETs are low density lipoproteins.³⁷

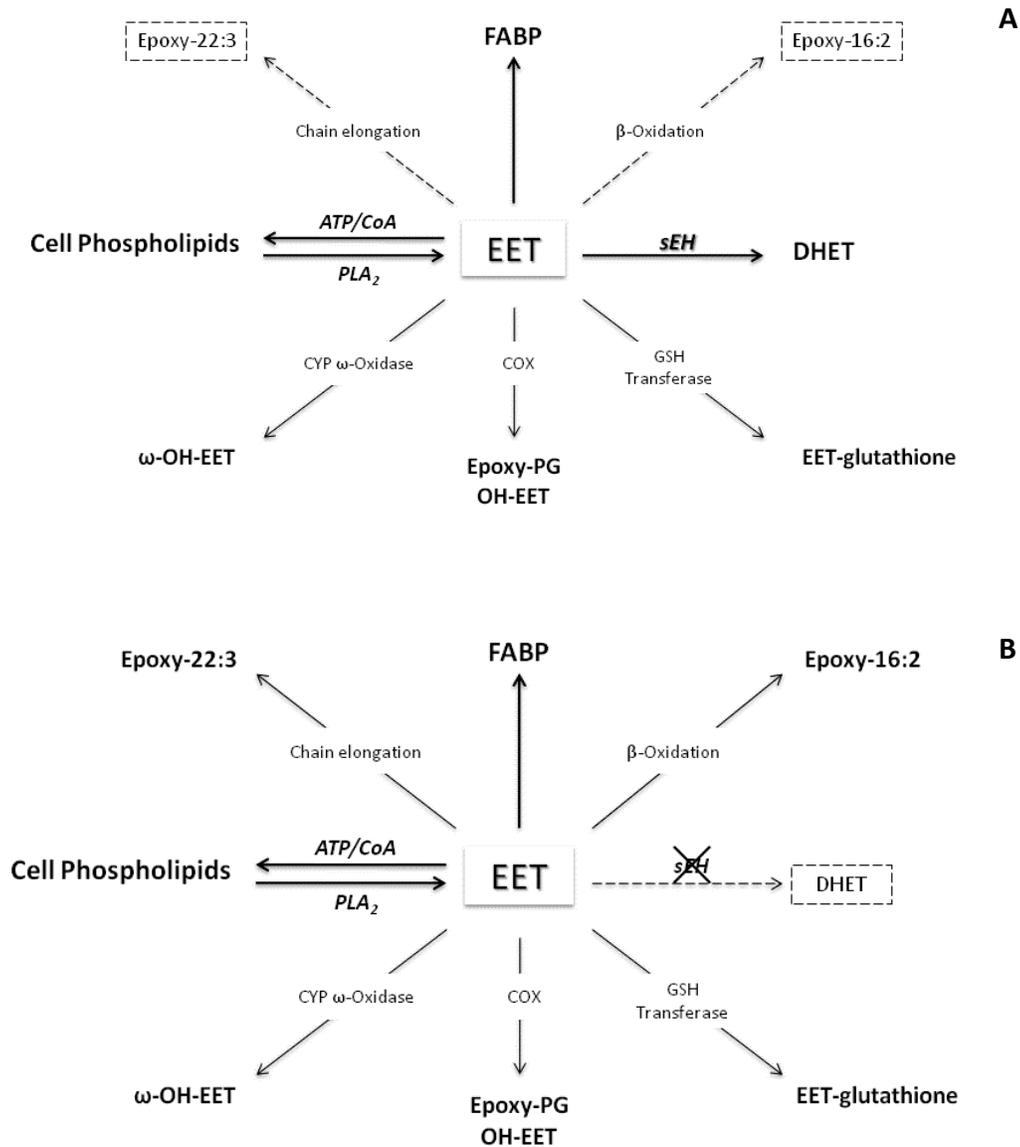


Figure 2. Metabolic fate of EETs. The diagram in A provides an overview of the main EET metabolic pathways under normal condition in which sEH is active. Alternative pathways in the presence of sEH inhibitors have been depicted in diagram B.

2) *EET Hydration*: Supported by several lines of evidence to be the main catabolic pathway of EETs, soluble epoxide hydrolase (sEH) modulates bioconversion of epoxyeicosatrienoics to the corresponding diols, dihydroxyeicosatrienoic acids (DHETs), through a hydration reaction (Figure 1).^{5,9,16,37} Interestingly, there is a marked difference in the affinity of this enzyme for the four EET regioisomers with the 14,15-EET being the preferred and the 5,6-EET the poorer substrates.⁴²⁻⁴⁴ The metabolism of EETs by sEH is also highly stereospecific for the 14R,15S-EET, 11S,12R-EET and 8S,9R-EET enantiomers.⁴⁵

Under conditions of sEH inhibition (Figure 2B), either inherently (e.g. owing to sEH gene polymorphisms) or in the presence of the pharmacological enzyme inhibitors, only the 11,12- and 14,15-EET undergo β -oxidation, producing a chain-shortened 16-carbon epoxy-fatty acid, and/or they can be converted to a 22-carbon product through chain elongation.^{36,46,47} Moreover, the 8,9-, 11,12- and 14,15-EET can be metabolized to the corresponding hydroxylated products by CYP ω -oxidases,⁴⁸ and the 5,6- and 8,9-EET are substrates for COX to be converted to vasoactive epoxy-derivative metabolites.^{49,50} Lastly, all four EET regioisomers can form glutathione conjugates, the biological significance of which is unknown.⁵¹ The majority of the aforementioned EET metabolites, including the sEH-derived DHETs, possess biological properties but generally less than those of the parent epoxides.^{5,16}

3) *EET Binding to Fatty Acid Binding Protein*: Fatty acid binding protein (FABP), is a low-molecular-weight cytoplasmic protein involving in the intracellular uptake and buffering of free fatty acids and other lipophilic substances such as eicosanoids and retinoids.^{52,53} Among the ten identified FABP isoforms,⁵⁴ heart-type FABP (H-FABP) has been more studied.³⁷ All four EET regioisomers can bind to cytosolic.⁵⁵ FABP could act as a transporter protein for EETs and hand

over them to intracellular target enzymes, ion channels, regulatory proteins, transcription factors and specific organelles.^{56,57} In addition, FABP can modulate the incorporation of EETs into the membrane phospholipids and also facilitate EET uptake into the cell.¹⁶ It has also been shown that the binding affinity of FABP to EETs limits the availability of EETs to sEH for converting to DHETs and consequently increases the intracellular bioavailability and the half-life of EETs (Figure 2A, 2B).⁵⁸

3. CYP epoxygenases expression and EET resources in cardiovascular system

Taking into the account that EETs act on the cells in which they are produced (autocrine effects) or adjacent cells (paracrine effects),^{5,22} and that functional effectiveness of each regioisomeric EET, and even two enantiomers of an individual EET regioisomer, differ from organ to organ,¹⁶ it is logical to speculate that the local EET intracellular and tissue levels would be biologically more important than those of serum alone.

There is mounting evidence that the total concentrations of EET as well as the relative abundance of each EET regioisomer are organ specific.^{18,59} These findings could be explained by the fact that CYP2C, CY2J and sEH enzymes are expressed heterogeneously in different tissues and that, as abovementioned, each CYP epoxygenase produces its own unique profile of EET regioisomers.^{18,59-61}

Endothelial cells (ECs) are the main sources for EETs in the cardiovascular system.^{17,18} In fact, ECs are the sole source of EETs in the vascular wall; even though endothelium-derived EETs diffuse into and paracrinely act on the contiguous smooth muscle cells (SMCs), none of the EET regioisomers are not produced by SMCs.^{17,62} Although the CYP2C8, CYP2C9 and CYP2J2 are

generally considered as the most frequently expressed epoxygenases in ECs,^{9,17,32,34} accumulating evidence suggests that these enzymes are not ubiquitously expressed across the vascular bed and that the vascular endothelium of different organs can express different epoxygenases;^{4,5,17,20} in coronary artery ECs, for instance, CYP2J2 and CYP2C9, but not CYP2C8, isoenzymes are the main source for EET-formation.⁶³ Therefore, the relative concentration of each EET regioisomer, and their corresponding enantiomers, in a particular vascular bed depend on the expression rates of the CYP epoxygenases in the EC line. Nevertheless, functional studies point that the 11,12-EET followed by 14,15-EET are the main EETs released from ECs in general; the secretion of these EETs by ECs can be stimulated by acetylcholine, bradykinin, shear stress and cyclic stretch.^{5,16-18}

In addition to the ECs, cardiomyocytes contribute to high concentrations of EETs in the heart (60 nanograms per gram of cardiac tissue).²⁰ It has been well demonstrated that CYP2J2 and, to a lower extent, CYP2C9 isoenzymes are the primary epoxygenases producing EETs in the heart.^{9,20,27} The results of the transcriptomic studies reveal that the relative myocardial CYP2J2 mRNA level is 1,000 fold higher than that of CYP2C9 and CYP2C8.⁶⁴ It has been shown that 14,15-EET (*R,S/S,R*: 64%/37%), 8,9-EET (*R,S/S,R*: 43%/57%) and 11,12-EET (*R,S/S,R*: 44%/56%) comprise, respectively, 39%, 37% and 24% of the total cardiac EET content.^{32,35,37,65} On the other hand, as it mentioned earlier, CYP2C8 forms very small amount of the 8,9-EET and the 11,12-EET is mostly synthesized by CYP2C8 as the *R*, *S*-form.⁹ Taken together, the regio-/stereoselectivity of EETs in the heart support the view that the CYP2J2 and CYP2C9, but not CYP2C8, are the main contributors in cardiac EET formation.

Circulating blood cells, including red blood cells (RBCs), platelets, macrophages and monocytes, have also found to be of the major sources of EET and are potentially capable to regulate EET levels in the human plasma.⁶⁶⁻⁷⁰ The fact that circulating cells, particularly RBCs, can generate, serve as reservoirs of and release EETs, arises the hypothesis that, in addition to autocrine and paracrine effects, EETs may act as endocrine hormones on the cardiovascular tissues, such as ECs, which are far from the organs that they are produced in.

4. Physiological roles of EETs in cardiovascular system

There is a large volume of evidence that EETs possess a wide range of protective effects on the vasculature, including coronary arteries, and myocardium.^{4,5,16-18} The beneficial contribution of EETs to cardiovascular physiology has progressed to a point where the epoxygenase pathway, including sEH enzyme, has become a potential therapeutic target for the prevention and treatment of a variety of cardiovascular pathologies including hypertension, atherosclerosis, vascular inflammation, cardiac hypertrophy and ischemic injuries. Over the past years, considerable efforts have been devoted to apply exogenous EETs as therapeutic agents and pharmacological tools; however, when applied exogenously, EETs are rapidly metabolized by sEH enzyme, incorporated into the cell membrane and are chemically labile.³⁷ Additionally, limited solubility and storage issues have hampered long-term therapeutic use of EET analogues.^{4,59} On the other hand, unlike EET agonists, sEH inhibitors have been more promising. The development of pharmacological sEH inhibitors has been reached to the stage where they have been testing human,⁷¹⁻⁷³ and it is expected that sEH-targeted medications, and also probably EET analogues, will be widely used in daily medical practice in the near future.

While the contribution of EETs to myocardial function is mostly limited to cardiomyocytes ion channels and consequently heart contractility, the numerous physiological effects of EETs on the vasculature could be grossly categorized as modulation of vascular tone and regulation of vascular homeostasis (Figure 3).

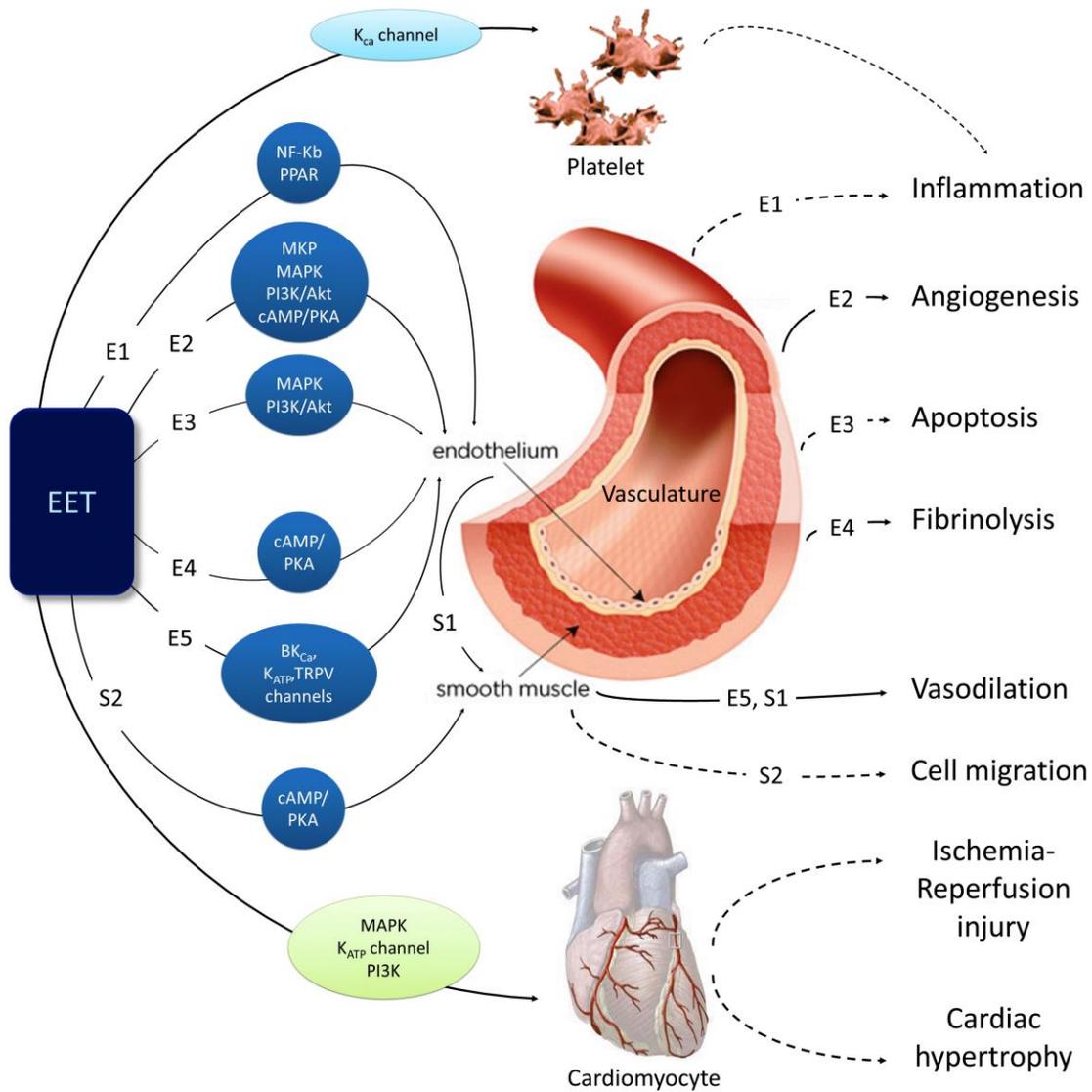


Figure 3. The main biological functions of EETs on the cardiovascular system and the corresponding cellular signaling pathways. Solid line represents stimulatory influence and dash line represents inhibitory influence. «E» represents the ECs pathway and «S» represents the SMCs pathways.

4.1. EETs and myocardial function

EETs have shown to exert protective effects on myocardium both under physiological and ischemic/reperfusion conditions through modulation of the myocytes contraction.⁵ Although the mechanisms by which EETs contribute to cardiac contractility have not been fully elucidated, several experimental and *ex vivo* studies have provided convincing evidence indicating that cardiomyocytes ion channels including Na⁺ channels, ATP-sensitive K⁺ channels (K_{ATP}) and L-type Ca²⁺ channels are the primary targets of EETs in the cardiac myocytes.⁷⁴⁻⁷⁸

EETs, mainly 8,9-EET, have been demonstrated to regulate myocyte action potential, and consequently myocyte contraction, through the inhibition of cardiac Na⁺ channels.⁷⁵ Activation of myocyte L-type Ca²⁺ channels is another mechanism by which EETs, particularly 11,12-EET, regulate cardiac contraction. Cell culture models suggest that EETs enhance Ca²⁺ currents through cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA)-mediate phosphorylation of the L-type Ca²⁺ channels.^{5,16,79,80} It is worth mentioning that cardiac L-type Ca²⁺ channels are the major pathway for Ca²⁺ entry during excitation, the process that results in the initiation and regulation of cardiac muscle contraction.⁵ EETs also activate K_{ATP} channels in cardiac myocytes by reducing channel sensitivity to ATP;⁷⁶⁻⁷⁸ considering the importance of K_{ATP} channels in the modulation of myocyte resting membrane and cardiac action potential, this effect of EET activating of K_{ATP} channels, may be a significant mechanism for the regulation of cardiac contractile function. Compared to other EET regioisomers, the effects of 11,12-EET for activating K_{ATP} channels, has been more explored.

Accumulating evidence suggests that EETs are also cardioprotective against ischemic-reperfusion (I/R) injury and improve post-ischemic recovery of left ventricular contractility.⁸¹⁻⁸⁵

In addition to the modulation of ion channels, most importantly K_{ATP} channels,^{76-78,81} post I/R EET-mediated cardioprotection involve various cellular pathways such as phospho-p42/p44 mitogen-activated protein kinase (MAPK),⁸¹ phosphatidylinositol-3-kinase (PI3K),⁸⁵ and brain natriuretic peptide (BNP)-protein kinase C (PKC)-mediated signaling.⁸⁶ It might be interesting to mention that plasma levels of H-FABP, the intracellular carrier protein for EETs, as well as BNP have been proven to have independent and additive diagnostic and prognostic values, in addition to troponin, in a number of cardiovascular diseases (CVDs) including acute coronary syndrome.^{87,88} The investigation of the interaction between EETs, H-FABP and BNP, therefore, may yield exciting and, clinically, practical results. Surprisingly, a recent study showed that while overexpression of epoxygenases in cardiomyocytes results in an increased functional recovery after I/R, CYP2C8 overexpression in ECs increases susceptibility to I/R injury in mouse heart.⁸⁹

4.2. *EETs and vascular function*

Based on our current knowledge of EETs metabolism and actions, the vascular system is, undoubtedly, the main target organ of CYP epoxygenase-derived EETs in the human body. The diverse effects of EETs on the vasculature could be discussed in two main categories: Regulation of vasodilation and vascular homeostasis. The contribution of EETs to vascular homeostasis could be subcategorized, in turn, into their effects on angiogenesis, SMC migration, EC proliferation and inflammation. Interestingly, it has been indicated that the threshold concentration of exogenously applied EETs for exerting a specific biological response, differs across their vascular effects; while vasodilation and inhibition of vascular cell adhesion

molecule 1 (VCAM-1) expression occur at low physiological concentrations (10^{-9} M and $<10^{-8}$ M, respectively), at least 10^{-7} M of EETs are required to exert fibrinolytic, anti-platelet and proangiogenic effects and SMC anti-migratory and antiapoptotic properties of EETs occur at their greater concentrations i.e. at 10^{-6} M and 10^{-5} M, respectively.¹⁷

4.2.1. EETs and vasodilation

It has been well established for many years that the endothelium regulates vascular tone through the synthesis and release of a wide range of vasoactive mediators.⁹⁰⁻⁹⁴ The main endothelium-derived vasodilators are nitric oxides (NO), prostacyclin (PGI_2) and also a chemically heterogeneous group of compounds, generally referred to as endothelium-derived hyperpolarizing factors (EDHFs), that share the ability to cause vasodilation through activation of large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) on vascular SMCs to elicit hyperpolarization and relaxation.¹⁷

The NO/PG-independent vasodilatory effects of EETs were originally identified, by Campbell et al. in 1996, in the coronary circulation.¹⁰ Subsequent studies using arteries from a variety of species, including human, have supported the notion and have clearly indicated that EETs act as EDHF in various arterial territories including coronary, renal, mesenteric and cerebral vasculatures.^{3-5,10,11,16-18,42,72,95-103} However, there is evidence from human and experimental studies suggesting that the EET-mediated vasodilation is not a consistent finding in all arterial beds and that in certain arteries either EETs are not synthesized or are with no activity.^{17,104-108} Additionally, the effect of EETs on vascular tone is highly regioisomeric and/or enantiomeric

specific.^{16,17} In coronary arteries, where the vascular actions of EETs have been extensively explored, EET-mediated vasodilation is not regioisomer-specific but is stereoselective; it has been shown that all four EET regioisomers have similar vasorelaxant potency in the bovine, canine and human coronary arteries,^{10,42,109,110} however, the effectiveness of two enantiomers of 14,15-EET differs with 14*S*,15*R*-EET being more potent than 14*R*,15*S*-EET.¹¹¹ Also, it has to be pointed out that considerable evidence suggests that DHETs exert vasodilatory activity in the coronary circulation.^{42,112-114} On the other hand, the vasorelaxation effect of EETs on the renal arteries represents both regioisomeric specificity and stereospecificity; in the rat and human kidneys, 11,12-EET, but not 14,15-EET, and 11*R*,12*S*-EET, but not *S*, *R* enantiomer, has renovasodilatory properties.^{17,115,116}

As an EDHF, EET elicits hyperpolarization and relaxation of SMCs, principally, via activation of K channels on the ECs or SMCs in an autocrine or paracrine manner, respectively.^{4,5,17,22}

The paracrine-induced vasodilatory effect of EETs (Figure 4A) is mediated by three main mechanisms and signaling pathways, two of which are receptor-dependent (Figure 4A, pathway A). It has been shown that as “transferable factors”,⁹¹ EETs generated and released by ECs and act on the SMCs to activate large conductance BK_{Ca} channels, resulting in K⁺ efflux from SMCs and subsequent an increase in E_m or hyperpolarization. The hyperpolarization inhibits activation of voltage-activated calcium channels reducing calcium entry and causing relaxation (Figure 4A, pathway A1).^{4,10,17,18,97,117,118} Experimental studies demonstrate that EETs require a guanine nucleotide binding protein (G protein) for activation of BK_{Ca} channels, and this effect cannot be achieved by direct binding to the channel’s extracellular domain.^{5,119,120} Convincing evidence

postulates that EETs, particularly 8,9-EET, 11,12-EET and 14,15-EET, activate vascular SMC BK_{Ca} channels via binding to an EET-specific Gα_s protein-coupled receptor;^{5,10,11,95,103} although specific binding sites have been identified on monocytic cell lines,^{3,121-126} the receptor has not yet fully characterized. Likewise, 11,12-EET and 14,15-EET activate SMC-ATP-sensitive K channels (K_{ATP}) via a receptor-dependent mechanism similar to that described for the activation of BK_{Ca} channels (Figure 4A, pathway A2). Stimulation of EET receptor provokes the production of cAMP via activation of adenylyl cyclase by ADP-ribosylation of the guanine nucleotide binding protein Gα_s. Subsequent activation of PKA by cAMP phosphorylates and activates both the K_{ATP} and BK_{Ca} channels resulting in K⁺ efflux and SMC hyperpolarization, inhibition of voltage-activated calcium channels and relaxation.^{5,17,76,127,128}

Several lines of evidence indicate that, in addition to the previously described receptor-dependent mechanisms, EETs may hyperpolarize and relax SMC through an additional receptor-independent pathway (Figure 4A, pathway B); EET has been identified as independent activator of transient receptor potential (TRP) channels on SMCs,¹²⁹ resulting in an increase in intracellular Ca²⁺ and subsequently hyperpolarization and relaxation of SMCs. As non-selective cation channels, TRP channels mediate the transmembrane influx of Ca²⁺. Amongst seven TRP subfamilies, vanilloid TRP (TRPV) and specifically TRPV4 channels are involved in the paracrine EET-induced SMC relaxation. Through activation of TRPV4 channels, EETs, particularly 5,6-EET and 8,9-EET, increase Ca²⁺ influx and subsequently SMC Ca²⁺ levels resulting in a rapid Ca²⁺ release from the endoplasmic reticulum, an event termed “Ca²⁺ sparks”. Ca²⁺ sparks then stimulate large conductance BK_{Ca} channels which promote K⁺ efflux from the SMC and eventually lead to the SMC hyperpolarization.^{5,17,130-135}

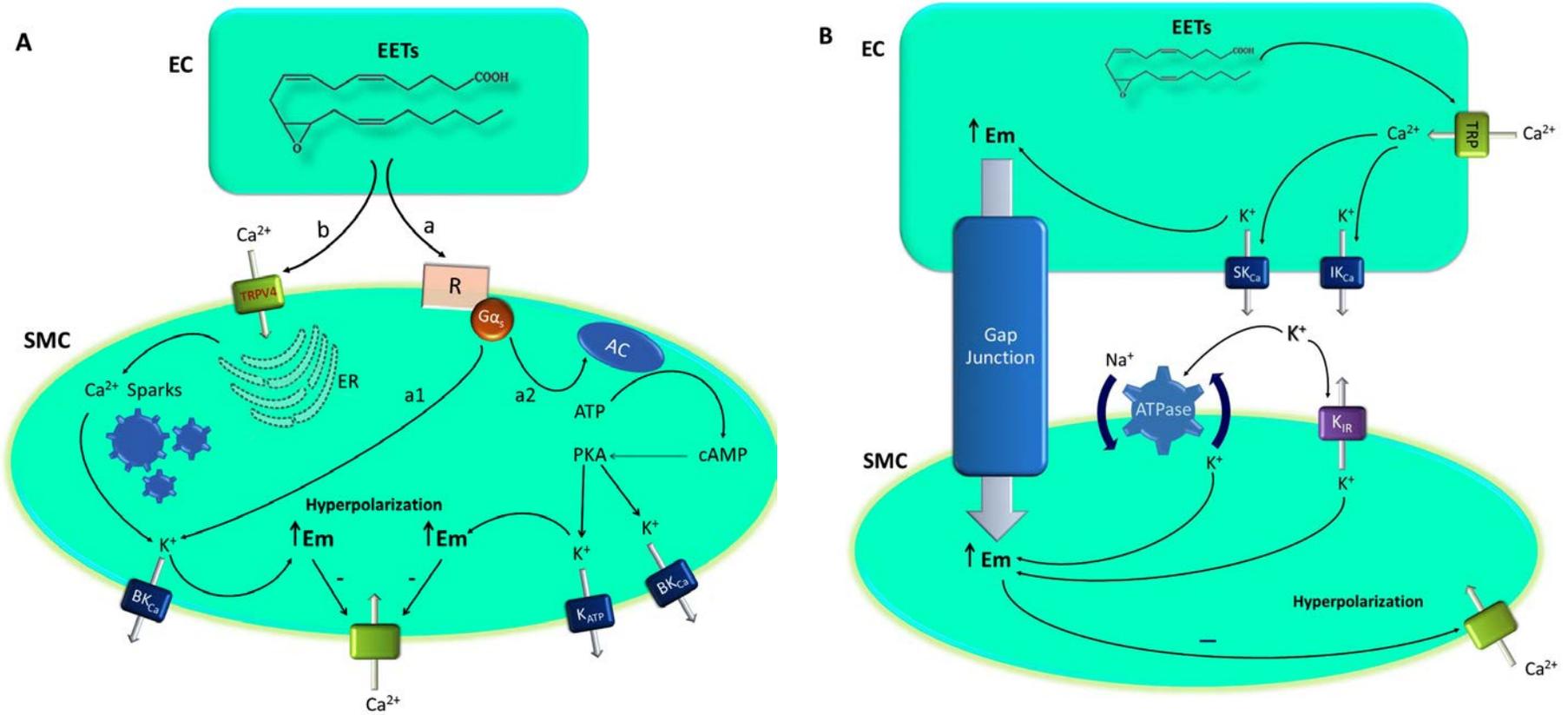


Figure 4. EETs act as an EDHF through two main mechanisms: **A.** Transfer of a factor mechanism: EETs function as transferable factors generated by ECs and acting on SMCs via receptor-dependent (a) and receptor independent (b) pathways. EETs released from ECs may activate a receptor on SMCs stimulating the BK_{Ca} channels which results in K efflux and an increase in the Em (hyperpolarization) and consequently relaxation (a1). The activation of EET receptor on SMCs may alternatively induce cAMP production (a2). Subsequently, cAMP activates PKA leading to the stimulation of the BK_{Ca} and K_{ATP} channels, K efflux and SMC hyperpolarization and relaxation. Through a receptor independent mechanism, the released EETs from ECs, activate TRPV4 channels on SMCs leading to Ca influx through these channels. An increased intracellular Ca causes Ca sparks from the endoplasmic reticulum which leads to the activation of BK_{Ca} channels and hyperpolarization. **B.** Transfer of hyperpolarization mechanism: EETs generated by ECs act in an autocrine manner on the TRP channels. Increased intracellular Ca activates the SK_{Ca} and IK_{Ca} channels on ECs resulting in hyperpolarization and also release of K ions in the subendothelial space. The released K ions activate sodium-potassium ATPase or Kir channels on SMCs leading to K efflux and hyperpolarization. The endothelial membrane hyperpolarization spreads to SMCs through gap junction resulting in SMC hyperpolarization and relaxation.

Endothelial hyperpolarization, through activation of TRP, apamin-sensitive small conductance (SK_{Ca}) and charybdotoxin-sensitive intermediate conductance (IK_{CA}) channels, is an alternative and well-explored mechanism by which EETs, autocrinely, regulate vascular tone (Figure 4B). In this regard, EETs, specifically 11,12-EET, activate two members of canonical endothelial TRP (TRPC) subfamily, TRPC3 and TRPC6 but not TRPV4 [136], evoking Ca^{2+} influx in ECs. Calcium stimulates SK_{Ca} and IK_{CA} channels, resulting in K^+ efflux from ECs. This increase in K^+ efflux hyperpolarizes ECs and increases the levels of K^+ in the subendothelial space. Via a mechanism termed “transfer of hyperpolarization”, endothelial hyperpolarizing current transmitted, through gap junctions, to SMCs. Furthermore, subendothelial K^+ ions stimulate the SMC Na^+/K^+ ATPase and inward rectifying K (K_{IR}) channels, leading to SMC hyperpolarization and relaxation.^{5,17,136-138}

4.2.2. EETs and vascular homeostasis

Large volume of evidence suggests that the CYP-derived epoxides contribute significantly to vascular homeostasis.^{3-5,16-18} Beyond vasodilation, EETs have been demonstrated to play a range of important non-vasomotor regulatory roles in the vascular biology. Endothelium-derived EETs stimulate EC proliferation and angiogenesis, protect ECs from apoptosis, have antimigratory effects on SMCs and also possess potent anti-inflammatory properties. Furthermore, considering the importance of circulatory blood cells, mainly RBCs and platelets, in the production and storage of EETs,⁶⁶⁻⁷⁰ the potential contributions of EETs to the endothelial homeostasis should not be neglected; this could be a hint for further studies

to investigate the biological roles of EETs as endocrine, and on a wider spectrum of autocrine and paracrine, agents.

4.2.2.1. *EETs and regulation of angiogenesis, fibrinolysis, apoptosis and SMCs migration*

Since the original reports of the mitogenic effects of EETs in renal epithelial cells,^{139,140} an ever growing number of human and experimental investigations have supported the notion that CYP-epoxygenase overexpression and exogenous application of EETs promote EC proliferation, migration and angiogenesis.^{3-5,16,18,78,93,118,139-148} Even though all four EET regioisomers have been demonstrated to enhance EC proliferation,^{145,147} various signaling pathways, depending on the species, type of vascular bed and EET regioisomer, are implicated in the EET-mediated angiogenesis.¹⁶ For instance, in human umbilical vein endothelial cells (HUVECs), the phosphatidylinositol 3-kinases (PI3K)/protein kinase B (Akt), the MAPK and the MAPK phosphatase-1 (MKP-1) are the cell signaling pathways implicated in the EET-induced angiogenesis.^{44,93,145,147-150} EET-mediated activation of these pathways leads to the inactivation of c-Jun NH₂-terminal kinase (JNK), leading to upregulation of cyclin D1 and cell proliferation.^{150,151} Alternatively, the 11,12-EET-induced activation of the PI3K/Akt-dependent phosphorylation, may result in overexpression of cyclin D1 through a JNK-independent signal transduction cascade involving a reduction in cyclin-dependent kinase inhibitor p27^{Kip1} and forkhead transcription factors FOXO1 and FOXO3a.¹⁴⁵ Upstream to the intracellular angiogenic signaling cascades, EETs, particularly 11,2-EET and 14,15-EET, appear to transactivate the epidermal growth factor (EGF) receptor.^{4,149} Through transactivation of matrix metalloprotease (MMP), EETs cause shedding of heparin-binding EGF-like growth factor (HB-EGF) from the plasma membrane of ECs and subsequent

activation of the EGF receptor, resulting in the triggering intracellular angiogenic cascades.^{152,153} It has also been proposed that 11,12-EET can stimulate cytosolic sphingosine kinase-1 (SK-1) which catalyzes the conversion of sphingosine to sphingosine-1-phosphate (S1P). Subsequently, S1P, in an autocrine manner, activates the G protein-coupled S1P receptor, leading to the stimulation of MMP, the release of HB-EGF and eventually the transactivation of the EGF receptor (Figure 5).¹⁵⁴

The fact that hypoxia is a potent inducer of both CYP2C8/9 and vascular endothelial growth factor (VEGF),^{141,142,155} a well-known key mediator of physiological and pathological angiogenesis, draw the attention to speculate that they could be involved in a common angiogenic pathway. Interestingly, it has been shown recently that hypoxia-induced angiogenesis also involves CYP activation and EET production and also that CYP2C-derived EETs participate as second messengers in the angiogenic response initiated by VEGF in human ECs.^{3,156} Needless to say that, the EET-VEGF interaction could be a potential target for pharmacological interventions in future.

There also exists an EGF receptor-independent angiogenic signal transduction pathway in HUVECs in which EETs, particularly 11,12-EET, increase cAMP levels and activate PKA resulting in the overexpression of the cAMP-response-element binding protein (CREBP) and COX-2 that promotes angiogenesis.¹⁵⁷ The EET-induced cAMP/PKA activation may also enhance the expression and activity of tissue-type plasminogen activator (t-PA) in ECs demonstrating EETs, in particular 5,6-EET and 11,12-EET, as having potential fibrinolytic activity (Figure 5).¹⁵⁸

There is also significant evidence that EETs protect human ECs from apoptosis, an enzymatically controlled and energy-dependent form of programmed cell death, through inhibition of extracellular signal-regulated kinase (ERK) dephosphorylation and activation of MAPK and PI3K/Akt signaling pathways (Figure 5).¹⁵⁹⁻¹⁶² The results of a recent study indicate that 14,15-EET, 11,12-EET and 8,9-EET, but not 5,6-EET, significantly enhance survival of human coronary ECs in a concentration-dependent manner.¹⁶¹ Further experimental studies also revealed that CYP epoxygenase overexpression inhibits tumor necrosis factor- α (TNF- α)-induced apoptosis in bovine and rat ECs.^{160,163} Furthermore, inhibition of sEH has also been shown to protect the brain from ischemic injury by preventing neuron death.¹⁶⁴⁻¹⁶⁶ These findings collectively suggest that EETs exert significant antiapoptotic effects on ECs which may additionally contribute to the promotion of angiogenesis and endothelial survival during atherosclerosis and related CVDs.

Although the effects of EETs on various aspects of EC biology have been extensively investigated, the biological actions of EETs on SMCs have been less explored. Of the *limited number of studies* of the effects of EETs on SMCs, the majority provide convincing evidence that EETs are antimigratory.⁵ Through a mechanism involving upregulation of cAMP/PKA signaling pathway, EETs, mainly 5,6-EET, 11,12-EET and 14,15-EET, inhibit rat aortic SMC migration induced by potentiated platelet-derived growth factor (PDGF) (Figure 5). Furthermore, CYP2J2 overexpression in vascular SMCs has been shown to be associated with the inhibition of PDGF-induced migration.¹⁶⁷ Additionally, there is some evidence suggesting that EETs also contribute to angiogenesis by acting on SMCs;⁵ the detailed signaling pathway, however, remains to be identified.

4.2.2.2. CYP epoxygenase-derived EETs and inflammation

Inflammation is a complex, highly orchestrated process that has a domino-like effect on the initiation and development of a wide range of systemic illnesses including CVDs;¹⁶⁸ coronary artery disease, as an example, is currently believed to be a disorder of inflammation and innate immunity.¹⁶⁹ In recent years, a growing body of *in vivo* and *in vitro* studies has provided support for the concept that EETs exhibit a broad spectrum of anti-inflammatory activity against acute and chronic inflammation.^{4,5,16-18,21} Similar to the aforementioned biological effects, there is a marked difference in the anti-inflammatory activity across the EET regioisomers with 11,12-EET being the most potent isomer, 5,6-EET and 8,9-EET being less active and 14,15-EET being without activity.^{170,171} The intricate mechanisms by which EET regulates vascular inflammation have been elucidated by several studies and could be discussed under two broad headings; inhibition of endothelial activation and leukocyte adhesion, and inhibition of platelet adhesion and aggregation.

4.2.2.2.1. CYP epoxygenase-derived EETs and inhibition of EC activation

Under physiological conditions, EC provides a critical balance between pro- and anti-inflammatory mediators and it is not surprising, therefore, that there exists a strong link between inflammation and endothelial dysfunction (ED).^{18,172} Indeed, ED (also known as endothelial “activation”) initiates and promotes vascular inflammation by inducing the expression of a variety of cell adhesion molecules (e.g. VCAM-1, E-selectin and intercellular adhesion molecule 1 (ICAM-1)) and cytokines (e.g. TNF- α and interleukin 1a (IL-1)) that leads to leukocyte-endothelial adhesion and subsequently leukocyte transmigration across the endothelium.¹⁷³ Given the crucial contribution of inflammation to the pathophysiology of CVDs including atherosclerosis, hypertension and heart failure, ED has been speculated to be

an important intermediate phenotype in the relationship between inflammation and CVDs and is independently associated with increased cardiovascular risk.¹⁷⁴⁻¹⁷⁷

In human ECs, EETs, particularly 11,12-EET, suppress the expression of cytokine-induced EC adhesion molecules and prevent leukocyte adhesion to the arterial wall, mainly by inhibiting nuclear transcription factor-kappa B (NF- κ B) activity. The EET-induced NF- κ B inhibition occurs either directly or through activation of peroxisome proliferator-activated receptor (PPAR) transcription factors and EGF receptor.^{15,21,163,171,178-181} In addition, other mechanisms including inhibition of COX-2 and LOX-5, induction of endothelial nitric oxide synthase (eNOS) and heme oxygenase (HO-1) expression are also implicated in the EET-induced anti-inflammatory actions through preventing EC activation.

4.2.2.2.1.1. NF- κ B and inflammation

4.2.2.2.1.1.1. NF- κ B; structure and activation pathways

NF- κ B refers to a mammalian ubiquitously expressed pleiotropic nuclear transcription factor that controls the expression of genes involved in a wide variety of pathophysiological processes including immune and inflammatory responses, cell proliferation, cell death (apoptosis) and cell survival.¹⁸²⁻¹⁸⁷ In regard to inflammation, stimulation of NF- κ B causes EC activation through the upregulation of endothelial cytokine, chemokine and CAMs expression, resulting in the subsequent adherence of leukocytes to the endothelium. NF- κ B can be activated by a number of stimuli including inflammatory cytokines such as TNF- α and IL-1, exposure of cells to lipopolysaccharide (LPS), pathogens, toxins, drugs and oxidants.¹⁸⁵

Inactive NF- κ B exists in the cytoplasm of all cells as heterogeneous group of homo- or heterodimeric transcription factors consisting of different subunits.¹⁸⁶ The subunits are members of the reticuloendotheliosis (Rel) structurally related family of proteins. All the five

mammalian Rel proteins share a highly conserved Rel homology domain (RHD) of approximately 300 amino acids at the N-terminal end which mediates dimerization, DNA binding and the interaction with inhibitor of NF- κ B (I κ B). These proteins are Rel A (also known as p65), Rel B, c-Rel, NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100). Of them, the first three proteins are synthesized as active transcription factors; the latter, however, are produced as large precursors (p105 and p100) which are further to the smaller and transcriptionally active forms.¹⁸²⁻¹⁸⁷

There are two signaling pathways leading to the activation of NF- κ B known as the canonical (or classical) and the non-canonical (or alternative) pathways.¹⁸⁸ The common regulatory step in both of these cascades is activation of an I κ B kinase (IKK) complex that occurs under the influence of activating triggers among which include inflammatory cytokines (TNF- α , IL-1, etc.).

The canonical NF- κ B activation cascade (Figure 6, left side) applies to dimers that composed of RELA (p65) and p50 which are held inactive in the cytoplasm by specific inhibitors called as I κ B. In the canonical pathway, the IKK complex composed of one regulatory subunit, IKK γ (also known as NF- κ B essential modulator (NEMO)) and two catalytic subunits, IKK α and IKK β ; the activated IKK complex phosphorylates I κ B and targets it for subsequent E1-E2-E3-mediated ubiquitylation and proteasomal degradation. The phosphorylation, ubiquitylation and degradation of I κ B release the p50-p65 NF- κ B dimers allowing them to translocate to the nucleus and bind to DNA. The IKK complex is stabilized by the linear ubiquitin chain assembly complex (LUBAC), which linearly adds ubiquitin (Ub) moieties to the IKK γ .

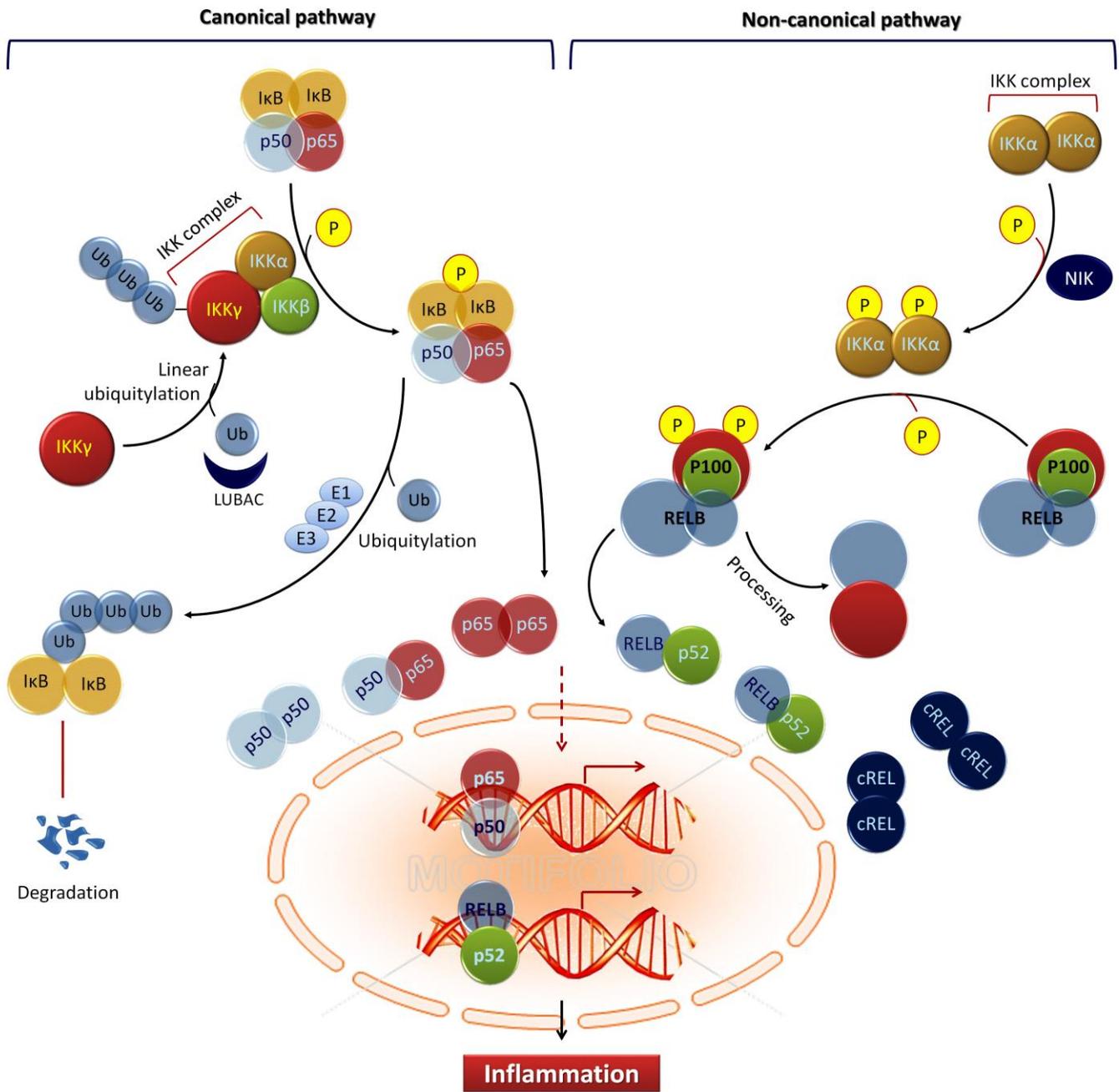


Figure 6. The canonical and non-canonical pathways of the NF- κ B system

The non-canonical pathway (Figure 6, right side) is responsible for the activation of p100/Rel B complexes. In the non-canonical pathway, the IKK complex which consists of IKK α dimers is activated by NF- κ B-inducing kinase (NIK)-mediated phosphorylation; active IKK α , subsequently, phosphorylates p100 to induce its proteolytic processing yielding p52, which can dimerize with other NF- κ B subunits and enter the nucleus.¹⁸⁸

Once entered in the nucleus, transcriptionally active NF- κ B dimers bind specific recognition (consensus) sequences in the promoter region of a variety of inflammation-related genes such as COX-2 and adhesion molecules.^{182,185}

4.2.2.2.1.1.2. CYP epoxygenase-derived EETs as NF- κ B inhibitors

EETs were originally described by Node et al. in 1999 as having vascular anti-inflammatory properties.¹⁵ Through a mechanism involving inhibition of NF- κ B and I κ B kinase, EETs at their physiological concentrations as well as CYP2J2 overexpression were found to significantly attenuate inflammation through inhibition of cytokine-induced expression of EC adhesion molecules which are required for leukocytes to adhere to and migrate across the endothelial lining of the vasculature. Since then, a large number of human and animal studies have provided evidence that depending on the species, type of endothelium, type of cytokine stimulus and the EET regioisomer, CYP-derived epoxides can target different steps of NF- κ B pathway that are operative in both the cytoplasm and the nucleus;^{15,21,163,171,178-181,189,190}

EETs regulate NF- κ B-mediated induction of proinflammatory genes through inhibition of IKK complex activity, I κ B degradation, Rel A nuclear translocation and NF- κ B binding.²¹

In human, in particular, administration of 11,12-EET resulted in marked attenuation of TNF- α -stimulated endothelial cell-surface expression of VCAM-1, E-selectin and ICAM-1 in HUVECs.¹⁵ As mentioned before, 11,12-EET was the most potent inhibitor of VCAM-1

upregulation, causing 72% inhibition of TNF- α -induced VCAM-1 expression, followed by 8,9-EET and 5,6-EET and 14,15-EET yielded no anti-inflammatory properties.¹⁵ This finding was supported by further report demonstrating that 11,12-EET can attenuate TNF- α -induced NF- κ B activation in HUVECs.¹⁹⁰ Also, 11,12-EET has also been shown by to have ability to suppress TNF- α -induced VCAM-1 expression in human saphenous vein endothelial cells.¹⁷⁹ These findings collectively demonstrate that CYP-derived epoxides inhibit cytokine-induced endothelial activation and leukocyte adhesion, at least in part, through inhibition of NF- κ B activation and thereby are important regulators of vascular inflammation (Figure 7).

Lastly, there exists evidence that the EET-induced inhibition of NF- κ B could be a mediator of other anti-inflammatory pathways. This is exemplified by the suppression of inflammation in response to direct stimulation of angiotensin II type 2 (AT₂) receptors (AT₂R). The AT₂R have been demonstrated to exert anti-inflammatory effects. In this way, 11, 12-EET acts as second messenger in AT₂R-mediated anti-inflammation. Direct stimulation of AT₂R increases CYP2C/2J-mediated production of 11,12-EET resulting in the modulation of NF- κ B activity and reduction of IL-6 (Figure 7).^{191,192}

4.2.2.2.1.2. CYP epoxygenase-derived EETs and activation of PPAR and EGF receptor

Accumulating results of several experimental studies indicate that epoxide-induced inhibition of NF- κ B is mediated, at least in part, through activation of multiple putative effectors including PPAR and EGF receptor.^{163,178,181,193}

The PPARs belong to the nuclear receptor superfamily which is ligand-activated transcription factors. The function of PPARs is modified by the precise shape of their ligand-binding domain induced by ligand binding and by a number of coactivator and corepressor proteins, the presence of which can stimulate or inhibit receptor function, respectively.¹⁹⁴ A wide

range of natural ligands, such as fatty acids and eicosanoids, as well as synthetic ligands including the lipid lowering fibrates and antidiabetic glitazones, could trigger the activation of PPARs.^{195,196} Different PPAR subtypes have been reported to regulate the expression of genes implicated in lipid and lipoproteins metabolism, glucose homeostasis and cellular proliferation and differentiation.^{195,196} Specifically, PPAR α and PPAR γ also can inhibit inflammatory response in the artery wall via repressing NF- κ B pathway (Figure 7). The activation of PPAR α and PPAR γ suppress the NF- κ B-induced expression of inflammation-associated molecules such as VCAM-1, ICAM-1 and endothelin-1 in cultured ECs.^{163,178,181,193,198,199} PPAR α is mainly expressed in the heart, liver and kidney and PPAR γ is predominant subtype expressed in the adipose tissue; however, both PPAR α and PPAR γ have found to be expressed in vascular SMCs and ECs.¹⁹⁷

In recent years, CYP-derived EETs and their corresponding hydration metabolites, DHETs, have been identified as novel PPAR α and PPAR γ activator. While 11,12-EET and, interestingly, 14,15-DHET have been the most potent activator of PPAR α [193], it seems that there is not regioisomeric specificity across the four EET regioisomer for the activation of PPAR γ .¹⁷⁸ These observations together indicate PPAR α and PPAR γ contribute to the anti-inflammatory effects of EETs mainly via inhibition of NF- κ B.

Another mechanism implicated in EET-induced inhibition of NF- κ B is activation of PI3K-dependent Akt and EGF receptor signaling pathways. In a very recent experimental study, Zhao et al. showed that both EETs and CYP2J2 overexpression reverses the TNF- α -induced inhibition of EGF receptor-mediated activation of PI3K/Akt pathway in rat cardiomyocytes (Figure 7).¹⁶³ This finding reveals the preventive role of CYP-derived epoxides in the TNF- α -induced cardiac injury and subsequently proposes CYP epoxygenase pathway as being a potential pharmacological target to enhance the cardiovascular protective functions of EETs.

4.2.2.2.1.3. CYP epoxygenase-derived EETs and inhibition of LOX-5 and COX-2

The key roles of PGs and LTs in the generation of inflammatory response have been extensively studied.^{21,200-204} Their biosynthesis is markedly enhanced in inflamed tissues and they contribute to the development of cardinal signs of acute inflammation. The production of PGs/TXs depends on the activity of COXs, which exists as distinct isoforms referred to as COX-1 and COX-2; LTs are generated from arachidonic acid by the action of LOX-5 isoenzyme.^{6,7,200-204} Even though there is some evidence suggesting that the two COX isoforms may contribute to the inflammatory response, COX-2 appears to be dominant source of PG synthesis in the presence of an inflammatory status in human.²⁰¹

It has been demonstrated by several studies that CYP epoxygenase-derived EETs may dramatically down regulate induced, but not constitutive, COX-2 and LOX-5 and their corresponding metabolites leading to decreased inflammatory response (Figure 7).^{21,181,205}

For instance, while an increase in 11,12-EET, in a dose-dependent manner, has been shown to attenuate LPS-stimulated PGE₂ production in rat monocytes,^{206,207} direct administration of 11,12-EET to HUVECs, in the absence of an inflammatory stimuli, has been associated with increased COX-2 expression and prostacyclin formation.¹⁵⁷ It should also be indicated that COX-1 isoenzyme contributes to PG production merely during an inflammatory status; however, COX-2 derived PGs appears to be important both in acute inflammatory process and also in the resolution phase.²⁰³ Seemingly, COX-2 has a dual role in the inflammatory process, initially contributing to the onset of inflammation and later helping to resolve the process.²⁰³ Therefore, it is tempting to speculate that an “*intellectual feedback*” mechanism between EET and COX-2 could exist to justify the various effects of EETs on COX-2 in the presence vs. absence of an inflammatory stimulus. It would be logical to hypothesize that in the presence of an active inflammatory reaction, EETs inhibit COX-2 in order to prevent,

specifically, production of proinflammatory PGs; in the absence of inflammation and/or under physiological conditions, however, CYP-derived epoxides increase COX-2 expression to stimulate production of COX-2-derived anti-inflammatory PGs. Further studies to elucidate the likely underlying mechanism are required.

EETs are able to inhibit COX-2-, LOX-5-mediated inflammatory response either directly via inhibition of their activity or transcriptionally through suppression of NF- κ B-induced upregulation of COX-2 and LOX-5.^{21,181} The concept of EETs as having direct inhibitory effect on COX-2 has been supported by the study in which 14,15-EET administration to cultured murine brain microvascular SMCs resulted in an decrease in PGE2 production without altering COX-2 expression.²⁰⁸

4.2.2.2.1.4. CYP epoxygenase-derived EETs and increase HO-1 expression

HO is a rate-limiting and microsomal enzyme that catalyzes the oxidative breakdown of heme to generate equimolar amounts of free ferrous iron, carbon monoxide (CO) and biliverdin; subsequently, free iron is sequestered by ferritin and biliverdin is converted to bilirubin by biliverdin reductase.²⁰⁹⁻²¹¹ Three isoforms of HO have been reported.^{212,213} Of them, HO-1 and HO-2 are expressed in human ECs.²¹⁴ HO-2 is constitutively expressed; whereas HO-1 is transcriptionally inducible by a wide range of agents including hypoxia, endotoxins and cytokines.²¹⁵ There is mounting evidence that HO-1 and the subsequent metabolites of heme exert potent protective effects against inflammatory, oxidation and atherosclerosis.^{209,216-219} Induction of HO-1 suppresses inflammation, in part, through the inhibition of EC adhesion molecule expression and leukocyte adhesion *in vivo* and *in vitro*.²²⁰⁻²²⁴ Upregulation of HO-1 with hemine has been demonstrated to markedly attenuate NF- κ B activation and ICAM-1/VCAM-1 expression on the vessel wall in various experimental

studies.²¹⁷⁻²²⁴ Furthermore, the products of HO-1 have also been well-known to have prominent anti-inflammatory and antioxidant actions, to the extent that some researchers believe that the cardioprotective properties of HO-1 are mediated by HO-1 metabolites rather than HO-1 by itself.^{214,225} It has been established for a long time that bilirubin suppresses inflammation and oxidative stress and that higher plasma levels of bilirubin correlate inversely with the incidence of coronary artery disease.^{209,226,227} The proposed mechanisms by which bilirubin exerts its protective functions include its ability to inhibit the protein phosphorylation and PKC activity,^{228,229} both of which result in the inactivation of various proatherogenic processes including inflammation.^{209,230} In addition, CO also has been posited to confer inhibitory effects against the LPS-induced pro-inflammatory cytokine production in macrophages via activation of the p38 MAPK pathway.^{209,218} Interestingly, a few animal studies have shown that both bilirubin and CO have also the ability to dramatically decrease NF- κ B activation (Figure 7).²²⁴ Additionally, ferritin, the iron storage protein product of HO-1, has been identified as a potent antioxidant agent.^{204,214,224,225} Collectively, the HO-1 system would be important for cellular homeostasis in many pathophysiological conditions including inflammation.

Recent studies have revealed that there exist functional and molecular interactions between CYP epoxygenase-derived EETs and the HO-1 system.²³¹⁻²³⁴ EETs, specifically 11,12-EET, stimulate HO-1 expression and activity in ECs,²³⁵ but HO-1 overexpression in rat kidney has been associated with a decrease in CYP epoxygenase expression and activity,²³⁶ suggesting the potential presence of a feedback mechanism.^{21,235} Furthermore, it has been shown that in rat mesenteric arteries, direct administration of 11,12-EET was associated with an increase in HO-1-mediated CO production and that the vasodilatory effect of 11,12-EET was mediated by activating HO-1.²³⁴

Recently, a novel molecular crosstalk between EETs and the HO-1 pathways has been identified that affects adipocyte formation and adipogenesis in human mesenchymal stem cells (MSCs).^{231,232,233,237} Growing evidence emphasizes the importance of adipocyte tissue in the development of a systemic inflammatory state that contributes to obesity-associated vasculopathy and cardiovascular disease.²³⁸ Through several mechanisms, including activation of HO-1-mediated pathways, EETs have been found to decrease MSC-derived adipocyte stem cell differentiation and adiposity (Figure 7). A very recent study showed that EET administration increases the expression of HO-1 in MSCs, at least in part, by inhibiting the negative transcriptional regulator of HO-1 expression, Bach 1.²³³ The EET-induced upregulation of HO-1 expression in MSCs, results in the activation of signaling cascades involving in adipogenesis such as AMP activated kinase (AMPK) and adiponectin-pAKT which in turn attenuates fatty acid synthase, thus leading to suppression of MSCs-derived adipocyte differentiation and lipid accumulation. Further, EETs have been shown to inhibit adipogenesis via the activation of anti-adipogenic factors, β -catenin and pACC, and also the reduction of expression of PPAR γ and C/EBP α , as adipogenic differentiation markers, in MSCs-derived adipocytes.^{232,233,237} These findings collectively suggest that the crosstalk between EETs and HO-1 regulates adipogenesis in MSCs, manifesting by a decrease in the number of large inflammatory adipocytes and subsequently the plasma levels of inflammatory cytokines including TNF- α , interferon (INF)- γ and IL-1. On the other hand, the EET-HO interaction leads to an increase in small healthy adipocytes and adiponectin levels.^{231,232,237} This interaction and the relevant pathways could be the potential targets of pharmacological interventions especially in diabetes type II, obesity and metabolic syndrome where the role of adipocytes in the development of a systemic inflammatory state has been well established.

4.2.2.2.1.5. CYP epoxygenase-derived EETs and increase eNOS expression

The role of eNOS and NO in the regulation of vascular homeostasis is interestingly complicated. In mammalian including human, NO is an extremely potent and important autocrine and paracrine signaling molecule that involved in a variety of biological processes. NO acts as a double-edged sword in the regulation of vascular function. At the physiological concentrations, NO causes vasodilation, downregulates adhesion molecule expression and also inhibits platelet aggregation and SMC proliferation.²³⁹⁻²⁴³ On the other hand, excess NO production, as occurs in different disease states, can adversely affect blood flow and other vascular functions.^{244,245} In ECs, NO is produced from L-arginine and oxygen by four isoforms of NOS, including eNOS.²⁴⁰ Even though eNOS expression is regulated by numerous stimuli,²⁴⁶ laminar shear stress has been identified as the most physiologically important inducer of eNOS expression and activity.²⁴⁷ It has been elucidated that the shear stress-induced overexpression of eNOS is mediated by the activation of NF-κB.²⁴⁸ On the other hand, via several mechanisms, NO significantly inhibits NF-κB activity.²⁴⁹⁻²⁵¹ It has been proposed that the interaction between shear stress, NF-κB activation and increased eNOS expression, NO production and eventually NF-κB inhibition, serves a negative feedback loop which halts sustained NF-κB activation and eNOS transcription.²⁵²

There is evidence that there exists a functional interaction between CYP-derived EETs and eNOS expression/NO biosynthesis pathways.^{253,254} Both overexpression of CYP epoxygenases and exogenous addition of EETs have been associated with a robust upregulation of eNOS expression and activity, consequentially raising NO biosynthesis in ECs *in vitro*.²⁵⁴⁻²⁵⁶ Cell signaling pathways that are implicated in EET-mediated eNOS overexpression in ECs include the MAPK pathway, the PI3K/Akt pathway and the PKC pathway.^{255,256} Interestingly, 11,12-EET has also been found to increase eNOS activity in human platelets *in vitro*.²⁵⁶ Despite the

aforementioned findings, the anti-inflammatory effect of EET-induced eNOS expression and NO generation is still a matter of uncertainty (Figure 7).

4.2.2.2.2. CYP epoxygenase-derived EETs and platelet inhibition

Apart from their contribution to thrombus formation, platelets have a central role in the induction and maintenance of inflammatory process. Upon activation, platelets express and release inflammatory mediators, induce an inflammatory response in adjacent leukocytes and respond with activation to several of the inflammatory mediators produced by these cells.²⁵⁷⁻²⁵⁹

It has been indicated by several studies that EETs can elicit inhibitory actions on platelets (Figure 7). Several mechanisms are involved in the antiplatelet effects of EETs. Original experimental study revealed that 8,9-EET and 14,15-EET, stereospecifically, downregulate the TXB₂ content and COX activity in platelets both of which leads to an inhibitory effect on platelet aggregation.²⁶⁰ Moreover, unlike their stereospecific effect on COX, all four EET isomers inhibited arachidonic acid-induced platelet aggregation without evident stereospecificity and without altering COX activity and thromboxane synthesis.^{260,261} Further *in vivo* studies demonstrated that EETs induce platelet membrane hyperpolarization through a mechanism involving activation of K_{Ca} channels resulting in the suppression of platelet adhesion molecule expression and platelet activity.^{262,263}

At last, even though seemingly beyond the scope of this part, it might be interesting to mention that it has been very recently shown that EETs, particularly 8,9-EET may inhibit B-lymphocyte function *in vitro* and *in vivo*, which suggests a new therapeutic strategy for

diseases with excess B cell activation.²⁶⁵ Further studies are needed to investigate the likely relevance of this effect on inflammation.

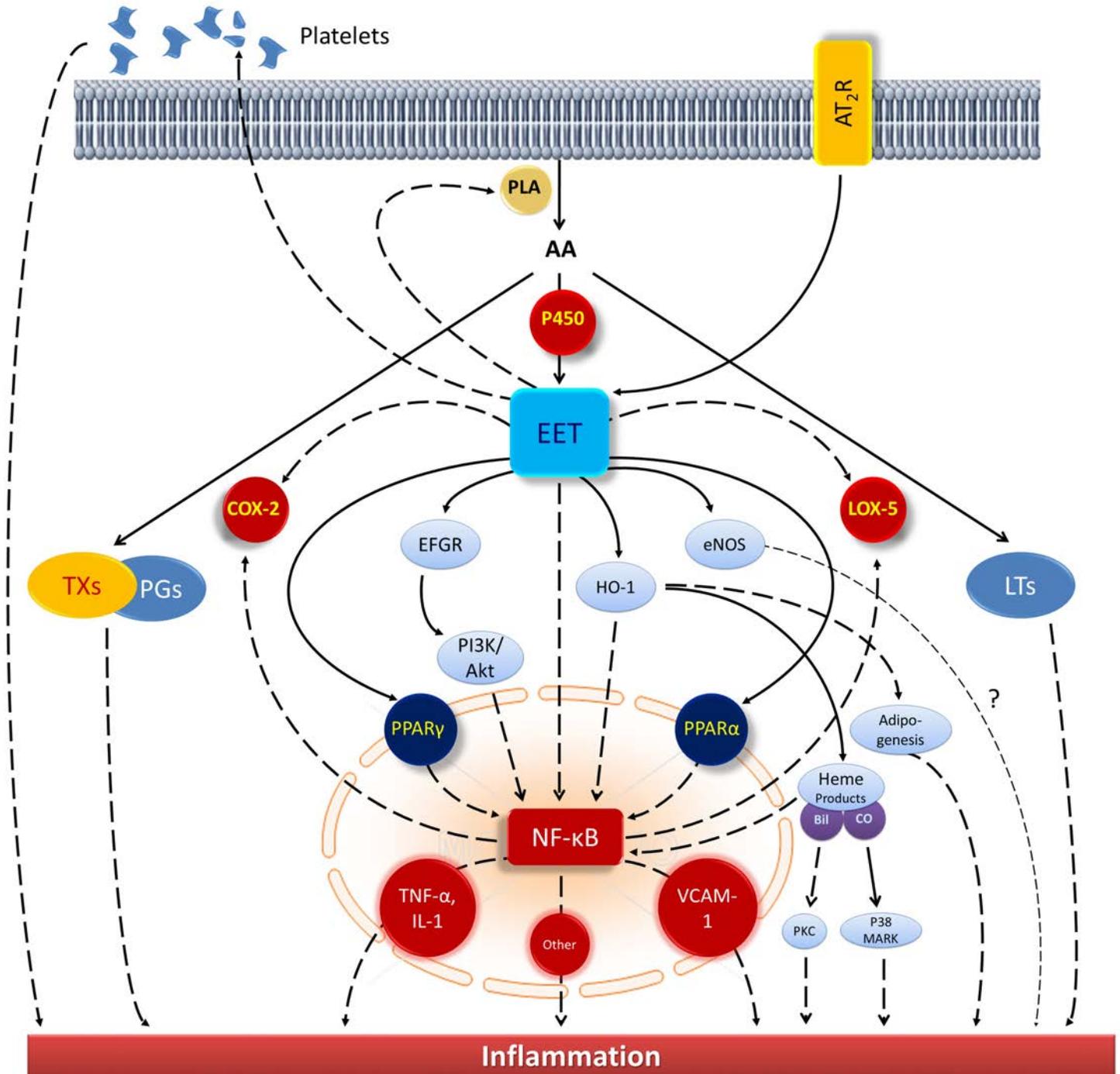


Figure 7. The intracellular pathways by which EETs exert their anti-inflammatory effects. Solid line represents stimulatory influence and dash line represents inhibitory influence.

4.2.2.3. *Effect of inflammation on CYP epoxygenase expression*

The phenomenon of hepatic and extrahepatic CYP down regulation by inflammation and its consequent effect on xenobiotics, including medications, metabolism has been extensively explored in experimental animal studies and, to a lesser extent, in human beings.²⁶⁵⁻²⁶⁸ The main underlying mechanism by which inflammation downregulates CYP expression has been found to be modulated mainly by inflammatory cytokines (IL-1, IL-6), TNF- α and INF- α and INF- γ ;^{231,232,237} however, cytokines have enzyme-specific effects on CYP expression so that the subsets of CYPs regulated by individual cytokines are different.²⁶⁵

Depending on the specific CYP gene, type of inflammatory stimuli and the time point in the response, multiple transcription-related mechanisms are implicated in the inflammation-induced downregulation of CYP activity. For most of CYPs studied, a pretranslational mechanism, involving depression of CYP mRNA levels and a subsequent decrease in CYP synthesis, mediates the impact of inflammation on CYP expression. Not surprisingly, NF- κ B, the master regulator of inflammation, is involved in the regulation of CYPs through interaction with their induction mechanisms.^{266,267} For instance, suppression by inflammatory stimuli of induced transcription of CYP1A1 and 1A2 is due to mutually antagonistic interaction of aryl hydrocarbon receptor (AhR) with NF- κ B; NF- κ B binds to AhR and these two transcription factors repress each other's function.²⁷¹ The interaction of NF- κ B with pregnan X receptor (PXR) and constitutive androstane receptor (CAR) are other mechanisms proposed to be involved in the downregulation of inducible CYP in response to inflammation.^{266,267,272,273} Even though pretranscriptional downregulation is the main factor in inflammation-induced suppression of many CYPs, there are also several post-transcriptional mechanisms that are implicated in this effect; it has been shown that

inflammation induces NO synthesis through the induction of NOS resulting in the downregulation of CYP2B1 and 2B6.^{274,275}

Recent *in vivo* studies have demonstrated that the LPS-induced activation of innate immune response, downregulates CYP epoxygenases resulting in the alteration of epoxygenase-mediated eicosanoid metabolism content in rat hepatic and cardiovascular tissues.^{267,277} This finding contains an important message; the inflammation-induced downregulation of CYPs is potentially associated with not only the alteration of xenobiotics metabolism, but also the modification of the formation of endogenous eicosanoids, including EETs.

5. Inflammation, CYP epoxygenase expression and EET generation; a vicious cycle?

As described earlier, accumulating evidence confirms that, via multiple mechanisms, CYP-derived EETs exert strong anti-inflammatory effects on the vascular system including the coronary vasculature. On the other hand, it is well-established, by experimental animal as well as human studies, that inflammatory states are associated with downregulation of hepatic and extrahepatic CYPs, including epoxygenases, expression and a decrease in EETs formation. Considering strong anti-inflammatory properties of EETs, it would make sense to speculate that under inflammatory conditions there exists a vicious cycle between inflammation and CYP epoxygenase expression/EETs generation in which the inflammation-induced downregulation of CYP epoxygenases causes a decrease in the EET production. Hypothetically speaking, insufficient EET synthesis would, in turn, lead to an ineffective EET-mediated anti-inflammatory effect, leading to an augmentation of systemic and regional inflammatory responses and further downregulation of CYP epoxygenase activity/EET production (Figure 8). This cycle might explain, at least in part, why inflammation establishes

following the onset of an inflammatory insult. This cycle may also help to better understanding of pathophysiology of chronic cardiovascular diseases, including atherosclerosis and hypertension, and also could be an emerging target for further pharmacological therapy of these disorders where increased inflammatory responses are known to occur. Additionally, considering the central role of ED in the triggering and development of inflammation and given the fact that the blood vessels in each organ can express different epoxygenases and each CYP isoform generates its own unique profile of EET regioisomers, the relative abundance of EET regioisomers, with clear difference in anti-inflammatory effects, differs across the vascular beds. Next, it would be logical, therefore, to assume that the contribution of EETs to the regulation of vascular inflammation varies among arterial ECs of different organs.

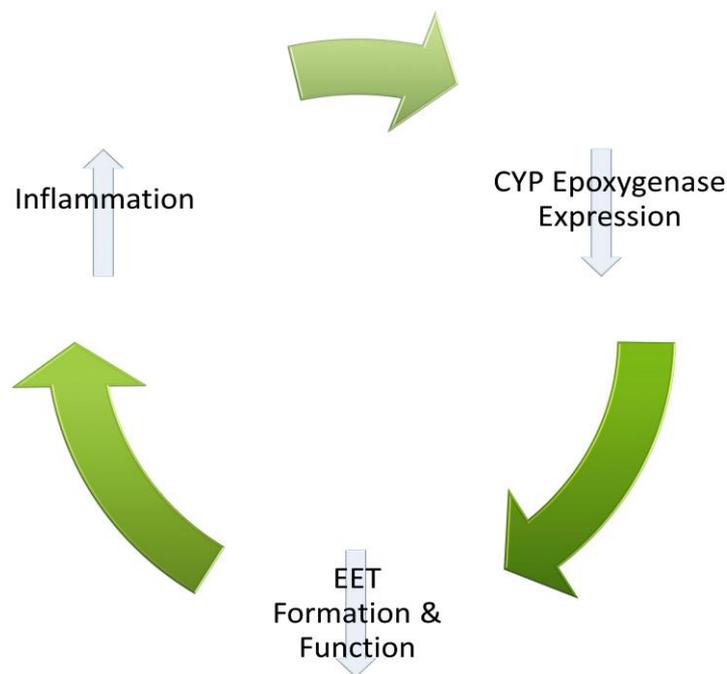


Figure 8. The inflammation-CYP epoxygenase-EET formation/production vicious cycle

Accordingly, the significance of the inflammation-epoxygenase-EET-inflammation cycle, if any, would be different between various endothelial cell lines in a given inflammatory state. This hypothesis may be a reasonable justification for the difference in the prevalence of an inflammation-associated phenotype, e.g. atherosclerosis, among the blood vessels across the body. For instance, atherosclerosis is a result of a systemic inflammation; however, it is detected more frequently in the coronary circulation and brain arterial system. This theory would provide the impetus for developing EET-based medications that selectively inhibit inflammation in a specific arterial territory, coronary arterial tree, for example. Additionally, as it mentioned earlier, there are large differences in functional CYP epoxygenases polymorphisms across various ethnics and populations. This could result in an inter-ethnic variability of EET levels which might explain, at least in part, the heterogenous pattern of the prevalence of the inflammation-associated cardiovascular diseases, including hypertension and atherosclerosis, among different ethnics. This hypothesis also could be a source of further multicentric, international genetic epidemiological studies

6. Conclusion and perspective

CYP epoxygenase-derived EETs play multiple key roles in the cardiovascular biology with anti-inflammatory effects being of special importance. The mechanism of inflammation inhibition is mainly by suppressing inflammatory cell recruitment and further targeting the PPAR and NF- κ B pathways which results in an inhibition of COX-2, LOX-5 and VCAM-1. Furthermore, we now know that inflammation is associated with the downregulation of CYPs expression; epoxygenases are not exception. The study of interaction between CYP epoxygenase-derived EET and inflammation in which CYP epoxygenase products affect negatively inflammation and inflammation suppresses the expression of epoxygenases may

provide interesting pharmacological targets for the prevention and treatment of wide range of inflammation-associated disorders such as chronic cardiovascular diseases. Further studies are required to evaluate whether this concept add more efficacy to the existing medications in treatment of inflammatory conditions.

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IV. Point-of-care pharmacogenetic testing

As mentioned in part I, the pharmacogenetics has a significant impact on the response to thienopyridines, and particularly to clopidogrel. It has been predicted that pharmacogenetics will have a great future and will revolutionize the prescribing and patient care across the whole of medications.

The introduction of novel technologies, including point-of-care (POC) genetic testing, has provided the opportunities that ease the application of pharmacogenetic data in the daily clinical practice. The use of POC tests have been reported originally for warfarin and more recently also for clopidogrel and other medications.

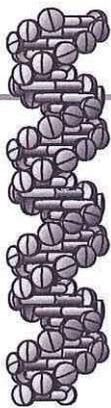
Through a short Editorial paper, we tried to demonstrate how POC genetic testings can contribute to encourage the clinicians and pharmacists to incorporate the pharmacogenetic testings in their clinical settings.

Interet clinique des tests pharmacogénétique aux points de service

“Le tout nouveau dosage pharmacogénétique auprès des patients fournit une solution rapide, facile à mettre en œuvre et meilleur marché pour obtenir à la fois plus sûrement (ex. la warfarine) et plus efficacement (ex. le clopidogrel) le bénéfice du traitement médicamenteux.”

“Les tests points de service ne sont considérés actuellement que comme des simples outils de recherche ; à terme, toutefois, on pourrait en faire un argument de motivation pour encourager les médecins dans intégrer la pharmacogénomique à la pratique médicale courante.”

“Les tests génétiques au lieu des soins ne nécessitent pas l’intervention de personnel spécialisé et justifiant d’une formation approfondie, s’effectuent relativement vite et, surtout, sont réalisables au chevet des patients.”



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Clinical interest of point-of-care pharmacogenomic testing: clopidogrel behind warfarin

“The recently emerging bedside point-of-care genetic assay has offered rapid, easy-to-use and a more affordable solution to achieving the benefit of drug therapy more safely (e.g., warfarin) and effectively (e.g., clopidogrel).”

KEYWORDS: clopidogrel ■ cytochrome P450 ■ genetics ■ pharmacogenomics ■ point-of-care testing ■ warfarin

Interindividual variability in drug efficacy and toxicity in various clinical settings remain major obstacles to optimal pharmacotherapy. For example, 35% of hypertensive patients receiving β -blockers, up to 30% of patients with schizophrenia who are treated with anti-psychotics and up to 30% of patients with depression who are treated with antidepressants do not respond satisfactorily to drug treatment, and only one-third of patients with multiple sclerosis respond effectively to interferon therapy [1,2]. There are also marked variations in both the response rate and incidence of adverse therapeutic events of cancer chemotherapy [3]. Even though demographic variables, diet, coexisting chronic diseases, inflammatory status and concurrent medications may contribute to interindividual differences in drug responsiveness, up to 95% of the variability in drug disposition is attributed to genetic variations [4]. For example, more than 60% of statin-related myopathy has been shown to be attributable to *SLCO1B1* gene variants [5]. Clinical pharmacogenomics provide a promising way to the adoption of personalized pharmacotherapy through a powerful and pragmatic approach for predicting drug response, avoiding adverse effects and optimizing medication dose. Currently, the US FDA has recommended genetic information, including polymorphic drug target and disposition genes, to be added to the product labels of more than 100 approved medications and to be incorporated in daily medical practice [10]. Accordingly, pharmacogenomic testing of drug-metabolizing enzyme genes as well as other determinative candidate genes involved in the pharmacokinetics and pharmacodynamics of widely prescribed medications are putatively used across a range of medical specialties, including oncology,

neurology, psychiatry and cardiology. Most of these genetic variations, however, are currently identified using the classic real-time PCR-based methods that are characteristically time consuming, operator dependent and relatively costly. All of these factors hamper the routine clinical application of pharmacogenomic testing in daily pharmacotherapy, particularly in conditions in which the time element is a paramount factor, such as in critical care settings. The recently emerging bedside point-of-care (POC) genetic assay has offered rapid, easy-to-use and a more affordable solution to achieving the benefit of drug therapy more safely (e.g., warfarin) and effectively (e.g., clopidogrel).

Warfarin: pioneer drug target of POC genetic testing

POC genetic testing was prototypically implemented and validated for prediction of response to warfarin in mid 2011 [6]. For the past 60 years, warfarin has been, worldwide, the mainstay oral anticoagulant agent used for long-term treatment and prevention of thromboembolic disorders [7,8]. However, approximately 20% of patients are hospitalized within 6 months of warfarin therapy initiation because of overcoagulation and related bleeding events [9]. A large body of evidence now exists to strongly suggest that genetic variations of CYP2C9, the main drug-metabolizing and inactivating enzyme of *S*-warfarin, and VKORC1, the target enzyme for warfarin, can explain up to 35% of interindividual variability in dose requirement and also provide explanations for warfarin narrow therapeutic index [10]. Accordingly, the FDA as well as the Clinical Pharmacogenomics Implementation Consortium, has recently recommended a genetic-guided approach for



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warfarin-dose selection [11,102]. This strategy may confer therapeutic benefits including fewer incidences of adverse events, and in particular, thromboembolic events and bleeds. The novel POC genetic testing provides precise information for a genetic-based warfarin therapy in less than 2 h [6].

Clopidogrel: subsequent station of POC genetic testing train, but still en route

Clopidogrel is the most widely prescribed antiplatelet therapy in the world [12] and is currently the thienopyridine of choice for dual antiplatelet therapy, along with aspirin, in patients with acute coronary syndrome and those treated with percutaneous coronary intervention and stenting [13]. More than 40 million patients are on clopidogrel worldwide [14]. Up to 30% of patients, however, have been found to be 'nonresponders' to clopidogrel [15]. Accumulating evidence suggests that persistent platelet reactivity on clopidogrel treatment is associated with marked increased risk of major adverse cardiovascular events [16].

“Currently point-of-care genetic testing is considered merely as a tool in research settings; however, it could eventually be used as a motivator to encourage physicians and pharmacists to integrate pharmacogenomics into routine medical practice.”

It has also been well documented that the response to clopidogrel can be influenced by pharmacokinetic factors, including intestinal absorption and biological activation, and pharmacodynamic variables, all of which are under the control of genetic polymorphisms. The polymorphisms of genes that are involved in clopidogrel absorption (*ABCB1*), bioactivation (*CYP3A4*, *CYP3A5*, *CYP2C9*, *CYP2C19* and *PON-1*) and biological activity (*P2RY12*, *ITGA2* and *ITGB3*) have been shown to be effective in responsiveness to this medication [17,18]. In particular, the loss-of-function *CYP2C19* allele, *CYP2C19*2*, is the strongest predictor of resistance to clopidogrel [17]. In March 2010, the FDA issued a 'boxed warning' recommending that healthcare professionals should consider genotyping of the nonfunctional *CYP2C19* allele prior to clopidogrel therapy and to judge use of alternative antiplatelet agents (e.g., prasugrel and ticagrelor) or to modify dosing strategies, accordingly [19]. Tests are available for identifying the patients' *CYP2C19* status; however,

these tests are not widely accessible, are time consuming and require well-trained personnel. Very recently, a well-validated POC genetic test has been introduced that can be performed effectively at the patient bedside and permits the determination of *CYP2C19*2* carriers in less than 1 h [20]. There are presently two ongoing clinical trials in Italy and Canada, seeking to assess the clinical implication of clopidogrel pharmacogenomics through the POC genetic testing for *CYP2C19*2*, *CYP2C19*17* (a relatively frequent polymorphism of the *CYP2C19* gene, which is associated with increased enzyme expression and activity and augments the risk of bleeding in clopidogrel takers) and *ABCB1* 3435 C>T alleles [103,104]. In this line, we are also about to commence a multinational (France, Portugal and Spain) collaborative research investigation project, on a large cohort of post-percutaneous coronary intervention patients receiving clopidogrel, in which the concordance of POC genetic testing for the detection of *CYP2C19* loss-of-function polymorphism with the laboratory-based genotyping methods, would be evaluated. Even though *CYP2C19*2* is the main culprit polymorphism for the variability in individual responsiveness to clopidogrel, for achieving an optimal response, it makes sense and seems logical to speculate that a well-validated POC genetic test covering all of the determinant genetic variants, including *PON-1* and *P2R12* polymorphisms, would be more ideal.

Future perspective

Currently POC genetic testing is considered merely as a tool in research settings; however, it could eventually be used as a motivator to encourage physicians and pharmacists to integrate pharmacogenomics into routine medical practice. POC genetic testing does not need to be performed by expert and thoroughly trained staff, is relatively quick to perform and most importantly, can be performed at the patients' bedside. These characteristics allow it to be carried out in both emergency and elective settings.

POC genetic testing could also contribute in the vital application role of system-based pharmacogenomics in drug discovery and development. As a cutting-edge technology, POC genetic testing could potentially facilitate the elaborate procedure and accelerate the time-consuming process of new drug testing by offering a genetic-based stratification of patients into risk-benefit groups and also by providing

surrogate theranostic biomarkers for evaluating the pharmacokinetic characteristics of newly invented medications.

On the other hand, whole-genome sequencing is rapidly transforming the landscape of clinical pharmacogenomics, and sequencing sets of genes is already being used to guide cancer treatment. The current cost of sequencing a patient's complete genome, is generally prohibitive to routine use; however, as the whole-genome sequencing cost is becoming affordable and the entire genome data are becoming more clinically available, the question is raised of whether POC genetic testing can resist and compete with full genome-sequencing developments. Furthermore, to be practically used in a large scale, the likely relevant financial debate including insurance coverage issues should be clarified, *a priori*.

The European Society of Pharmacogenomics and Theranostics, an active association in integrating translational pharmacogenomics in clinical pharmacotherapy, will attempt to further evaluate clinical use of POC pharmacogenomic testing and to encourage healthcare

professionals, by this means, to consider the clinical implementation of pharmacogenomics in their daily practice.

“Point-of-care genetic testing does not need to be performed by expert and thoroughly trained staff, is relatively quick to perform and most importantly, can be performed at the patients’ bedside.”

Could the Trojan horse of pharmacogenomics, POC genetic testing, enter the Troy of the clinicians/pharmacists and persuade them to consider the indispensable role of genetics in their practical pharmacotherapy? Only time will tell!

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CHAPTER 2 – HYPOTHESES AND OBJECTIVES

Low grade, chronic inflammation, as indicated by levels of the inflammatory markers including CRP, Hp and orosomucoid acid, is associated with the initiation and development of a wide range of systemic illnesses including CVDs.

I. Hypothesis, Part - 1

We identified a common SNP, rs2000999, in the Hp gene that was found to be a strong predictor of plasma Hp levels ($P = 8.1 \times 10^{-59}$) and also was demonstrated to explain 45.4% of its genetic variability. Considering the important role of reference values in clinical chemistry and laboratory medicine, we speculated that (hypothesis (H) 1) the newly-discovered Hp SNP, rs2000999, could be used in the partitioning of human plasma Hp reference intervals.

II. Hypothesis, Part - 2

A growing body of studies provided support for the concept that CYP epoxygenases-derived EETs exhibit potent anti-inflammatory effect. Also, the most common CYP epoxygenase SNPs, *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C8*2* and *CYP2J2*7* are associated with partial or complete loss of activity of the corresponding enzyme. We, therefore, hypothesized (H2) that these polymorphisms might lead to a decrease in the EET formation and consequently to difference in the EET-mediated levels of inflammatory markers.

III. Hypothesis, Part – 3, 4

There exists a marked inter-individual variability in response to the anti-platelet effects of thienopyridine-derivatives, clopidogrel and prasugrel, that is attributed mainly to the loss- and gain-of-function CYP2C19 polymorphisms (*CYP2C19*2* and *CYP2C19*17*). Moreover, some studies have reported a phenomenon that has been termed “smoker’s paradox” whereby clopidogrel-treated smokers appears to have a greater platelet inhibition and lower aggregation when compared to non-smoker patients. In addition, several studies proposed that thienopyridines may exert pleiotropic anti-inflammatory effects; however, considerable inter-study controversy has surrounded the effects of anti-platelet drug use on the level of inflammatory markers. Since activated platelets play central roles in the inflammation, we hypothesized that (H3) *CYP2C19*2* allele is associated with higher levels of platelet reactivity and consequently higher levels of inflammatory markers in on-thienopyridines post-PCI patients. We also made a hypothesis that (H4) the “smoker’s paradox” phenomenon might be associated with inter-individual variability in response to the anti-inflammatory effects of thienopyridines.

IV. Hypothesis, Part - 5

It has been shown that the patients with “high platelet reactivity” despite treatment with thienopyridines are more likely to suffer therapeutic failure manifested by ischemic cardiovascular events and ISR. Nevertheless, there is inconsistency in the literature regarding the optimal definition of “high platelet activity” in on-thienopyridine patients. While absolute or relative *changes* in platelet aggregation from baseline, seems to be the most reliable indicator of anti-platelet efficacy, the absolute level of platelet reactivity during treatment (i.e., *on-treatment* platelet reactivity) has been suggested to be a more accurate way of detecting the patients who are at high risk of thrombotic events. Based on a recent consensus definition, the high on-treatment platelet reactivity can be determined by four criteria, one of which is platelet reactivity index (PRI) > 50% by vasodilatory-stimulated phosphoprotein (VASP) analysis. Again, since activated platelets play a crucial role in the initiation and maintenance of inflammation, it would be logical to hypothesize that (H5) the patients with high on-treatment platelet reactivity (VASP PRI > 50%) have high levels of inflammatory markers.

Based on the mentioned above, in this thesis, we followed five main objectives:

V. Objectives

Objective 1: To determine the reference values of Hp on the basis of its main sources of biological variation including the rs2000999 in healthy subjects

Objective 2: To assess the association between the most common polymorphisms of CYP epoxygenases on the plasma levels of inflammatory markers of healthy subjects

Objective 3: To assess if the loss- and gain-of-function CYP2C19 polymorphisms (*CYP2C19*2* and *CYP2C19*17*) are associated with the levels of inflammatory markers in post-PCI on-thienopyridine patients.

Objective 4: To investigate if there the levels of inflammatory markers are associated with smoking status in post-PCI patients who treated with thienopyridines (another aspect of “smoker’s paradox” phenomenon).

Objective 5: To investigate whether high on-treatment platelet reactivity (VASP PRI > 50%) in on-thienopyridine patients is associated with higher levels of inflammatory markers.

CHAPTER 3 – POPULATIONS AND METHODS

Generally speaking, in this thesis we had three main objectives: A) partitioning the reference values of Hp in the function of newly-discovered Hp SNP, rs2000999; B) pharmacogenetic approach to the anti-inflammatory effects of thienopyridines; and C) genetic approach to the inflammation-reducing properties of EETs. The reference ranges of Hp was determined on the population of the STANISLAS cohort (SC) and we conducted two collaborative studies, with two distinguished research centers in Paris and Marseille, to achieve to the other objectives. The hypotheses have been tested in each population using biochemical, hematological and genetic tests. Here, we present a brief description of the populations and the employed methods; more detailed information regarding each study population and also the laboratory techniques used for genotyping and determining the biochemical profile have been presented in the relevant papers found in Chapter 4.

1. SC

The SC is a 10-year longitudinal cohort involving 1,006 volunteer families of European ancestry whose members were free of chronic disease (cardiovascular or cancer) with recruitment taking place from 1993–95. The SC samples and data are part of the Biological Resources Centre (BRC) “Interactions Gène - Environnement en Physiopathologie CardioVasculaire” (IGE-PCV) in Nancy, France.

In our study, we first identified the main biological variables of Hp in 3,129 apparently disease-free individuals of the SFS. Hp reference ranges were subsequently established in a subgroup of 2,923 selected healthy subjects, as the reference population by taking into account the newly-discovered Hp polymorphism, rs2000999.

1.1. *Sample reference population*

The sample consisted of 1,421 men and 1,502 women individuals aged 6-67 years who met all of the following criteria: Z-BMI <3%, cigarette consumption <20 /day, alcohol consumption <88 g/d, plasma level of Hp <3.5 g/L, TG <10 mmol/L, total cholesterol <10 mmol/L, glucose <8.5 mmol/L, albumin <55 g/L, bilirubin <50 µmol/L, AST <100 IU/L, ALT <150 IU/L, ALP <600 IU/L, GGT <200 IU/L, WBC <15 x 10⁹/L, creatinine <150 µmol/L, CRP <20 mg/L, alpha-1 glycoprotein <2 g/L and also SBP <140 mm Hg and DBP <90 mm Hg.

1.2. *Genotyping*

Data regarding Hp genotype was recovered from a GWAS which had been performed by our team and laboratory [Gene expression for Hp was measured using the Affymetrix Human U133 Plus 2.0 platform and DNA was isolated from peripheral blood and genotypes were generated using Illumina 610-Quad arrays]. Genotyping quality control was performed systematically for all of the participants.

1.3. *Hp measurement*

Blood samples were collected between 8:00 and 9:00 am or 11:30 and 12:30 pm by venipuncture after overnight fasting. Hp protein levels were measured in blood plasma samples by high sensitivity immunophotometry analyses using the BNTM II Siemens analyzer (Siemens, Marburg, Germany) and Siemens reagents and following the manufacturer's instructions.

2. Populations on Thienopyridines

To achieve our objectives classified as B and C, we prepared a detailed proposal for a clinical trial on the pharmacogenetic response to the anti-inflammatory effects of thienopyridines. In this regard, we have contacted, in a time order, with the following clinical research centers to run a collaborative clinical investigation:

1. Centre Hospitalière Universitaire (CHU), Nancy, France – Prof. Yves Juillière
2. Charles University, Hradec Kralove, Czech Republic – Prof. Pudil Radek
3. Centre Hospitalière Régional (CHR), Metz-Thionville, France – Prof. Khalifé Khalifé
4. Saint Antoine Hospital, Pierre et Marie Curie University (UPMC), Paris, France – Prof. Tabassome Simon, Prof. Nicolas Danchin
5. Eli Lilly Corporate Center, Indianapolis, United States – Dr. Snadra Close Kirkwood
6. DSAR–Drug Disposition, Sanofi-Aventis Recherche Développement, Montpellier, France – Dr. Fabrice Hurbin
7. Genetic and Molecular Epidemiology Laboratory, McMaster University, Hamilton, Canada – Dr. Guillaume Paré
8. Centre Hospitalière Saint-Charles, Toul, France – Dr. Laetitia Albertini
9. Department of Cardiology, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal – Prof. Pedro Monteiro
10. CICAB Clinical Research Centre, Hospital Universitario Infanta Cristina, University of Extremadura, Badajoz, Spain – Prof. Adrián Llerena
11. Université Paris Descartes, Sorbonne Paris Cité, AP-HP, Hôpital Européen Georges Pompidou, Paris, France – Prof. Pascale Gausseme
12. Centre Hospitalière de la Timone, Marseille, France – Prof. Marie-Christine Alessi, Prof. Pierre Morange
13. The Wolfson Centre for Personalized Medicine, Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, Liverpool, UK (see also “*Perspectives*”) – Prof. Munir Pirmohammed

From all mentioned above, just the research centers of the Hôpital Européen Georges Pompidou in Paris (#11) and the Centre Hospitalière de la Timone in Marseille (#12) could provide us the biological sample and it should be acknowledged that the results of our thesis have obtained from collaborative studies with these centers. Also, we are now involved in a collaborative clinical trial on the anti-inflammatory effects of clopidogrel with the University of Liverpool (#13; see also "*Perspectives*"); the results of this study will be released within the next few months.

2.1. *Paris Study*

This study was performed on forty nine volunteer young healthy males Caucasians (aged 18-35 years) who met the following conditions were enrolled in the study: non-smokers, normal physical and laboratory results, no history of use of medications as well as normal platelet count, normal maximal platelet aggregation in response to four platelet agonists and normal PFA-100 assay.

The subjects were asked to come to the Clinical Investigations Center every day at 9 am, where they were given a standard dose of clopidogrel 75 mg once daily for 7 consecutive days in the presence of medical staff. The plasma concentration of a selected number of inflammatory proteins including CRP, Hp, orosomucoid acid and CD-40 were measured in all subjects before and 7 days after clopidogrel administration. In addition, in order to determine the appropriateness of platelet-inhibition response to clopidogrel (responders vs. non-responders), the ADP-induced platelet aggregation as well as VASP were measured on day 1 (baseline) and 7 of clopidogrel intake.

At baseline, and before taking clopidogrel, the levels of inflammatory markers were compared across the population, in the function of the most frequent CYP epoxygenase gene

polymorphisms in Caucasians: *CYP2C8*3* (rs10509681), *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*2* (rs4244285) and *CYP2J2*7* (rs890293).

Additionally, the changes in the levels of inflammatory markers, from baseline to end of clopidogrel therapy, were compared between the carriers vs. non-carriers of *CYP2C19*2* and *P2Y12 H* alleles and also between responders vs. non-responders to platelet anti-aggregatory effect of clopidogrel.

The study was approved by the Committee for the Protection of Human Subjects in Biomedical Research, which functions for the Cochin University Hospital (Paris, France), and all the subjects gave their written informed consent to participate, in accordance with the Declaration of Helsinki.

2.1.1. Measurement of inflammatory markers

The plasma levels of CRP, Hp and orosomucoid acid were determined, immunonephelometrically, using BN II (Siemens Healthcare Diagnostics, Germany); the intra-assay variation coefficient for CRP, Hp and orosomucoid acid was 2.6%, 3.2% and 2.6%, respectively. Moreover, soluble CD-40 was measured using commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instruction.

In respect to EETs, in spite of our great efforts and due to the technical and financial problems, we failed to measure plasma levels of EETs in our study populations.

2.1.2. Genotyping

Genotyping for *CYP2C8*3* (rs10509681), *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*2* (rs4244285) and *CYP2J2*7* (rs890293) was performed by LGC Genomics and

KBioscience Laboratory using KASP™ genotyping assay. Genotyping quality control was performed systematically for all of the participants.

2.2. *Marseille Study*

This study, recruited 1128 patients with ACS who underwent a successful PCI and were discharged between April 2007 and December 2012 with either with clopidogrel 75 mg/d (n = 118); clopidogrel 150 mg/d (n = 631); or prasugrel 10 mg/d (n = 309). In addition, all of the patients received aspirin 75 mg/d simultaneously.

All consecutive patients with acute coronary syndrome who underwent successful PCI between April 2007 and December 2012 at the Centre Universitaire Hospitalier Timone, Marseille, France recruited in the study. Exclusion criteria for the current study were a history of bleeding diathesis, previous stroke or transient ischemic attack, contraindications to anti-platelet therapy, platelet count <100 g/L and creatinine clearance <25 ml/min.

The demographic and clinical data were collected through patient interview. After interview, systolic and diastolic blood pressures (SBP and DBP) were measured following a standard protocol and mean arterial pressure (MAP) was calculated as $[(2 \times \text{DBP}) + \text{SBP}]/3$.

The study protocol was approved by the local ethics committee and all the patients gave written informed consent before participating in the study.

2.2.1. *Biochemical profile*

At 1-month follow-up visit hemogram, platelet count and selected parameters of biochemistry including CRP, lipids (triglyceride, total cholesterol, low density lipoprotein and high density lipoprotein), blood glucose, hemoglobin A1C, insulin blood level and serum level of creatinine were performed. All parameters were determined by standard laboratory

methods at the Department of Hematology and Biochemistry of the Centre Universitaire Hospitalier Timone, Marseille, France and the UMR INSERM U1122, Université de Lorraine, Nancy, France. The biochemical parameters were measured in blood samples using different autoanalyzers; ADVIA 120 (Bayer Diagnostics, NY, USA) for hemoglobin and platelet counts; *Beckman DX 800* (Beckman Coulter, Inc., Fullerton, CA) for serum lipids, glucose, creatinine and CRP; *Variant II Turbo* (Bio-Rad, USA) for hemoglobin A1C; and COBAS (Roche Diagnostics, Burgess Hill, West Sussex, UK) for insulin. Also, Hp and orosomucoid acid were determined, immunonephelometrically, using BN II (Siemens Healthcare Diagnostics, Germany).

2.2.2. Genotyping

Genomic DNA was extracted from peripheral blood samples using the salting-out method. *CYP2C19*2* (rs4244285) and *CYP2C19*17* (rs12248560) genotyping was carried out using the amplification refractory mutation system-polymerase chain reaction (PCR) in duplex reaction. The primers used (Invitrogen, Carlsbad, California) were as follows: *2 forward: CAg AgC TTg gCA TAT TgTATC; *2 reverse: TAT CgC AAg CAg TCA CAT AAC; *2G specific (sens): ACT ATC ATT gAT TAT TTC CCg; and *2 A specific (antisens): gTA ATT TgT TAT ggg TTC CT; *17 forward: aag aag cct tag ttt ctc aag, *17 reverse: aaacacctttaccatttaaccc; *17 T specific (sense): tgt ctt ctg ttc tca aag ta and *17 C specific (antisense): atta tct ctt aca tca gag atg. Size of the PCR products generated: *CYP2C19*2* forward/reverse: 373 bp, *2 allele G (normal): 283 bp, and *2 allele A (mutated): 129 bp; *CYP2C19*17* forward/reverse: 507 bp, *17 allele C (normal): 330 bp, and *17 allele T (mutated): 218 bp. The reactions were made in a final volume of 12.5 ml consisting of 200 nM of primer (300 nM for *2 A); 0.15 U of Taq DNA polymerase and its Buffer (Qbiotaq, MP Biomedicals, Santa Ana, California); 200

mM of deoxynucleotide triphosphates; and 25 ng of genomic DNA. The cycling conditions were as follows: a first denaturing step for 1 minute, 30 seconds at 94 °C and then 35 cycles of 30 seconds at 95 °C; 30 seconds at 60 °C; 50 seconds at 72 °C; and a final extension step for 3 minutes at 72 °C, with cooling to 15 °C. The genotypes were determined according to the sizes of the PCR products on 2% agarose gel. Genotyping quality control was performed systematically for all of the participants.

2.2.3. Platelet function measurement

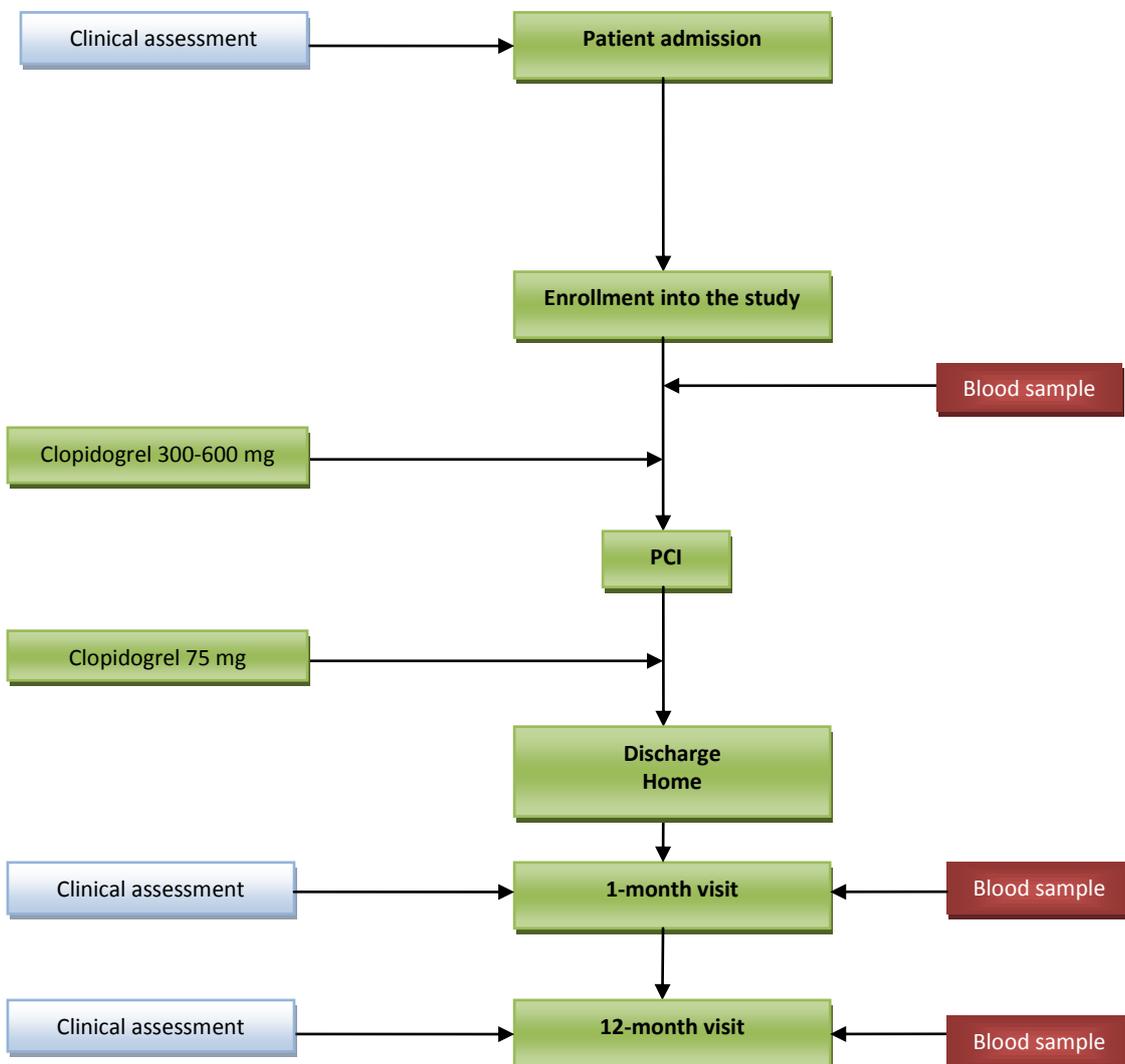
At 1-month follow-up visit after PCI, the P2Y12 receptor inhibition was quantitated using VASP PRI. The level of phosphorylated VASP was determined using a standardized flow cytometric assay (platelet VASP, Diagnostica Stago/BioCytex, Asnières, France), which is an altered version of the method described previously by Schwarz. The VASP PRI was calculated from the median fluorescence intensity (MFI) of samples incubated with prostaglandin E1 (PGE1) or PGE1 and adenosine diphosphate (ADP) according to the formula:
$$\text{VASP PRI} = \left[\text{MFI}_{(\text{PGE1})} - \text{MFI}_{(\text{PGE1} + \text{ADP})} / \text{MFI}_{(\text{PGE1})} \right] \times 100.$$

2.3. Liverpool Study

Currently, we are involved in a collaborative study with the “Wolfson Centre for Personalized Medicine of the Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine in University of Liverpool”, on the anti-inflammatory effects of clopidogrel. In this prospective study, the inflammation reducing properties of clopidogrel in the function of the genetic variants in genes involved in PK/PD of clopidogrel (2.3.1.) and also in the function of platelet function will be investigated in a large population of patients

consisting of approximately 1350 subjects who have had an ACS as their main diagnosis in their index hospital admission.

Participants will be followed up over a period of one year. This will require 3 visits at the admission, at one month and 12 months of follow-up as demonstrated below:



Schema of Liverpool Collaborative Study

- Inclusion criteria:
 - a. Subject is willing to take part
 - b. Index hospital admission with ACS as defined as either a positive Troponin (> 0.01) or ECG changes* with a history consistent of ACS.

- Exclusion Criteria
 - a. Unwilling to take part
 - b. Subject is unable to consent
 - c. Subject has a diagnosis or other pathology likely to account for symptoms or Troponin rise
 - d. ST-elevation myocardial infarction
 - e. Subject has a diagnosis or other pathology that may lead to death within 1 year other than cardiac (e.g. terminal lung cancer)
 - f. Subject has no fixed address
 - g. Subject has no current general practitioner
 - h. Subject is, in the opinion of the Investigator, not suitable to participate in the study.

* ECG changes defined as:

1. ST-segment depression

2. Transient ST-segment elevation (in which ST elevation appears, not meeting criteria for thrombolysis and then resolves on subsequent ECGs)
3. T wave inversion
4. ST-segment flattening

2.3.1. Genotyping

The following SNPs will be determined in the recruited patients:

1. SNPs of genes involved in PK/PD of Clopidogrel

1.1. CYP3A4

- rs2740574 (*1B)

1.2. CYP3A5

- rs28365083 (*2)
- rs776746 (*3)

1.3. CYP2C9

- rs1799853 (*2)
- rs1057910 (*3)

1.4. CYP2C19

- rs4244285 (*2)
- rs4986893 (*3)
- rs28399504 (*4)
- rs12248560 (*17)

1.5. ABCB1

- rs1128503
- rs2032582
- rs1045642

1.6. P2Y12

- rs16846673
- rs6809699
- rs6785930

1.7. ITGB3

- rs5918
-

It is noteworthy to mention that, in this pharmacogenetic approach to the anti-inflammatory effects of clopidogrel, we will also have the opportunity to evaluate whether the interaction

of genes involved in the PK/PD of clopidogrel with those that encode markers of inflammation (ex. Hp and CRP), are associated with response to anti-inflammatory effects of clopidogrel. Accordingly, the newly-discovered Hp SNP (rs2000999) and a selected number of CRP SNPs will be determined in the enrolled subjects.

2.3.2. Inflammatory markers

In order to assess the potential anti-inflammatory effects of clopidogrel, at the first stage, the markers of systemic inflammation (CRP, Hp and orosomucoid acid) will be measured immunonephelometrically, using BN II (Siemens Healthcare Diagnostics, Germany), before and at one month and 12 months after taking clopidogrel. Also, a selected number of adhesion molecules and cytokines (see below) will be measured in a subgroup of recruited patients using RANDOX biochip array technology (RANDOX Laboratories, UK).

Inflammatory markers which will be measured in the study:

A. Markers of systemic inflammation

1. CRP
2. Hp
3. Orosomucoid acid

B. Adhesion molecules

1. E-selectin
2. L-selectin
3. P-selectin
4. Intercellular adhesion molecule-1 (ICAM-1)
5. Vascular cell adhesion molecule-1 (VCAM-1)

C. Cytokines

1. Interferon- γ (INF- γ)
2. Tumor necrosis factor- α (TNF- α)
3. Interleukin (IL)-1 α
4. IL-1 β
5. IL-2
6. IL-4
7. IL-6

8. IL-8
9. IL-10
10. Vascular endothelial growth factor (VEGF)
11. Epidermal growth factor (EGF)
12. Monocyte chemoattractant protein-1 (MCP-1)

CHAPTER 4 – RESULTS

The results of this thesis are presented in the context of five original articles. These papers have been peer reviewed and/or are currently under review in the distinguished international medical journals. Briefly, the first paper (I) deals with the fact that genetic polymorphisms should be considered in the partitioning of reference values of plasma inflammatory markers and that the genetically-determined reference intervals should be applied for the interpretation of values in the daily medical practice. The next three papers (II, III and IV) have a pharmacogenetic approach to the anti-inflammatory effects of thienopyridines. Also, in the second paper (II), we have tested the hypothesis that the CYP epoxygenase polymorphisms are associated with changes in the EET production and in the EET-mediated regulation of systemic inflammation. The three mentioned articles have been performed using three main markers of systemic inflammation including CRP, Hp and orosomucoid acid; the profile of measured inflammatory markers, will be completed in our ongoing collaborative clinical trial on the anti-inflammatory effect of clopidogrel that is about to be finished and the results will be released within the next few months (see “*Perspectives*”). In addition, while the second article (II) was carried out on a small healthy population, the other two papers (III and IV), have been done on a large population of post-PCI, on-thienopyridine patients. Additionally, in the fifth paper (V), we have found a drug-drug interaction between clopidogrel and calcium channel blockers for the regulation of blood pressure. This study has been performed on an existing dataset of a large on-clopidogrel population. These five original papers are accompanied with two additional review papers as well as an Editorial letter which have been already presented as a part of the introduction.

I. Clinical Necessity of Partitioning of Human Plasma Haptoglobin Reference Intervals by Recently-discovered rs2000999

Published: Clin Chim Acta. 2012;413:1618-24.

Nécessité clinique du cloisonnement des intervalles de référence de l'haptoglobine plasmatique humaine par le rs2000999 récemment découvert

Contexte :

Tout récemment, nous avons identifié le rs2000999, un polymorphisme original situé dans le gène de l'haptoglobine (*HP*), comme puissant déterminant génétique des taux d'haptoglobine (Hp). Nous nous sommes fixé pour objectif de déterminer les valeurs de référence de Hp sur la base de ses principales sources de variation biologique, dont le rs2000999, dans une vaste population d'origine française, la STANISLAS Family Study (SFS).

Méthodes :

A travers la mise en œuvre d'une analyse de régression par étapes, les principales variables biologiques des taux de Hp ont été identifiées chez 3.129 sujets de la SFS "apparemment" exempts de pathologies. La fourchette de référence de Hp a été établie ultérieurement dans un sous-groupe de 2.923 sujets sains sélectionnés, en qualité de population de référence.

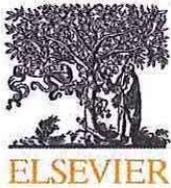
Résultats :

Les valeurs de référence plasmatiques de Hp allaient de 0,08 à 1,97 g/l chez les hommes et de 0,08 à 2,19 g/l chez les femmes. Il est apparu que le sexe, l'âge, le tabac, les taux plasmatiques d'hémoglobine et le polymorphisme *HP* nouvellement découvert, rs2000999, étaient les plus solides prédicteurs des concentrations de Hp dans le plasma humain. Les taux de Hp, dans les deux sexes et toutes les tranches d'âge, étaient négativement associés à la présence et à la quantité d'allèle mineur rs2000999.

Conclusion :

Si l'on veut pouvoir l'interpréter d'une manière fiable dans la pratique médicale quotidienne, le polymorphisme *HP*, rs2000999, doit être envisagé pour le cloisonnement de ses valeurs de référence. Ce polymorphisme pourrait également aider à définir des limites de décision pour l'interprétation médicale des concentrations de Hp.

Mots clés : Haptoglobine, polymorphismes génétiques, facteurs de variation biologique, rs2000999, valeurs de référence



Clinical necessity of partitioning of human plasma haptoglobin reference intervals by recently-discovered rs2000999

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ABSTRACT

Background: Very recently, we identified a novel polymorphism, rs2000999, located in haptoglobin gene (*HP*) as a strong genetic determinant of the haptoglobin levels (Hp). We aim to determine the reference values of Hp on the basis of its main sources of biological variation including the rs2000999 in a large French origin population, the STANISLAS Family Study (SFS).

Methods: Through a stepwise regression analysis, the main biological variables of Hp levels were identified in 3129 “apparently” disease-free individuals of the SFS. Hp reference ranges were subsequently established in a subgroup of 2923 selected healthy subjects, as the reference population.

Results: The plasma reference values of Hp ranged 0.08–1.97 g/L in males and 0.08–2.19 g/L in females. Gender, age, smoking, plasma levels of hemoglobin and the newly-discovered *HP* polymorphism, rs2000999, were found to be the strongest biological predictors of the Hp concentrations in human plasma. Hp levels, in both genders and in all age groups, were negatively associated with the presence and number of rs2000999 minor allele.

Conclusion: To be reliably interpretable in daily medical practice, the *HP* polymorphism, rs2000999, should be considered for partitioning its reference values. This polymorphism may also help for setting decision limits for medical interpretation of Hp concentrations.

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1. Introduction

Haptoglobin (Hp) is an abundant plasma glycoprotein, which was initially discovered in 1938 by Polonovski and Jayle as a “plasma hemoglobin (Hb) binding substance” [1]. The human Hp is a tetrameric protein composed of two light chains (α) and two heavy chains (β) covalently linked to each other by disulfide bindings [2]. The α and β chains in Hp molecule are encoded by two distinct loci on the Hp gene (*HP*) located on the long arm of chromosome 16 (16q22). The *HP* gene at the α , but not at the β , chain loci is polymorphic, with two common alleles denoted *Hp1* and *Hp2*. These alleles give rise to three major phenotypes, Hp 1–1, Hp 2–1 and Hp 2–2 [3,4] distinguished by their electrophoretic patterns on starch or polyacrylamide gel electrophoresis [5].

The originally-described biological function of Hp, binding with high affinity to “free” Hb released into the circulation during physiological and pathological hemolysis, has been well established [2–5]. The heme iron in free Hb represents a highly potent oxidant; however, the rapidly formed Hp-Hb complex, which subsequently is internalized by CD-163-expressing macrophages, prevents Hb-mediated renal parenchymal injury and protects blood vessels from the toxic effects of Hb [2]. In clinical settings, the Hp assay is used to screen for and monitor

of massive or localized hemolysis and hypohaptoglobinemia is a diagnostic marker of red cells destruction of any cause including intra- and extravascular hemolysis, mechanical damage (following insertion of artificial heart valve) and trauma (hematoma) [6,7].

Hp has also been shown by accumulating evidence to be a reliable indicator of acute and chronic systemic inflammatory states. For decades and as an acute phase protein (APP), Hp plasma concentration has been used for the detection of recent inflammatory processes [6]. In response to inflammatory stimulators mainly interleukin (IL)-6 the plasma Hp level increases rapidly and dramatically in the presence of acute inflammatory conditions such as infection, burn and surgery [5,6]. Additionally, a large body of data has also shown that Hp could be used, clinically, not only as a marker of acute inflammatory flare-ups, but also as an inflammatory marker for risk stratification, diagnosis and prognosis of many inflammation-associated chronic diseases including neoplasia [5,8], metabolic syndrome [9,10], diabetes mellitus [11], as well as a wide range of cardiovascular diseases including hypertension [12], abdominal aortic aneurysm [13], congestive heart failure [14–16], and atherosclerosis-associated diseases including coronary artery disease and ischemic stroke [15–20]. However, neither the reported Hp plasma concentrations in healthy subjects [2,5,8,21–24], nor the determined predictive cut-off points and values of Hp levels [12–20,25], have been consistent in all trials and the results appear even sometimes contradictory. Furthermore, the reported pathological range of Hp concentrations falls mostly within its previously reported “normal” intervals. All these show that the clinical interpretation of Hp levels requires a well-partitioned,

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population-based reference values. The pre-analytical, analytical and biological sources of variation of plasma Hp concentration have been explored in previous published studies [5,21]. The relation between plasma level and phenotypes of Hp has also been studied previously [5,22,26,27].

Very recently, our team has identified, through a genome-wide association study (GWAS), a novel polymorphism, rs2000999, in the *HP* gene as a strong genetic determinant of the Hp level [28]. In the present study we originally evaluated the possible effects of 28 census selected variables that would most affect the plasma levels of Hp in a large general “apparently” healthy Caucasian population-based sample, a subset of the STANISLAS Family Study (SFS) [29]. We then established, for the first time, the reference ranges of the Hp in a subgroup of selected healthy subjects, the sample reference population, based on its main sources of biological variation including the novel *HP* polymorphism, rs2000999.

2. Materials and methods

2.1. Study population

2.1.1. General study cohort

The inclusion and exclusion criteria and detailed characteristics of the SFS have been described previously [29]. The SFS consists of a total of 4556 apparently healthy individuals of French origin. Of them, 3129 subjects, as the “general study cohort”, were enrolled in the primary analysis of the present study. Descriptive demographic and biological data of the general study cohort are provided in Table 1. All subjects gave informed consent to participate in the study and the study was approved by the local ethic committee. The possible effects of the following factors on the plasma levels of Hp in the general study cohort were evaluated: gender, age, body mass index (BMI), alcohol and cigarette consumption, plasma levels of triglyceride (TG), total cholesterol, high density lipoprotein (HDL)-cholesterol, apolipoprotein (Apo) A1 and B, glucose, albumin, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ glutamyltransferase (GGT), white blood count (WBC), total blood Hb, creatinine, C-reactive protein (CRP), alpha-1 glycoprotein and concomitant treatment with antihypertensive agents, oral hypoglycemics, lipid lowering agents, oral contraceptives, anti-inflammatory drugs and antibiotics.

2.1.1.1. Analytical methods. Blood count was determined using MAXIM analyser (Beckman Coulter, Paris, France). Serum glucose, albumin, bilirubin, AST, ALT, ALP, GGT, total cholesterol and TG were measured in fresh serum using established enzymatic methods on an AU5000 automated analyzer (Olympus Merck, Paris, France). CRP, alpha-1-glycoprotein, Hp, Apo A1 and Apo B were determined on a Behring automated nephelometer (Dade-Behring, Marburg, Germany) and serum HDL-cholesterol was measured on a Cobas-Mira analyzer (Roche Diagnostics, Meylan, France).

2.1.2. Sample reference population

Based on the guideline of the international federation of clinical chemistry and laboratory medicine (IFCC), the reference values of Hp was determined in a subset of the general study cohort, the “sample reference population”, consisting of 2923 selected healthy subjects, including 1421 men and 1502 women (29). The sample consisted of individuals aged 6–67 years who met all of the following criteria: Z-BMI <3%, cigarette consumption <20/day, alcohol consumption <88 g/d, plasma level of Hp <3.5 g/L, TG <10 mmol/L, total cholesterol <10 mmol/L, glucose <8.5 mmol/L, albumin <55 g/L, bilirubin <50 μ mol/L, AST <100 U/L, ALT <150 U/L, ALP <600 U/L, GGT <200 U/L, WBC <15 $\times 10^9$ /L, creatinine <150 μ mol/L, CRP <20 mg/L, alpha-1-glycoprotein <2 g/L and also systolic blood pressure <140 mm Hg and diastolic blood pressure <90 mm Hg. The Hp reference values were pro-

Table 1
Demographic and biological characteristics of the general study cohort.

	Fathers n = 769	Mothers n = 755	Sons n = 799	Daughters n = 805
Age (years)	41.90 (4.8)	39.79 (4.8)	13.82 (3.8)	13.93 (4.1)
BMI Z-score	0.12 (0.99)	0.17 (0.93)	0.20 (1.12)	0.30 (1.14)
BMI index (%)	101.7 (13.0)	103.7 (17.9)	103.7 (13.9)	104.7 (15.3)
Alcohol consumption (g/day)	23.3 (26.4)	4.4 (8.7)	0.2 (1.1)	0.3 (1.1)
Cigarette consumption (cigarette/day)	5.4 (9.4)	2.7 (6.9)	0.6 (2.9)	0.9 (3.5)
Triglyceride (mmol/L)	1.41 (0.88)	0.93 (0.50)	0.80 (0.42)	0.85 (0.41)
Total cholesterol (mmol/L)	6.01 (1.08)	5.50 (0.89)	4.59 (0.82)	4.88 (0.83)
HDL (mmol/L)	1.40 (0.35)	1.71 (0.42)	1.49 (0.38)	1.56 (0.35)
Apo B (g/L)	1.15 (0.27)	0.98 (0.22)	0.80 (0.19)	0.85 (0.19)
Apo A1 (g/L)	1.55 (0.23)	1.70 (0.26)	1.48 (0.22)	1.52 (0.27)
Glucose (mmol/L)	5.19 (0.55)	4.89 (0.71)	4.87 (0.33)	4.79 (0.38)
Albumin (g/L)	45.6 (1.9)	44.4 (2.0)	46.2 (1.9)	45.7 (2.1)
Bilirubin (μ mol/L)	12.7 (6.3)	11.0 (5.4)	12.8 (8.0)	10.6 (5.0)
AST (U/L)	25.0 (11.9)	18.3 (7.9)	25.6 (6.4)	21.8 (7.4)
ALT (U/L)	34.7 (20.3)	19.9 (14.9)	21.2 (17.0)	17.7 (8.2)
ALP (U/L)	69.0 (20.3)	56.4 (15.6)	224.1 (107.2)	175.0 (108.3)
GGT (U/L)	38.1 (37.4)	19.0 (16.3)	14.1 (6.6)	11.7 (3.8)
WBC (10^9 /L)	6.93 (1.91)	7.08 (1.87)	6.65 (1.56)	7.10 (1.81)
Hemoglobin (g/L)	152.8 (8.6)	135.3 (9.7)	143.4 (12.1)	135.8 (7.6)
Creatinine (μ mol/L)	91.3 (12.4)	74.5 (9.7)	70.4 (14.6)	65.8 (11.1)
Concomitant medications (%)				
Antihypertensive agents	3.1	1.6	0.0	0.0
Oral hypoglycemics	0.3	0.0	0.0	0.0
Lipid-lowering agents	6.4	1.9	0.6	0.4
Oral contraceptives	-	22.1	-	13.9
Anti-inflammatory	4.0	2.4	2.0	2.4
medications				
Antibiotics	1.3	1.7	3.0	4.5
CRP (mg/L)	1.90 (4.16)	2.10 (3.82)	1.03 (3.08)	1.18 (3.30)
Alpha-1-glycoprotein (g/L)	0.79 (0.19)	0.71 (0.18)	0.72 (0.21)	0.71 (0.22)
Haptoglobin (g/L)	1.08 (0.49)	1.07 (0.42)	0.66 (0.34)	0.84 (0.43)

Values are mean (SD) or frequency (%). ALT: aspartate aminotransferase; AST: alanine aminotransferase; ALP: alkaline phosphatase; Apo: apo-lipoprotein; CRP: C-reactive protein; GGT: γ -glutamyltransferase; HDL: high density lipoprotein-cholesterol; WBC: white blood cells.

2.2. HP genotyping and measurement of Hp levels in plasma

The rs2000999 genotyping and the measurement of plasma Hp concentrations were performed as previously described [28]. In our paper, “R” represents the “major” and “r” symbolizes the “minor” alleles.

2.3. Statistical analysis

Statistical analyses were performed by using the SAS software package version 9.2 (SAS Institute, Inc., Cary, NC). When the distribution of values for determinants exhibited a long-tailed positive skewness and kurtosis, \log_{10} transformation was used. In the 3 groups of age

and related biological analytes (drug use, tobacco or alcohol consumption and basic blood constituents as lipid and glucose concentrations, enzyme activities and blood cell counts...). Only statistically significant regression coefficients in at least one group of individuals were presented in Table 2. Because individuals within a family are not independent, multiple regressions were based on the estimating equation (EE) technique using the SAS GENMOD procedure with a repeated statement. For all analyses, statistical significance was taken at $p \leq 0.05$. Reference limits were calculated with non-parametric method.

3. Results

3.1. Allelic and genotypic frequencies of HP

The frequencies of the HP genotypes in the whole study cohort (Table 3) and in the selected reference sample population (Table 4) were in Hardy-Weinberg equilibrium ($p < 0.001$). The genotype distribution of the HP gene polymorphisms between the general and the reference sample populations did not differ significantly ($p = 0.98$). In addition, the frequency of the *r* allele in the general population (Table 3), the selected sample reference group and also in both males and females subgroups of these populations was the same (Minor Allele Frequency ~0.20). These relative allele frequencies were similar to those observed in the other Caucasian populations [30].

3.2. Sources of biological variation of Hp levels and other related blood constituents

The stepwise regression analysis on the whole study cohort revealed that age, BMI, cigarette consumption, total blood Hb and rs2000999 were the major sources of biological variation of the Hp plasma levels in this study cohort ($p < 0.05$). There were also statistically significant correlations between plasma Hp concentrations and levels of other blood constituents such as WBC and plasma inflammatory proteins (Table 2).

The plasma levels of Hp increased significantly with age in children (7–17 years old) of both genders, but not in adults (18–56 years old);

Table 2
Sources of biological variation of plasma Hp levels and related blood constituents in a multiple regression analysis.

	Fathers n = 769	Mothers n = 755	Sons n = 799	Daughters n = 805
Age (years)	0.004 (0.003)	0.003 (0.003)	0.019 (0.005)***	0.015 (0.004)***
BMI index (%)	-0.001 (0.001)	0.001 (0.001)	-0.001 (0.001)	-0.002 (0.001)*
Cigarette consumption (cig/day)	0.009 (0.002)***	0.006 (0.002)***	0.002 (0.004)	0.003 (0.003)
Apo A1 (g/L)	-0.007 (0.057)	0.009 (0.044)	-0.177 (0.050)***	-0.098 (0.048)
Apo B (g/L)	0.200 (0.052)***	0.083 (0.056)	-0.071 (0.057)	0.055 (0.056)
Glucose (mmol/L)	0.072 (0.024)**	-0.025 (0.016)	0.033 (0.032)	0.029 (0.028)
Albumin (g/L)	0.011 (0.006)	0.015 (0.006)*	0.028 (0.006)***	0.016 (0.005)**
Bilirubin (μmol/L)	-0.003 (0.002)	-0.004 (0.002)	-0.003 (0.001)*	-0.005 (0.002)**
AST (U/L) ^a	-0.018 (0.134)	-0.093 (0.119)	-0.232 (0.109)	-0.471 (0.117)***
ALT (U/L) ^a	-0.289 (0.113)*	0.008 (0.078)	-0.052 (0.094)	0.069 (0.084)
ALP (U/L) ^a	0.001 (0.001)	0.003 (0.001)**	0.000 (0.000)**	0.000 (0.000)**
GGT (U/L) ^a	0.165 (0.060)*	0.115 (0.060)	0.090 (0.097)	0.179 (0.093)
WBC (10 ⁹ /L)	0.042 (0.008)***	0.018 (0.007)**	0.000 (0.007)	0.028 (0.006)***
Triglyceride (mmol/L) ^a	0.021 (0.067)	-0.048 (0.074)	0.091 (0.061)	0.168 (0.062)**
CRP (mg/L) ^a	0.260 (0.032)***	0.183 (0.031)***	0.205 (0.030)***	0.255 (0.029)***
Alpha-1-glycoprotein (g/L)	0.583 (0.076)***	0.871 (0.080)***	0.656 (0.065)***	0.692 (0.062)***
Hemoglobin (g/L)	-0.006 (0.002)***	-0.006 (0.001)***	-0.004 (0.001)**	-0.005 (0.001)**
Effect of one rs2000999 allele	-0.309 (0.021)***	-0.234 (0.019)***	-0.188 (0.018)***	-0.236 (0.018)***
Concomitant medications (%)				
Analgesics	-0.091 (0.064)	0.106 (0.052)	0.217 (0.080)*	0.005 (0.058)
Antibiotics	0.051 (0.107)	0.060 (0.087)	-0.008 (0.060)	0.140 (0.049)*
Oral contraceptives	-	0.106 (0.035)**	-	-0.045 (0.045)

Values are regression coefficient ± SE; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

AST: alanine aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; Apo: apo-lipoprotein; CRP: C-reactive protein; GGT: γ -glutamyltransferase; WBC: white blood cells.

Table 3
Genotype distribution and minor allele frequency of HP in the general cohort study.

	RR	Rr	rr	r allele frequency (%)
General population (n = 3128)	1962 (62.7)	1,032 (33.0)	134 (4.3)	20.8
Males (fathers and sons) (n = 1568)	978 (62.4)	524 (33.4)	66 (4.2)	20.9
Females (mothers and daughters) (n = 1560)	984 (63.1)	508 (32.6)	68 (4.4)	20.6
Fathers (n = 769)	488 (63.5)	244 (31.4)	37 (4.8)	20.7
Mothers (n = 755)	469 (62.1)	251 (33.2)	35 (4.6)	21.2
Sons (n = 799)	490 (61.3)	280 (35.0)	29 (3.6)	21.1
Daughters (n = 805)	515 (64.0)	257 (32.0)	33 (4.1)	20.1

Values are n (%); "R" represents the major allele and "r" represents the minor allele, rs2000999.

therefore, we presented the Hp reference intervals with and without age stratification, in children and adults, respectively (Table 4).

The rs2000999, was the most powerful source of variation of Hp concentrations in plasma ($p \leq 0.001$ in all four subgroups) (Table 2). The negative effect of having one *r* allele on the plasma concentration of Hp was more noticeable in father (regression coefficient: -0.309 ± 0.021) and less in sons (regression coefficient: -0.188 ± 0.018). The regression coefficient for mothers and daughters was -0.234 ± 0.019 and -0.236 ± 0.018 , respectively.

3.3. Reference values for Hp concentrations in plasma

In the general population, the plasma level of Hp ranged from 0.08 to 3.83 g/L. Figs. 1 and 2, respectively, show the percentiles of Hp concentrations and the distribution of values of plasma Hp levels in the whole study cohort.

The established gender-, age- and genotype-specific reference ranges for Hp levels in the sample reference population are summarized in Table 4. Of a total of 2,923 subjects of the sample reference population, 1,834 (62.7%) subjects were RR genotype carriers, 967

Table 4
Reference values for plasma Hp level by genotype, age and gender in the selected sample reference population.

Age (yr)	Male (n = 1421)							Females (n = 1502)						
	n	Mean (SD)	2.5	5	50	95	97.5	n	Mean (SD)	2.5	5	50	95	97.5
<i>HP RR genotype (n = 1834)</i>														
7–13	242	0.59 (0.32)	0.10	0.16	0.52	1.21	1.41	253	0.77 (0.38)	0.20	0.31	0.70	1.46	1.69
14–17	150	0.84 (0.41)	0.17	0.27	0.83	1.48	1.66	152	1.05 (0.41)	0.41	0.48	0.99	1.86	1.99
18–56	498	1.11 (0.43)	0.40	0.48	1.08	1.86	1.97	539	1.14 (0.40)	0.50	0.59	1.09	1.86	2.19
<i>HP Rr genotype (n = 967)</i>														
7–13	133	0.45 (0.24)	0.11	0.13	0.39	0.86	0.96	119	0.53 (0.27)	0.18	0.22	0.47	1.01	1.41
14–17	83	0.59 (0.29)	0.08	0.18	0.57	1.18	1.25	75	0.75 (0.43)	0.15	0.29	0.65	1.59	1.71
18–56	258	0.83 (0.41)	0.28	0.34	0.77	1.61	1.75	299	0.94 (0.37)	0.38	0.41	0.90	1.72	1.88
<i>HP rr genotype (n = 122)</i>														
7–13	12	0.23 (0.14)	–	0.08	0.19	0.54	–	15	0.31 (0.26)	–	0.08	0.22	0.89	–
14–17	11	0.49 (0.38)	–	0.08	0.36	1.48	–	8	0.60 (0.24)	–	0.38	0.49	0.96	–
18–56	34	0.48 (0.27)	–	0.16	0.40	0.80	–	42	0.68 (0.32)	–	0.29	0.65	1.40	–

Values are g/L (SD); "R" represents the major allele and "r" represents the minor allele, rs2000999.

(30.0%) individuals carried Rr genotype and the frequency of rr genotype was 4.17% (122 subjects).

Regardless of HP genotypes, the overall plasma concentrations of Hp ranged between 0.08 g/L and 2.19 g/L in our reference population. The plasma reference values of Hp ranged 0.08–1.97 g/L in males and 0.08–2.19 g/L in females. Moreover, in each age group and regardless of HP genotypes, males exhibited lower plasma levels of Hp than those of females. Furthermore, there was a trend of increase in the concentration of Hp in plasma, in both genders and either in all three genotype groups, by the increase of age.

In both genders and also in all age groups, the plasma concentration of Hp was associated, negatively, with the presence and number of the r allele; the highest and lowest Hp levels in each age group of both males and females were, respectively, observed in the RR and the rr genotypes carriers (Table 4).

4. Discussion

In the present study, we established, for the first time, the reference values of Hp concentrations in plasma based on its components of biological variation, including the major genetic determinant of plasma Hp levels, in a large family-based population of French origin. The main findings of this study are as follow: Age, smoking, total blood Hb and HP polymorphisms rs2000999, were found to be the strongest biological predictors of Hp concentrations in human plasma. There were also significant correlations between plasma level of Hp and WBC count and a number of blood constituents including plasma proteins mostly CRP and alpha-1-glycoprotein.

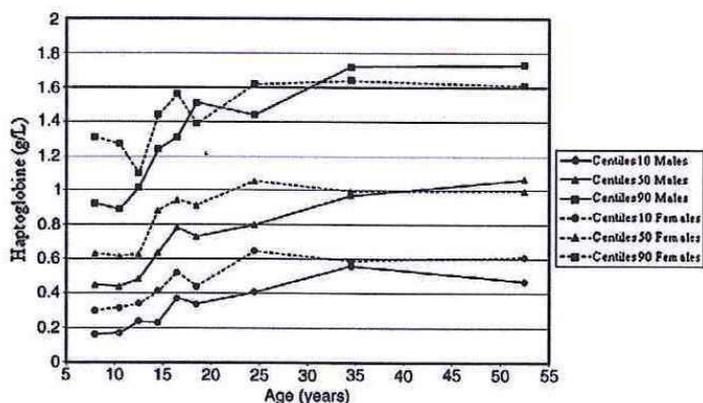


Fig. 1. Percentiles of Haptoglobin concentrations by age and gender in individuals before

4.1. Biological variations and related blood constituents

4.1.1. Age and smoking

In our study we observed that plasma Hp levels increase by age and that the values are lower in children than in adults. This finding has been reported in previous studies [5,6]. This is likely attributable to several mechanisms. First, it has been shown that plasma Hp concentrations are extremely sensitive to the release of Hb content into the circulation [23]. Releasing smaller amount of heme iron, as the result of insignificant hemolysis, can more commonly occur in the young subsequent to minor traumas, impact sports such as jogging and boxing and even playing a number of musical instruments [31]. Our further finding in this study of a significant negative correlation between bilirubin and Hp concentrations in children, but not in adults, corroborates this hypothesis. After CD163-mediated internalization of Hp-free Hb complex by macrophages, Hb-derived heme is converted into bilirubin and secreted into the plasma [2]. Therefore, it is natural to speculate that slight increases in plasma free Hb due to trivial physical hemolysis or concealed bruising, which is more frequent in juveniles, manifest as insignificant lower Hp levels and higher bilirubin concentrations. Secondly, Lavoie et al. recently demonstrated that physical activity energy expenditure (PAEE) is an independent predictor of Hp levels in plasma. The results of this study revealed that increased PAEE is associated with significant reduction in plasma Hp concentrations in healthy subjects [32]. Children generally have higher PAEE and consequently lower Hp levels than adults. Lastly, it has been shown that tobacco smoking induces overexpression of IL-6 in adipose tissues [33]. This effect is particularly more pronounced in parents, probably because of higher levels of smoking exposure, than in offspring [33]. This could also largely explain the significant and positive correlations observed in our reference population between cigarette consumption and plasma levels of Hp in parents and children, respectively.

4.1.2. Total blood Hb, WBC

In agreement with a previous study by Maes et al. which showed that healthy individuals who have higher setpoints of plasma Hp, also exhibit higher number of peripheral blood leukocytes (21), we found in the present study a strong positive relationship between plasma WBC and plasma Hp levels. It is logic to presume that IL-6 is also produced, further than adipose tissues, by WBCs. In a recent study, Leng et al. identified positive *in vivo* associations of total WBC with circulating IL-6 concentrations [34].

As mentioned earlier, Hp levels in plasma are exceptionally sensitive to free Hb concentrations [24]. Therefore, it is not surprising to observe

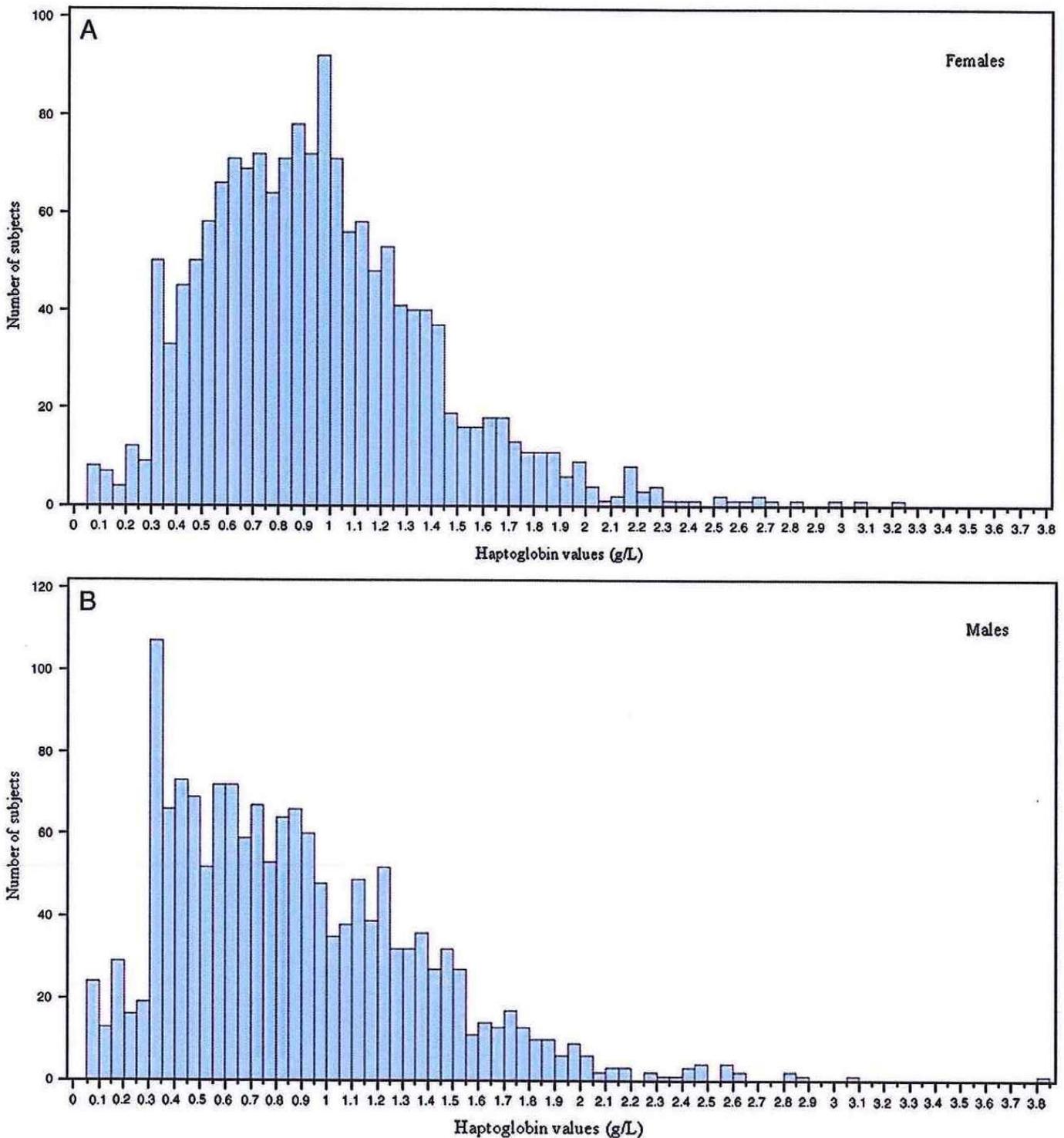


Fig. 2. Distribution of plasma Haptoglobin concentration in the general study cohort in males and females.

correlations between plasma Hp levels and total blood Hb concentrations in serum.

4.1.3. Genetic variation

Partitioning reference values on the basis of genetic differences could reduce inter-individual variation and improve the quality of laboratory data in clinical diagnosis. However, genetic-based reference value partitioning would be clinically practical only if a genetic polymorphism has an attributable effect of 4% or greater on the total variance of measured analyte [35].

in a total of 5022 individuals including a subsample of the SFS [28]. This genetic variant is located within the *HP* gene at the position 70,665,594 (relative to Human Genome NCBI Build 36) where an adenine (A) is substituted for the original guanine (G) (G>A) [30]. In the present study we demonstrated that plasma Hp levels, in both genders and all age groups, were negatively associated with the presence and number of the r allele. In our reference sample population, the subjects with the RR and rr genotypes had the highest and lowest values of plasmatic levels of the Hp, respectively. Indeed, the median Hp plasma levels were up to 3.9 folds higher in individuals with RR genotypes than in those with

This polymorphism would also be used for setting specific decision limits of Hp. Further studies for identifying genetic-based Hp decision limits are warranted.

4.1.3.1. rs2000999 and Hp phenotypes. Plasma Hp levels vary among three phenotypes being higher in individuals with phenotypes Hp 1–1 and Hp 2–1 than in those with Hp 2–2 [5,22,26,27]. According to the original report by Langlois and Delanghe [5], the mean concentration value of Hp in plasma of subjects with Hp 2–2 genotype is about 25% and up to 50% lower than that of individuals with Hp 2–1 and Hp 1–1, respectively. In addition to the three mentioned phenotypes, a number of further phenotypes with significant effects on plasma level of Hp have been reported in the blacks. The Hp 2–1 Modified (Hp 2–1 M) phenotype, for instance, which has been reported originally in African-Americans is associated with marked decrease in the Hp plasma levels [27]. It is, therefore, not-surprising to observe lower plasma concentrations of Hp in Africans. Starch or polyacrylamide gel electrophoresis, the gold standard method for determining Hp phenotype [8], is an operator-dependent, time-consuming and thereby sometimes unreliable technique. In addition, in this method, the Hp concentration in plasma should be at least 0.10 mg/ml which is considered another disadvantage of this method [8]. Moreover, different phenotypes of Hp may be composed of the same subunits but different copy numbers. This brings about the intra-phenotype differences in the molecular mass and renders electrophoretic determination of Hp phenotype difficult in some cases [36]. Therefore, the electrophoresis-based phenotype analysis of Hp seems to be not recommendable for partitioning reference values of Hp in daily medical practice. On the other hand, a PCR-based assay of *HP* polymorphism at the α chain loci has been described previously [37]. Recognition of *Hp2* alleles is relatively straightforward by this method; however, due to difficulties in producing a 1.7 kb fragment which is needed for diagnosis of *Hp1* allele, the detection of this allele has been found to be technically problematic and presently is judged to be less accurate when compared to the gel standard method [8].

The reported Hp plasma levels across phenotypic subgroups (Hp 1–1: 0.57–2.27 g/L vs. Hp 2–1: 0.44–1.83 g/L vs. Hp 2–2: 0.38–1.50 g/L) [5], are interestingly comparable with our findings in three genotypic subgroups (RR: 0.59–2.19 g/L vs. Rr: 0.45–1.88 g/L vs. rr: 0.23–1.40 g/L). This raises the hypothesis that there may be a relationship between rs2000999 and Hp phenotypes. Despite strong efforts we failed to evaluate this hypothesis in our study, which is acknowledged as a shortcoming of our work. Further studies are needed to elucidate the potential relation between the Hp polymorphism, rs2000999, and the Hp phenotypes.

4.1.4. Gender

Gender, as demonstrated previously by Langlois and Delanghe [5], could influence Hp concentrations in human plasma. In our study, females presented, even not significantly, higher plasma levels of Hp than males. This finding might be explained by the fact that IL6, the major cytokine regulating the hepatic production of Hp, is mainly produced by adipose tissue [38], that is, generally, greater in females than in males [39]. This finding also presumably attributable to the higher rate of strenuous physical activity in males, compared with females [40], which is associated with slightly higher amount of non-significant hemolysis and consequently lowers levels of plasma Hp concentrations in men.

4.1.5. Correlation with other plasma constituents

Our results also provide strong evidence that there are significantly positive correlations between other positive APPs, CRP and alpha-1-glycoprotein, and Hp levels in plasma. It has been well documented that IL-6 is the chief stimulator of the production of most APPs [41]. It therefore seems likely and makes sense that these proteins are coordinately upregulated under both normal physiologic and inflammatory

plasmatic levels of Hp, CRP and alpha-1-glycoprotein are not affected by administration of analgesic medications including over-the-counter preparations [42]. In accordance with this study, we found no strong correlation between taking painkillers and Hp levels in the present study.

In this study, in certain subgroups of population, plasma Hp levels were noted to have significantly negative correlation with plasma concentrations of Apo A1 and significantly positive correlation with Apo B plasmatic levels. These findings as well as our further observations that plasma Hp levels correlate positively with blood glucose and TG concentrations, although in a subset of the reference population, are in accordance with the results of Faraj and associates [43]; they found that Hp levels in plasma have significantly positive correlations with plasma Apo B, glucose and TG concentrations and also correlate negatively with plasma Apo A1 levels. Of interest, they also showed that plasma Apo B is a strong independent predictor of plasma IL-6 levels and that, individuals with hyperapobetalipoproteinemia (hyperapo B) have higher both IL-6 and Hp levels compared with normoapo B subjects. In addition, they demonstrated that reduction in plasma Apo B levels associated with significantly decrease in Hp concentrations. The negative association of Apo A1 with Hp might be attributable to their key roles in maintenance of a process referred to as reverse cholesterol transport (RCT), in which unesterified cholesterol in peripheral tissues is effluxed to HDL and is transported to the liver for excretion in the bile. In RCT, Apo A1, the major protein component of HDL, activates per se a critical enzyme, lecithin cholesterol acyl-transferase (LCAT). It has been shown by Salvatore et al. that Hp binds to Apo A1 to protect it from free radical-mediated damage [44]. That binding of Hp to Apo A1 has been demonstrated, by Spagnuolo and associates, leads to decrease in the amount of free Apo A1 for enzyme stimulation [45]. Therefore, it would be logical to expect that increase in Apo A1 levels is associated with decrease in Hp plasmatic concentrations.

In the present study, there was also a statistically significant positive correlation between plasma ALP and Hp levels in the major part of the reference population. The plausible mechanism might be that ALP expression in endothelial cells induced by IL-6 [46]. It therefore appears reasonable to anticipate that ALP concentrations increase in line with Hp levels in healthy subjects.

5. Conclusion and perspectives

To be reliably interpretable in daily medical practice, well-established reference intervals of Hp based on a large sample size as well as all sources of biological variation including the traditional as well as the novel genetic polymorphisms, rs2000999, is crucial. Due to technical problems, we failed to determine the previously well-known *HP* genotypes (Hp 1–1, Hp 1–2 and Hp 2–2) in our study population and, as a result, we did not proceed to assess the joint effects of the traditional and novel *HP* polymorphisms on its plasma levels. Our findings, however, provide a fundamental background for further studies to evaluate this likely relationship. The new *HP* polymorphism, and the potentially new-traditional polymorphisms relationship, may also be helpful for setting decision limits of Hp for the early detection of low- and high-grade inflammatory states and also for the determination of therapeutic reference range in patients with inflammation-associated disorders.

6. Author contribution (alphabetic)

- 6.1 Bernard Herbeth: data analysis and interpretation
- 6.2 Gérard Siest: study design, study supervision, Haptoglobin measurement, data interpretation
- 6.3 Payman Shahabi: study design, literature review, paper writing
- 6.4 Ndeye Coumba Ndiaye: haptoglobin genotyping, data interpretation

Conflict of interest

The authors declare that there is no conflict of interest.

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II. Influence of Genetic Variations on Inflammation-Reducing Effects of Epoxyeicosatrienoic Acids and Clopidogrel in Healthy Subjects; Results of a Preliminary Study

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Influence des variations génétiques sur les effets réducteurs d'inflammation des acides époxy-eicosatriénoïques et du clopidogrel chez des sujets sains ; résultats d'une étude préliminaire

Notre objectif consistait à évaluer l'association entre les polymorphismes les plus courants des époxygénases du cytochrome P450 (CYP) sur les taux plasmatiques de marqueurs inflammatoires dans une population de sujets sains. Nous avons également cherché à déterminer si le polymorphisme *CYP2C19*2* est associé à la réponse anti-inflammatoire au clopidogrel. Dans une population de 49 jeunes sujets sains de sexe masculin, les taux plasmatiques initiaux de marqueurs inflammatoires, dont la protéine C réactive, l'haptoglobine, l'acide orosomucoïde et le CD-40, ont été comparés chez les porteurs vs. non-porteurs des polymorphismes les plus fréquents des époxygénases du CYP : *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C8*2*, *CYP2J2*7*. On a comparé aussi la variation des marqueurs inflammatoires de l'état initial à 7 jours après administration de 75 mg par jour de clopidogrel chez les porteurs vs. non-porteurs de l'allèle *CYP2C19** et également chez les répondeurs vs. hypo-répondeurs au clopidogrel, déterminés par les tests de réactivité plaquettaire. Il n'a été trouvé aucune association significative entre les polymorphismes des époxygénases et les taux initiaux de marqueurs inflammatoires. De même, l'allèle *CYP2C19** n'était pas associé à la réponse anti-inflammatoire au clopidogrel. Nos constatations n'ont pas étayé l'idée que les variations génétiques des époxygénases du CYP soient associées au taux de marqueurs inflammatoires. En outre, nos résultats n'ont pas non plus corroboré l'hypothèse selon laquelle le polymorphisme *CYP2C19*2* serait associé à la variabilité de la réponse aux propriétés anti-inflammatoires du clopidogrel.

Mots clés : Inflammation, cytochrome P450, polymorphisme, acides époxy-eicosatriénoïques, clopidogrel, protéine C réactive, haptoglobine

*Brief Report***Influence of Genetic Variations on Levels of Inflammatory Markers of Healthy Subjects at Baseline and One Week after Clopidogrel Therapy; Results of a Preliminary Study****Payman Shahabi**¹, **Gérard Siest**¹, **Bernard Herbeth**¹, **Daniel Lambert**¹, **Christine Masson**¹, **Jean-Sébastien Hulot**², **Sébastien Bertil**³, **Pascale Gaussem**^{3,4,5,†} and **Sophie Visvikis-Siest**^{1,†,*}¹ UMR INSERM U 1122, IGE-PCV, Faculté de Pharmacie, Université de Lorraine, 30 Rue Lionnois, Nancy 54000, France; E-Mails: payman.shahabi@inserm.fr (P.S.);

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Abstract: We aimed to assess the association between the most common polymorphisms of cytochrome P450 (CYP) epoxygenases on the plasma levels of inflammatory markers in a population of healthy subjects. We also sought to determine whether *CYP2C19**2 polymorphism is associated with the anti-inflammatory response to clopidogrel. In a population of 49 healthy young males, the baseline plasma levels of inflammatory markers including C-reactive protein, haptoglobin, orosomucoid acid, CD-40 were compared in carriers vs. non-carriers of the most frequent CYP epoxygenase polymorphisms: *CYP2C9**2, *CYP2C9**3, *CYP2C19**2, *CYP2C8**2 and *CYP2J2**7. Also, the variation of inflammatory markers from baseline to 7 days after administration of 75 mg per day of clopidogrel were compared in carriers vs. non-carriers of *CYP2C19** allele and also in

responders vs. hypo-responders to clopidogrel, determined by platelet reactivity tests. There was no significant association between epoxygenase polymorphisms and the baseline levels of inflammatory markers. Likewise, *CYP2C19** allele was not associated with anti-inflammatory response to clopidogrel. Our findings did not support the notion that the genetic variations of CYP epoxygenases are associated with the level of inflammatory markers. Moreover, our results did not support the hypothesis that *CYP2C19*2* polymorphism is associated with the variability in response to the anti-inflammatory properties of clopidogrel.

Keywords: inflammation; cytochrome P450; polymorphism; epoxyeicosatrienoic acids; clopidogrel; C-reactive protein; haptoglobin

1. Introduction

Our knowledge of the contribution of inflammation to the pathophysiology of cardiovascular disorders has progressed to the point that, currently, coronary artery disease (CAD) and arterial hypertension (HTN) are considered to be diseases of inflammation characterized by interaction among platelets, leukocytes and endothelial cells [1–3].

Cytochrome P450 (CYP) enzymes play an indispensable role in the regulation of inflammation through the biosynthesis of endobiotics that modulate inflammation, such as arachidonic acid (AA) derivatives epoxyeicosatrienoic acids (EETs). Indeed, the main human CYP epoxygenases, *i.e.*, CYP2C8, CYP2C9, CYP2C19 and CYP2J2, catalyse the epoxidation of AA to EETs that have been shown to display potent anti-inflammatory properties [4,5]. The genes encoding the epoxygenases are polymorphic; therefore, it is logical to hypothesize that the CYP epoxygenase loss-of-function polymorphisms are associated with a decrease in the relative abundance and also the inflammation-reducing properties of EETs. The first part of the present study was designed to analyze plasma levels of a selected number of inflammatory markers as a function of the most frequent epoxygenase gene polymorphisms, in a population of young healthy male volunteers.

CYPs also play central roles in the metabolism and disposition of a wide range of drugs and xenobiotics with potential anti-inflammatory effects such as clopidogrel [6]. Clopidogrel is the thienopyridine of choice for dual antiplatelet therapy in patients with acute coronary syndrome (ACS) and those treated with percutaneous coronary intervention (PCI) [7]. Clopidogrel is a pro-drug that is required to be converted to its active metabolite through a two-step oxidative process. Both steps involve several hepatic CYP isoenzymes, including CYP2C19, CYP3A4/5, CYP2C9, CYP2C8 and CYP2B6 [8]. The ability of clopidogrel to inhibit platelet-mediated thrombogenicity is partly influenced by the polymorphisms of the clopidogrel-CYP-metabolizing enzymes and also the P2Y₁₂ receptor gene polymorphism [9,10]. We originally reported that the *CYP2C19*2* loss-of-function allele is involved in a reduced response to clopidogrel in healthy subjects [11]. In our small population of healthy volunteers, neither the polymorphism of CYP3A4 gene (IVS10 + 12G > A), nor the P2Y₁₂ receptor gene polymorphism (*H2* allele) were associated with the platelet response to clopidogrel, although there was a slight trend for *H2H2* homozygous subjects to being less responders to

clopidogrel [12,13]. Being primarily a platelet aggregation inhibitor, clopidogrel has also been shown to exhibit pleiotropic anti-inflammatory properties [6]. However, the inflammation-reducing effects of clopidogrel have not been a persistent finding in all trials and the results of different studies in this field are sometimes contradictory [14–19]. We hypothesized that the gene variations associated with a poor responsiveness to antiplatelet effects of clopidogrel could also contribute to the variability in response to its potential anti-inflammatory properties. Accordingly, the second aim of this study was to investigate whether the anti-inflammatory effect of standard-dose clopidogrel is affected by the functional variants of CYP2C19 and P2Y12 genes (*CYP2C19*2* and *H2* alleles) in healthy subjects taking clopidogrel.

2. Results and Discussion

Demographic and baseline biochemical characteristics of subjects are presented in Table 1. In order to minimize the influence of female hormones and to be free from the inflammatory response that is known to occur during CAD and the interventional procedures and also to eliminate the likely anti-inflammatory effects of other medications, this study included only healthy male volunteers.

Table 1. Patients' characteristics.

Variables	General population (<i>n</i> = 49)
Demographic characteristics	
Age (year) †	25.8 ± 3.8
Male sex, (%)	49 (100)
Body mass index (kg/m ²) †	23.2 ± 2.11
Vital signs	
Pulse rate (n) †	66.9 ± 9.4
Systolic blood pressure (mmHg) †	121.7 ± 8.4
Diastolic blood pressure (mmHg) †	68.6 ± 6.8
Hemogram	
White blood cells (×10 ³ /μL) †	5.41 ± 1.13
Red blood cells (×10 ⁶ /μL) †	4.72 ± 0.25
Hemoglobin (g/L) †	14.21 ± 0.55
Hematocrit (%) †	41.66 ± 1.60
Platelet (×10 ³ /μL) †	220.72 ± 37.32
Biochemistry	
Glucose (mmol/L) †	4.76 ± 0.38
Aspartate aminotransferase (U/L) †	20.00 ± 5.97
Alanine aminotransferase (U/L) †	25.93 ± 10.19
Alkaline phosphatase (U/L) †	55.37 ± 11.39
Gamma-glutamyltransferase (U/L) †	20.38 ± 8.23
Creatinine (μmol/L) †	83.56 ± 11.05
Fibrinogen (g/L) †	2.28 ± 0.35

† Mean ± SD; SD: standard deviation.

Table 2 presents the plasma concentrations of the inflammatory markers in carriers and non-carriers of the AA-metabolizing CYP polymorphisms. There was no significant association between plasma biomarker levels and *CYP2C19*2*, *CYP2J2*7*, *CYP2C8*3*, *CYP2C9*2* and *CYP2C9*3* polymorphisms in the healthy population analyzed.

Table 2. Baseline inflammatory marker levels ¹ in function of cytochrome P450 (CYP) epoxygenase polymorphisms.

Allele	Orosomuroid (g/L)	Haptoglobin (g/L)	CRP (mg/L)	CD40 (ng/L)
<i>CYP2C19*2</i> (681 G > A); <i>rs4244285</i>				
<i>CYP2C19*1/*1</i> (n = 30)	0.58 (0.10)	0.74 (0.28)	0.32 (0.16–0.65)	382 (153)
<i>CYP2C19*1/*2</i> (n = 19)	0.62 (0.13)	0.85 (0.31)	0.31 (0.14–0.67)	323 (66)
<i>CYP2J2*7</i> (–50 G > A); <i>rs890293</i>				
<i>CYP2J2*1/*1</i> (n = 42)	0.60 (0.12)	0.79 (0.31)	0.38 (0.32–0.44)	359 (132)
<i>CYP2J2*1/*7</i> (n = 7)	0.57 (0.09)	0.78 (0.34)	0.32 (0.15–0.69)	351 (96)
<i>CYP2C8*3</i> (1196 A > G); <i>rs10509681</i>				
<i>CYP2C8*1/*1</i> (n = 36)	0.60 (0.12)	0.82 (0.31)	0.33 (0.15–0.72)	328 (63)
<i>CYP2C8*1/*3</i> (n = 13)	0.58 (0.10)	0.76 (0.27)	0.27 (0.17–0.43)	426 (190)
<i>CYP2C9*2</i> (430 C > T); <i>rs1799853</i>				
<i>CYP2C9*1/*1</i> (n = 37)	0.60 (0.12)	0.80 (0.32)	0.34 (0.16–0.76)	341 (97)
<i>CYP2C9*1/*2</i> (n = 12)	0.59 (0.10)	0.84 (0.26)	0.26 (0.16–0.41)	424 (191)
<i>CYP2C9*3</i> (1075 A > C); <i>rs1057910</i>				
<i>CYP2C9*1/*1</i> (n = 42)	0.60 (0.12)	0.80 (0.30)	0.30 (0.15–0.57)	361 (133)
<i>CYP2C9*1/*3</i> (n = 7)	0.62 (0.06)	0.88 (0.37)	0.73 (0.21–2.48)	366 (6)

¹ Arithmetic mean (SD) or Geometric mean (range of 1 SD).

Our findings did not provide support to the hypothesis that, under physiological conditions, the CYP epoxygenase loss-of-function polymorphisms are associated with an inter-individual variability in the plasma levels of systemic inflammatory markers. However, it should be considered that the examination of this hypothesis in a population of young healthy individuals with the already low levels of circulating inflammatory markers might not be straightforward. Also, we acknowledge that our study was performed on a small population size and a lack of power cannot be excluded. Accordingly, further larger well-designed studies are necessary to better assess the potential associations between the most common epoxygenase polymorphisms and the inflammatory markers in healthy individuals. Furthermore, due to small sample size, we failed to investigate if the co-existence of multiple epoxygenase defective alleles is associated with the levels of markers of inflammation in healthy subjects; this theory also requires to be tested by the future studies.

Our observations in this study are, somehow, consistent with those of our previous study in which, we investigated whether *CYP2C19*2* polymorphism is associated with inflammatory markers of a healthy population, consisting of 178 men and 181 women. In that study we found that the plasma

level of hs-CRP is significantly higher in healthy females carriers of *CYP2C19*2* allele, but not in males [20]. In the present study, we observed that not only *CYP2C19*2*, but also other CYP epoxygenase polymorphisms are not associated with levels of systemic inflammatory markers of healthy males.

Additionally, our findings are also consistent with those of Hoffmann *et al.* [21], who failed to show statistically significant relationship between *CYP2J2*7* polymorphism and the levels of hs-CRP, fibrinogen and leukocyte count in a large number of patients with CAD. Likewise, the results of a large population-based prospective study by Kaur-Knudsen *et al.* [22], showed that there is not a significant association between *CYP2C9*2* and *CYP2C9*3* polymorphisms and the level of CRP.

In regard to the variation of inflammatory marker levels in response to clopidogrel, in general and regardless of the genetic polymorphisms, clopidogrel administration was not associated with changes in the inflammatory marker levels (Table 3). In addition, no significant association was found between the changes of inflammatory markers and either *CYP2C19*2*, or *P2Y12 H2* Polymorphisms (Table 3). Also, the other studied CYP polymorphisms, including *CYP2C8*3*, *CYP2C9*2* and *CYP2C9*3*, were not associated with the difference of the levels of markers from baseline to 7 days after taking clopidogrel (data not presented). Likewise, as presented in Table 4, no significant difference in the variation of plasma biomarkers was observed between responders vs. hypo-responders to clopidogrel, at baseline and one week after clopidogrel intake.

Clopidogrel is currently the most widely prescribed platelet P2Y12 receptor inhibitor [23]. However, there is a significant inter-individual variability in the response to the antiplatelet effect of clopidogrel, with up to 40% of patients being classified as poor responders to this drug [24–27]. This variability is partly attributed to the loss-of-function *CYP2C19*2* and, in some studies and to lesser extent, to the *P2Y12 H2* polymorphisms. Since, in addition to thrombus formation, platelet has a central role to the initiation and maintenance of inflammation [28], it not surprising to observe that clopidogrel has also pleiotropic anti-inflammatory properties [6]. The inflammation-reducing effects of clopidogrel could be clinically beneficial since on-clopidogrel patients with higher levels of inflammatory markers have been demonstrated to have an increased incidence of adverse cardiovascular events [29]. However, controversy has surrounded the anti-inflammatory of clopidogrel [14–19]. Since the genetic variations of applicable genes (especially *CYP2C19* and *P2Y12*) have been shown to be the main culprit in the variability in the responsiveness to the clopidogrel, it is logical to speculate that the same relevant polymorphisms could lead to an inter-individual variability in response to anti-inflammatory effects of clopidogrel.

Table 3. Variation of inflammatory marker levels¹ in response to clopidogrel in the whole population and in function of *CYP2C19**2 and *P2Y12 H2* Polymorphisms.

	Orosomucoïd (g/L)		Haptoglobine (g/L)		CRP (mg/L)		CD40 (ng/L)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
Whole population (n = 49)	0.61 (0.12)	0.60 (0.19)	0.78 (0.29)	0.79 (0.33)	0.32 (0.14–0.82)	0.29 (0.11–0.65)	358 (126)	360 (115)
<i>CYP2C19</i>*2 (681 G > A); rs4244285								
<i>CYP2C19</i> *1/*1 (n = 30)	0.58 (0.10)	0.60 (0.12)	0.74 (0.28)	0.76 (0.33)	0.32 (0.16–0.65)	0.31 (0.17–0.57)	382 (153)	382 (133)
<i>CYP2C19</i> *1/*2 (n = 19)	0.62 (0.13)	0.59 (0.19)	0.85 (0.31)	0.78 (0.33)	0.31 (0.14–0.67)	0.28 (0.14–0.57)	323 (66)	331 (78)
<i>P2Y12 H2</i>								
<i>P2Y12 H1/H1</i> (n = 33)	0.61 (0.12)	0.62 (0.15)	0.78 (0.31)	0.79 (0.35)	0.30 (0.16–0.57)	0.32 (0.16–0.65)	376 (150)	378 (131)
<i>P2Y12 H1/H2</i> & <i>H2/H2</i> (n = 16)	0.58 (0.08)	0.54 (0.12)	0.80 (0.26)	0.70 (0.26)	0.34 (0.14–0.82)	0.24 (0.15–0.40) ²	323 (49)	327 (68)

¹ Arithmetic mean (SD) or Geometric mean (range of 1 SD); ² Significant interaction between 7-day changes in CRP concentration and *P2Y12* (p = 0.038).

Table 4. Variation of inflammatory marker levels¹ in response to clopidogrel in function of platelet activity.

Platelet aggregation	Orosomucoïd (g/L)		Haptoglobine (g/L)		CRP (mg/L)		CD40 (ng/L)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
Responders (n = 32)	0.58 (0.10)	0.60 (0.12)	0.74 (0.28)	0.76 (0.33)	0.32 (0.16–0.65)	0.31 (0.17–0.57)	360 (135)	379 (132)
Hypo-responders (n = 17)	0.65 (0.12)	0.64 (0.19)	0.85 (0.31)	0.78 (0.33)	0.31 (0.14–0.67)	0.28 (0.14–0.57)	366 (132)	341 (78)

¹ Arithmetic mean (SD) or Geometric mean (range of 1 SD).

In general, our results did not provide evidence that clopidogrel exhibits anti-inflammatory effects in healthy male individuals. Yet, it should be considered that since the recruited individuals in our study have been all from young and healthy people, the concentrations of inflammatory markers in their plasma, even at baseline and before taking clopidogrel, were relatively low; the interpretation of results in this situation might be inconclusive. Our findings in the current study are inconsistent with those of Klinkhardt *et al.* [30], who showed that clopidogrel could attenuate significantly inflammation in 10 healthy subjects. Possible sources of discrepancy would be small sample sizes of the both studies and the differences in the determined inflammatory markers and study settings. Therefore, further well-conducted investigations with enough sample size are warranted to assess the pleiotropic anti-inflammatory effects of clopidogrel. Additionally, our current findings did not support the theory that either the CYP2C19*2 or the P2Y12 H2 alleles influence the anti-inflammatory potential of clopidogrel in healthy subjects. Nevertheless, here again, the low baseline levels of plasma inflammatory markers in our young healthy population may make interpretation of the results indecisive and unreliable. Also, since in this study the subjects were not genotyped for the gain-of-function CYP2C19 polymorphism (CYP2C19*17), it should be acknowledged that those who have been categorized under CYP2C19*1/*1 genotype group (also known as extensive metabolizers of clopidogrel), might also include the carriers of CYP2C19*17 allele who are ultra-metabolizers of clopidogrel.

Also, certain clopidogrel-metabolizing CYPs, specifically CYP2C9 and CYP2C19 are also involved in the metabolic transformation of AA to EETs. Therefore, another hypothesis that may explain our findings is that clopidogrel might act as a competitive inhibitor of CYP2C9/CYP2C19 resulting in a decrease in the production and in the anti-inflammatory effects of EETs in on-clopidogrel patients. In addition, clopidogrel has been shown to be a moderate inhibitor of CYP2C8 [31], a main human EET-producing epoxygenase, resulting in a decrease in the EET formation and in the EET-induced inflammation reduction. Together, as a paradigm of a xenobiotic-endobiotic interaction, the likely clopidogrel-EET interaction needs further studies to elucidate its clinical implication.

Last but not least, it should be pointed out that the current study, which has been performed exclusively on healthy males, is the pilot study of larger research projects carried out on post-PCI and hypertensive patients, including both males and females. The results of these studies will be released in the near future.

3. Experimental Section

3.1. Subjects and Study Protocol

The study design and the inclusion criteria for the subjects were described in details elsewhere [13,32]. Briefly, forty nine healthy male Caucasians (aged 18–35 years) who met the following conditions were enrolled: non-smokers, normal physical and laboratory results, no history of use of medications as well as normal platelet count, normal maximal platelet aggregation in response to platelet agonists and normal PFA-100[®] assay. The subjects were given a standard dose of clopidogrel 75 mg once daily for 7 consecutive days in the presence of medical staff. For each subject,

the plasma concentrations of a selected number of inflammatory proteins including high-sensitivity C-reactive protein (hs-CRP), haptoglobin (Hp), orosomucoid acid (Oroso) and CD-40 were compared before and 7 days after clopidogrel administration. In addition, the responsiveness to the antiplatelet effect of clopidogrel (responders vs. hypo-responders), was compared, in each participant, at day 1 (baseline) and 7 of clopidogrel intake using the ADP-induced platelet aggregation as well as the phosphorylated vasodilator-stimulated phosphoprotein (VASP).

Additionally, at baseline, and before taking clopidogrel, the levels of inflammatory markers were compared across the population, in the function of the most frequent CYP epoxygenase gene polymorphisms in Caucasians: *CYP2C8**3 (rs10509681), *CYP2C9**2 (rs1799853), *CYP2C9**3 (rs1057910), *CYP2C19**2 (rs4244285) and *CYP2J2**7 (rs890293). Also, the changes in the levels of inflammatory markers, from baseline to end of clopidogrel therapy, were compared between the carriers vs. non-carriers of *CYP2C19**2 and *P2Y12 H2* alleles and also between responders vs. non-responders to clopidogrel.

The study was approved by the Committee for the Protection of Human Subjects in Biomedical Research, which functions for the Cochin University Hospital (Paris, France), and all the subjects gave their written informed consent to participate.

3.2. Genotyping and Protein Quantification

Genotyping was performed by LGC Genomics and KBioscience Laboratory using KASPTM genotyping assay. The primers used for genotyping are presented in Table 5.

Table 5. Genotyping data.

SNP (allele); db SNPAccession no.	Primers used for sequencing
CYP2C8	
*3 (1196 A > G); rs10509681	F: B-5'-GTGTTCTCCCAGTTTCTGCCC-3' R: 5'-GACGCAGAGTAGAGTCACCCAC-3'
CYP2C9	
*2 (430 C > T); rs1799853	F: 5'-GTATTTTGGCCTGAAACCCATA-3' R: B-5'-CACCTTGGTTTTTCTCAACTC-3'
*3 (1075 A > C); rs1057910	F: B-5'-TGCACGAGGTCCAGAGAT-3' R: 5'-GATACTATGAATTTGGGACTTC-3'
CYP2C19	
*2 (681 G > A); rs4244285	F: 5'-CAGAGCTTGGCATATTGTATC-3' R: 5'-GTAAACACACAAAACACTAGTCAATG-3'
CYP2J2	
*7(-50 G > A); rs890293	F: 5'-CATAGGAGAGACGGTGATTGAACC-3' R: 5'-GGCGTCTTCTCGTCCTCCTGCA-3'
P2Y12	
<i>H2</i> Haplotype	As described in reference [33]

The plasma levels of CRP, Hp and Oroso were immunonephelometrically determined, using BN II (Siemens Healthcare Diagnostics, Marburg, Germany); the intra-assay variation coefficient for CRP,

Hp and Oroso was 2.6%, 3.2% and 2.6%, respectively. Moreover, soluble CD-40 was measured using commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instruction.

3.3. Platelet Function Studies

Platelet aggregation studies and the determination of the level of phosphorylated VASP were performed as detailed elsewhere [13,33]. Clopidogrel hypo-responsiveness was defined as a maximal 10 μ M ADP-induced aggregation at 7 day above 50% and/or a VASP index above 60% after one week 75 mg/d clopidogrel administration [33].

3.4. Statistical and Data Analysis

ANOVA with repeated measures was used to test the 7-day changes of inflammatory markers, the average effect of polymorphisms and the polymorphisms/7-day changes interaction. Data were log transformed for CRP concentrations. Results are presented as arithmetic means (SD) or geometric means (range of 1 SD). Statistical analyses were performed using SAS 9.3 software (SAS Institute, Cary, Cary, NC, USA). Statistical significance was set at $p \leq 0.05$.

4. Conclusions

Our findings did not support the hypothesis that, under physiological conditions, the most common polymorphisms of the main human CYP epoxygenase polymorphisms are associated with altered plasma level of inflammatory proteins. Moreover, we did not find a significant association between either the *CYP2C19*2/P2Y12 H2* polymorphisms or the antiplatelet responsiveness to clopidogrel and the inflammation-reducing properties of clopidogrel in healthy male subjects.

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Conflict of interest

The authors declare no conflict of interest.

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III. Genetic-determined Low Response to Thienopyridines is Associated with Higher Systemic Inflammation in Smokers

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Genetic-determined Low Response to Thienopyridines is Associated with Higher Systemic Inflammation in Smokers

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Abstract

Objectives: This study sought to investigate whether the interactions of cytochrome P450 (CYP) 2C19 loss- and gain-of-function polymorphisms (*CYP2C19*2* and *CYP2C19*17*) with smoking are associated with the levels of P2Y12 receptor inhibition and C-reactive protein (CRP), in patients undergoing percutaneous coronary intervention (PCI) treated with aspirin and either clopidogrel or prasugrel.

Background: **2* and **17* alleles influence the response to the anti-platelet effects of both clopidogrel and prasugrel. Also, some, but not all, studies suggested that smoking positively modify response to clopidogrel, but not prasugrel. Moreover, post-procedural CRP level is a reliable predictor of post-PCI complications like in-stent restenosis.

Methods: In total, 1128 patients with acute coronary syndrome who underwent a successful PCI were recruited. At one-month clinical follow-up, the interactions of smoking and CYP2C19 polymorphisms on the levels of on-treatment platelet reactivity, assessed by vasodilator-stimulated phosphoprotein platelet reactivity index (VASP PRI) and CRP were explored in three metabolizing groups as follow: poor metabolizers (PM) (**2* carriers/**17* non-carriers); intermediate metabolizers (IM) (**2* carriers/**17* carriers or **2* non-carriers/**17* non-carriers); and ultrametabolizers (UM) (**2* allele non-carriers/**17* carriers).

Results: There was a significant difference in VASP PRI, but not CRP, levels between the three metabolizing groups ($p < 0.001$ and $p = 0.412$, respectively). The interactions of metabolizing status and smoking was significant for CRP ($p = 0.004$) but not for VASP PRI ($p = 0.720$).

Conclusion: Interaction between CYP2C19 polymorphisms and smoking modifies the on-treatment CRP level of post-PCI, on-thienopyridine patients. This effect seems to be independent to the level of P2Y12 receptor inhibition.

Key words: Cytochrome P450, polymorphism, smoking clopidogrel, prasugrel, C-reactive protein

La faible réponse génétiquement déterminée aux thiénoxyridines est associée à une inflammation systémique plus importante chez les fumeurs

Objectifs : Cette étude cherchait à évaluer si les interactions des polymorphismes 2C19 de perte et de gain de fonction (*CYP2C19*2* et *CYP2C19*17*) du cytochrome P450 (CYP) avec le tabac sont associées aux niveaux d'inhibition du récepteur P2Y12 et aux taux de protéine C réactive (PCR) chez des patients subissant une intervention coronarienne percutanée (ICP) traités par aspirine et soit clopidogrel, soit prasugrel.

Contexte : Les allèles *2 et *17 influencent la réponse aux effets antiagrégants plaquettaires tant du clopidogrel que du prasugrel. Certaines études, mais pas toutes, ont laissé entrevoir, aussi, que le tabac modifie positivement la réponse au clopidogrel mais non au prasugrel. En outre, le taux de PCR après l'intervention est un prédicteur fiable de complications post-ICP telles que la resténose intra-stent.

Méthodes : Un total de 1128 patients atteints de syndrome coronarien aigu, ayant subi une ICP avec succès, a été recruté. Lors du suivi clinique à 1 mois, les interactions du tabac et des polymorphismes CYP2C19 sur les niveaux de réactivité plaquettaire sous traitement, évalués par l'indice de réactivité plaquettaire stimulée par vasodilatateur (IRP VASP) et la PCR, ont été explorées dans trois groupes de métabolisme comme suit : mauvais métaboliseurs (MM) (porteurs de *2 / non-porteurs de *17) ; métaboliseurs intermédiaires (MI) (porteurs de *2 / porteurs de *17 ou non-porteurs de *2 / non-porteurs de *17) ; et ultramétaboliseurs (UM) (non-porteurs de *2 / porteurs de *17).

Résultats : Une différence significative s'est fait jour entre les trois groupes de métabolisme en ce qui concerne les taux d'IRP VASP, mais non de PCR (respectivement $p < 0,001$ et $p = 0,412$). Les interactions du statut de métabolisme et du tabac étaient significatives pour la PCR ($p = 0,004$) mais non pour l'IRP VASP ($p = 0,720$).

Conclusion : L'interaction entre les polymorphismes CYP2C19 et le tabac modifie le taux de PCR sous traitement en post-ICP sous thiénoxyridines. Cet effet semble être indépendant du niveau d'inhibition du récepteur P2Y12.

A notre connaissance, c'est la première fois qu'a été rapportée une interaction entre CYP2C19 et tabac sur le taux de PCR de patients en post-ICP sous thiénoxyridines. Des taux plus élevés de PCR ont été associés à une interaction entre le tabac et l'état de mauvaise métabolisation du CYP2C19. Des études complémentaires s'imposent afin de valider ces résultats, ainsi que d'évaluer leur application dans le contexte clinique.

Mots clés : Cytochrome P450, polymorphisme, tabac, clopidogrel, prasugrel, protéine C réactive

Dual antiplatelet therapy with aspirin and a P2Y₁₂ receptor inhibitor, primarily thienopyridine-derivatives clopidogrel or prasugrel, is currently the accepted standard for the prevention of thrombotic complications and ischemic events after percutaneous coronary intervention (PCI) (1,2). However, there exists a marked inter-individual variability in the pharmacodynamic response to thienopyridines (3-5), and multiple studies have demonstrated that the post-PCI patients with higher on-treatment platelet reactivity have a higher incidence of major adverse cardiovascular events as well as in-stent restenosis (ISR), and also in those with the lowest on-thienopyridine reactivity, the risk of bleeding is significantly increased (6). The cytochrome P450 (CYP) 2C19 loss-of-function (*CYP2C19*2*) and gain-of-function (*CYP2C19*17*) polymorphisms have been shown to be associated, respectively, with an attenuated and strengthened response to both clopidogrel and prasugrel (7,8). Moreover, some, but not all, studies have reported a phenomenon that has been termed “smoker’s paradox” whereby clopidogrel-, but not prasugrel, treated smokers appears to have a greater platelet inhibition and lower aggregation when compared to non-smoker patients (9-11). However, the potential impact of the interaction between CYP2C19 polymorphisms and smoking on the on-thienopyridine platelet reactivity has not previously been explored.

Accumulating evidence indicates that local and systemic inflammatory responses to the angioplasty-induced vascular injury trigger multiple thrombotic and proliferative processes and have been proposed as the key pathophysiological mechanisms of ISR (12-14). A number of studies have indicated that inflammatory markers, particularly C-reactive protein (CRP), in patients undergoing PCI are reliable predictors of post-PCI complications, such as ISR (15-22). It has also been well established that, in addition to its contribution to the thrombus formation, activated platelet plays a central role in the initiation and maintenance of

inflammation (23). In this reason, some studies proposed that, as anti-platelet agents, thienopyridines may exert pleiotropic anti-inflammatory effects. However, the inflammation-reducing effects of these drugs have not been a persistent finding in all trials and the results of different studies in this field are sometimes contradictory (24-29). We hypothesized that CYP2C19 polymorphisms and/or a CYP2C19 polymorphisms-smoking interaction may contribute to these discrepancies.

In this study, we examine whether interactions between CYP2C19 polymorphisms and smoking is potentially associated with the levels of platelet activation and the CRP level of a large post-PCI patient population, after one month treatment with aspirin and either clopidogrel or prasugrel. We also assess if the platelet reactivity and CRP level are correlated in this population.

Methods

Study population. All consecutive patients with acute coronary syndrome who underwent successful PCI between April 2007 and December 2012 at the Centre Universitaire Hospitalier Timone, Marseille, France and were discharged with either clopidogrel 75 mg/d (Clo75) or 150 mg/d (Clo150) or prasugrel 10 mg/d (Pra) were prospectively enrolled in the study. In addition, all of the patients received aspirin 75 mg/d simultaneously. Exclusion criteria for the current study were a history of bleeding diathesis, previous stroke or transient ischemic attack, contraindications to anti-platelet therapy, platelet count <100 g/L and creatinine clearance <25 ml/min.

The demographic and clinical data were collected through patient interview. After interview, systolic and diastolic blood pressures (SBP and DBP) were measured following a standard protocol and mean arterial pressure (MAP) was calculated as $[(2 \times \text{DBP}) + \text{SBP}]/3$.

The study protocol was approved by the local ethics committee and all the patients gave written informed consent before participating in the study.

Laboratory methods

Platelet function measurement. At 1-month follow-up visit after PCI, the P2Y₁₂ receptor inhibition was quantitated using vasodilator-stimulated phosphoprotein (VASP) platelet reactivity index (PRI). The level of phosphorylated VASP was determined using a standardized flow cytometric assay (platelet VASP, Diagnostica Stago/BioCytex, Asnières, France), which is an altered version of the method described previously by Schwarz (30). The VASP PRI was calculated from the median fluorescence intensity (MFI) of samples incubated with prostaglandin E₁ (PGE₁) or PGE₁ and adenosine diphosphate (ADP) according to the formula: $\text{VASP PRI} = [\text{MFI}_{(\text{PGE}_1)} - \text{MFI}_{(\text{PGE}_1 + \text{ADP})} / \text{MFI}_{(\text{PGE}_1)}] \times 100$.

Genotyping. All patients were genotyped for the loss-of-function and gain-of-function CYP2C19 polymorphisms. Genomic DNA was extracted from peripheral blood samples using the salting-out method. *CYP2C19*2* (rs4244285) and *CYP2C19*17* (rs12248560) genotyping was carried out using the amplification refractory mutation system-polymerase chain reaction (PCR) in duplex reaction. The primers used (Invitrogen, Carlsbad, California) were as follows: *2 forward: CAg AgC TTg gCA TAT TgTATC; *2 reverse: TAT CgC AAg CAg TCA CAT AAC; *2G specific (sens): ACT ATC ATT gAT TAT TTC CCg; and *2 A specific (antisens): gTA ATT TgT TAT ggg TTC CT; *17 forward: aag aag cct tag ttt ctc aag, *17 reverse:

aaacacctttaccatttaaccc; *17 T specific (sense): tgt ctt ctg ttc tca aag ta and *17 C specific (antisense): atta tct ctt aca tca gag atg. Size of the PCR products generated: *CYP2C19**2 forward/reverse: 373 bp, *2 allele G (normal): 283 bp, and *2 allele A (mutated): 129 bp; *CYP2C19**17 forward/reverse: 507 bp, *17 allele C (normal): 330 bp, and *17 allele T (mutated): 218 bp. The reactions were made in a final volume of 12.5 ml consisting of 200 nM of primer (300 nM for *2 A); 0.15 U of Taq DNA polymerase and its Buffer (Qbiotaq, MP Biomedicals, Santa Ana, California); 200 mM of deoxynucleotide triphosphates; and 25 ng of genomic DNA. The cycling conditions were as follows: a first denaturing step for 1 minute, 30 seconds at 94 °C and then 35 cycles of 30 seconds at 95 °C; 30 seconds at 60 °C; 50 seconds at 72 °C; and a final extension step for 3 minutes at 72 °C, with cooling to 15 °C. The genotypes were determined according to the sizes of the PCR products on 2% agarose gel.

Biochemical profile. At 1-month follow-up visit hemogram, platelet count and selected parameters of biochemistry including CRP, lipids (triglyceride, total cholesterol, low density lipoprotein and high density lipoprotein), blood glucose, hemoglobin A1C, insulin blood level and serum level of creatinine were performed. All parameters were determined by standard laboratory methods at the Department of Hematology and Biochemistry of the Centre Universitaire Hospitalier Timone, Marseille, France. The biochemical parameters were measured in blood samples using different autoanalyzers; ADVIA 120 (Bayer Diagnostics, NY, USA) for hemoglobin and platelet counts; *Beckman DX 800* (*Beckman Coulter, Inc.*, Fullerton, CA) for serum lipids, glucose, creatinine and CRP; *Variant II Turbo* (*Bio-Rad*, USA) for hemoglobin A1C; and COBAS (Roche Diagnostics, Burgess Hill, West Sussex, UK) for insulin.

Statistical analysis. For those variables of which the distribution exhibited a long-tailed positive skewness and kurtosis (triglyceride and CRP), \log_{10} transformed values were used for analysis in order to normalize their distribution. The Gaussian distribution was verified by using normal probability plots. Regression coefficients (for crude values and after adjustment for covariates) were calculated to assess the associations between inflammation markers and all parameters of interest. ANOVA with interaction term was used to study association of VAS PRI and CRP concentration with *2 and *17. Statistical analyses were performed by using the SAS software package version 9.3 (SAS Institute Inc., Cary, NC, USA) and for all analyses, statistical significance was set at $p \leq 0.05$.

To investigate the hypothesis that an interaction between CYP2C19 polymorphisms and smoking is potentially associated with the altered response to the anti-platelet and also pleiotropic anti-inflammatory effects of thienopyridines, we investigated, in the general population and in a stepwise manner, whether the CRP level differs between three treatment groups; if smoking is independently associated with the level of platelet inhibition (VASP PRI) and the CRP level; whether there is an association between CYP2C19*2/*17 and VASP PRI and the CRP level; if the interaction between CYP2C19 polymorphisms and smoking is associated with VASP PRI and the CRP level; and eventually if VASP PRI and the CRP level are correlated. We also assessed the hypothesis on the study population by classifying the patients into three groups according to their genotype and metabolizing status (MS) as follow: poor metabolizers (PM; *2 carriers/*17 non-carriers), intermediate metabolizers (IM; *2 carriers/*17 carriers) and ultrametabolizers (UM; *2 non-carriers/*17 carriers).

Results

The characteristics of the entire population are shown in Table 1. In total, 1128 patients were recruited in the study, of whom 118 patients on clopidogrel 75 mg/d; 631 patients were treated with clopidogrel 150 mg/d; and 309 patients received prasugrel 10 mg/d. Also, genotype distribution was in Hardy-Weinberg equilibrium

Predictors of CRP level. Based on the multiple regression analysis, the CRP level was positively associated with age ($p \leq 0.001$), MAP ($p \leq 0.001$), blood glucose ($p = 0.007$), creatinine ($p \leq 0.001$) and platelet count ($p \leq 0.001$), and was also negatively associated with male gender ($p = 0.017$) and high density lipoprotein ($p \leq 0.001$). Furthermore, the univariate regression analysis revealed that current smoking (CS) significantly affects the level of CRP ($p = 0.001$) and this impact remained statistically significant after multivariate analysis and adjustment for other covariates ($p \leq 0.001$).

In the general population, in the univariate regression analysis, the level of platelet inhibition, assessed by VASP PRI, associated with CRP levels ($p = 0.026$). This correlation, however, did not reach a statistical significance in the multivariate regression analysis ($p = 0.882$). Likewise, neither *CYP2C19*2* nor *CYP2C19*17* polymorphisms were found to be associated with the CRP level ($p = 0.756$ and $p = 0.616$, respectively) and no difference was observed in CRP levels between the three metabolizer groups (PM vs. IM vs. UM) ($p = 0.412$). Additionally, CRP levels did not differ significantly between patients treated with bare metal stent versus those treated with drug eluting stent ($p = 0.741$); statin therapy and treatment with other cardiovascular medications did not influence CRP levels; and no significant difference was found in the CRP level between the three treatment groups (Clo75 vs. Clo150

vs. Pra) ($p = 0.13$) (Table 2). Since the variation of CRP level between the groups was trivial, further analyses were carried out on the whole population.

Predictors of VASP PRI. In general, there was a significant difference in the level of on-treatment platelet activity between the three treatment groups ($p < 0.001$), with Pra group and Clo75 group having the lowest and highest levels of VASP PRI, respectively.

After multiple regression analysis and inclusion of variables showing relevant influence in univariate analysis (data not presented), there was a significant association between the level of on-treatment platelet activation and both *CYP2C19*2* and *CYP2C19*17* alleles ($\beta = 12.17$, $p < 0.001$ and $\beta = -2.18$, $p = 0.018$, respectively). Likewise, VASP PRI was significantly associated with the patients' MS with PMs and UMs having the highest and lowest level of platelet activation, respectively ($p \leq 0.001$).

Additionally, in the general population consisting of the three treatment groups, smoking was positively associated with VASP PRI ($p = 0.032$). More specifically, in Pra and Clo75 groups ($p = 0.041$), but not in Clo150 group ($p = 0.514$), VASP PRI was significantly higher in smokers compared to non-smokers.

Association of smoking and CYP2C19 polymorphisms with VASP PRI and CRP levels. The associations of smoking and *CYP2C19*2*; smoking and *CYP2C19*17*; and smoking and MS with on-treatment platelet activity and CRP levels are represented in Tables 3A, 3B and 3C, respectively. The interaction between *CYP2C19*2* and smoking was not significant for VASP PRI, neither in the two-allele (Figure 1A), nor the dominant allele models ($p = 0.429$ and $p = 0.369$, respectively). On the other hand, the interaction of *CYP2C19*2* and smoking was found to be significant for the CRP level both in the two-allele model ($p = 0.004$) (Figure 1B),

and the dominant allele model ($p = 0.001$). Also, there was not a significant interaction between *CYP2C19*17* and smoking on VASP PRI neither in the two-allele model ($p = 0.560$), nor in the dominant allele model ($p = 0.608$). In addition, in the allele model, no significant interaction was observed between the **17* and smoking on the CRP level ($p = 0.196$); however, in the dominant allele model, the interaction was associated with a borderline significant decrease in on-treatment CRP levels ($p = 0.077$), whereby the CRP level was 2.01 (0.66 – 6.06) mg/L and 2.63 (0.79 – 8.71) mg/L in smokers who carry and do not **17* allele (CT + TT vs. CC), respectively.

In regard to the MS, while the interaction between MS and smoking was not significant for VASP PRI ($p = 0.719$) (Figure 2A), the MS-smoking interaction was strongly significant for the CRP level in the population ($p = 0.001$), whereby among the smokers, the highest CRP levels were observed in PMs (3.39 (0.98 – 11.75) mg/L) followed by IMs (2.34 (0.76 – 7.24) mg/L) and UMs (1.95 (0.63 – 6.03) mg/L) (Figure 2B).

Discussion

Systemic and vascular inflammation after PCI is dependent on heterotypic interactions between endothelial cells, immune cells and activated platelets as evidenced by an increase in the circulatory levels of CRP, chemokines, cytokines and adhesion molecules (14,31). It has been shown that PCI evokes an acute inflammatory cell response within the first 3 days following the procedure which subsides by 4 weeks and is replaced with a chronic persistent inflammatory response (15,32).

In addition to platelet inhibition, thienopyridines have been reported to exhibit anti-inflammatory effects. The ability of clopidogrel and prasugrel in the reduction of

inflammation would be particularly important in the clinical setting since it has been demonstrated that post-PCI inflammatory response is the primary causative mechanism of ISR (12-14), and that elevated inflammatory markers is associated with an increased incidence of adverse cardiac events in post-PCI patients (33). Of note, although a number of inflammatory markers, including matrix metalloproteinases, pregnancy-associated plasma protein, soluble CD-40, plasminogen activator inhibitor-1 and eosinophils have been used as predictors of outcome after PCI, owing to its higher stability, CRP has been found to be a more reliable inflammatory marker in predicting future cardiovascular events (34) and, therefore, the effects of thienopyridines on the CRP level has been more extensively explored. However, considerable controversy has surrounded the effects of anti-platelet drug use on the level of inflammatory markers (24-29).

In the present study we found that the CYP2C19 loss- and gain-of-function polymorphisms interact significantly with CS for the CRP level in post-PCI on-thienopyridine patients. This effect seems to be, at least in part, independent to the level of on-treatment platelet inhibition. Our results did not favor the hypothesis that CYP2C19 polymorphisms are independently associated with CRP levels in patients treated with thienopyridines, whereas supported the theory that an interaction between the polymorphisms and CS would be a major source of the inter-individual variability in response to the anti-inflammatory effects of thienopyridines.

The latter finding could be explained, at least partly, by the fact that both clopidogrel and smoking have been discovered to modify the activation of nuclear factor-kappa B (NF- κ B). NF- κ B refers to a ubiquitously expressed pleiotropic nuclear transcription factor that controls a wide variety of pathophysiological processes including immune and inflammatory responses, cell proliferation, cell death (apoptosis) and cell survival (35). Indeed, NF- κ B is a

master switch in the inflammatory cascade (36) and, therefore, is involved in numerous cardiovascular diseases including atherosclerosis and heart failure and also in the ISR process (35). In patients with unstable angina, CRP levels have been reported to correlate with monocyte NF- κ B activity and amount of chemokines release (37). It has been shown that clopidogrel administration reduces inflammation via inhibition of NF- κ B (38). Also, strong evidence suggests that the mechanisms behind the modulation of inflammation by smoking mainly involve NF- κ B (39). Accordingly, it is not surprising that among on-thienopyridine patients, the highest CRP levels were observed in smokers who were CYP2C19 PM, with probably the lowest thienopyridine active metabolite exposure. Furthermore, multiple studies suggested that clopidogrel inhibits reactive oxygen species (ROS) production (39), and also improves endothelial nitric oxide (NO) bioavailability (41) and that ROS and NO promotes and inhibits NF- κ B activation, respectively (35,42). Besides, smoking is associated with a decreased ability of the body to synthesize NO (43), and also with an increase in the ROS production (44). It, therefore, makes sense that the interaction of smoking and CYP2C19 poor MS is associated with increased CRP levels. The interaction of CYP2C19 polymorphisms and smoking on the inflammation might also be explained by the linkage between inflammation and thrombosis. It has been well established that inflammation can cause thrombosis and thrombosis can mediate inflammation (45). These processes have several connecting points of which tissue factor (TF) has been shown to be critical for and plays a central role in the inflammation-thrombosis circuit (46). The role of TF in inflammation is mediated by blood coagulation. Upon tissue injury, such as post-PCI endothelial injury, TF upregulation results in the initiation of the extrinsic blood coagulation and activation of a series of coagulation factors including FVIIa, FXa and FIIa which have been shown to exert

pro-inflammatory effects (46). Thienopyridine therapy and CS have been found to be associated with a decrease and an increase in the circulating TF, respectively (47-49).

Regardless of the underlying mechanism, and considering the prognostic value of CRP, the interaction of CYP2C19 polymorphisms and smoking could make a significant contribution to identifying the patients who are at high risk of post-PCI clinical and procedural complications. At this time, there is no convincing data to support that routine CYP2C19 genotyping, as a tailored thienopyridine treatment, would be an effective strategy in clinical practice (50). The results of the current study may provide additional incentive for the clinicians to incorporate pharmacogenetics data into their daily clinical practice.

In this study, we observed that for none of the three thienopyridine metabolizer groups, there was a significant association between CS and on-treatment platelet inhibition; this findings did not support the notion that smoking may positively modify the beneficial anti-platelet effects of thienopyridines (9-11). On the other hand, we found that there is a trivial difference in the CRP levels between UMs who smoke and UM non-smokers. Since accumulating evidence reveals that smoking is associated with increased level of CRP (51), it seems that our findings may provide supporting evidence for an additional, and more complicated, aspect of the "smoker's paradox" in which CS may be associated with increased CRP levels but also with the smoking-related increase in the anti-inflammatory effects of thienopyridines. However, considering the fact that smoking is a well-established independent cardiovascular risk factor, further studies are needed to evaluate the clinical relevance of this potential phenomenon in well-validated prospective clinical trials. As such, the authors encourage smoking cessation rather than relying on the positive effect of so-called paradox.

Study limitations. Although CRP is *the most* commonly used, most extensively studied and best standardized systemic inflammatory marker, it is influenced by several clinical and biological factors and, thereby, it would not appropriately reflect the real levels of platelet activity. Indeed, specific markers of platelet activation, such as soluble P-selectin and soluble CD-40, would be better choices to evaluate our hypothesis. In addition, a long-term follow-up regarding relationship between the levels of CRP determined by the CYP2C19 polymorphisms-smoking interaction and clinical outcome was not performed. Therefore, the potential clinical significance of this relationship needs to be assessed by further investigations.

Conclusion. To the best of our knowledge, this is the first report of an interaction between CYP2C19 and smoking on the CRP level of post-PCI on-thienopyridine patients. Higher CRP levels were associated with an interaction between smoking and CYP2C19 poor metabolizing state. Further studies are needed to validate these findings and also to assess their application in the clinical setting.

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Table 1. Patients' characteristics

Variables	General Population (n = 1128)
Demographic characteristics	
Age (year) [†]	63.5 ± 12.3
Male sex, (%)	81.0
BMI (kg/m ²) [†]	26.9 ± 4.3
Clinical characteristics, (%)	
Diabetes mellitus	27.7
Hypercholesterolemia	56.1
Hypertension	54.0
Current smoking	38.3
Presenting coronary syndrome, (%)	
STEMI	32.8
NSTEMI, high troponine	25.6
NSTEMI, low troponine	28.6
Medication, (%)	
Thienopyridine	
Clopidogrel (75 mg/d)	16.7
Clopidogrel (150 mg/d)	55.9
Prasugrel (10 mg/d)	27.4
ACE inhibitors	70.9
Angiotensin receptor blockers	11.97
β-blockers	70.5
Calcium channel blockers	13.0
Statins	93.8
Target vessel, (n)	
Left main coronary artery	38
Left anterior descending artery	497
Circumflex artery	129
Marginal artery	80
Right coronary artery	371
Stent characteristics	
Bare-metal stent (%)	45.5
Drug-eluting stent (%)	54.5
Stent length (mm) [†]	20.6 ± 11.2
Biochemistry	
Triglyceride (g/L) [‡]	1.08 (0.67 - 1.76)
Total cholesterol (g/L) [†]	1.49 ± 0.40
Low density lipoprotein (g/L) [†]	0.85 ± 0.33
High density lipoprotein (g/L) [†]	0.42 ± 0.12
Glucose (mmol/L) [†]	6.34 ± 1.92
Insulin (mIU/L) [†]	13.3 ± 16.8
Hemoglobin A1C (%) [†]	6.29 ± 1.04
Creatinine (μmol/L) [†]	91.0 ± 50.4
Hemoglobin (g/dL) [†]	139.1 ± 14.2

Platelet count ($\times 10^3/\mu\text{L}$)†	246.0 \pm 66.8
Platelet function test	
VASP PRI (%)†	39.2 \pm 19.0
C-reactive protein (mg/L)‡	2.10 (0.63-6.93)
CYP2C19 Genotyping	
A (*2) allele frequency (%)	14.4
AA (*2/*2) (%)	2.6
AG (*2/wide type) (%)	23.8
GG (wide type/wide type) (%)	73.6
T (*17) allele frequency (%)	19.1
TT (*17/*17) (%)	4.2
CT (wild type/*17) (%)	29.9
CC (wild type/wild type) (%)	65.9
Metabolizing status	
Poor metabolizer (%)	21.72
Intermediate metabolizer (%)	48.67
Ultrametabolizer (%)	29.61

† Mean \pm SD; ‡ Geometric mean (range of one SD); SD: standard deviation

ACE: angiotensin converting enzyme;

BMI: body mass index;

CYP: cytochrome P450;

VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index;

STEMI: ST segment elevated myocardial infarction;

NSTEMI: non ST segment myocardial infarction

Poor metabolizers: (*2 loss-of-function allele carriers/*17 gain-of-function allele non-carriers); Intermediate

metabolizers: (*2 loss-of-function allele carriers/*17 gain-of-function allele carriers or *2 loss-of-function allele non-carriers/*17 gain-of-function allele non-carriers); and ultrametabolizers: (*2 loss-of-function allele non-

carriers/*17 gain-of-function allele carriers)

Table 2. Predictors of CRP level[‡] using univariate and multivariate regression analysis

Parameter	Univariate		Multivariate	
	Regression coefficient \pm SE	<i>p</i>	Regression coefficient \pm SE	<i>p</i>
Age (years)	0.0033 \pm 0.0013	0.008	0.0057 \pm 0.0014	\leq 0.001
BMI (kg/m ²)	0.0148 \pm 0.0036	\leq 0.001	-0.0058 \pm 0.0054	0.277
Male vs. female	-0.0764 \pm 0.0394	0.053	-0.0964 \pm 0.0404	0.017
MAP (mm Hg)	0.0084 \pm 0.0013	\leq 0.001	0.0081 \pm 0.0020	\leq 0.001
Current smoker vs. non smoker	0.1018 \pm 0.0317	0.001	0.1541 \pm 0.0323	\leq 0.001
Clopidogrel 150 vs. Clopidogrel 75 mg/d	-0.0864 \pm 0.0431	0.045	-0.0684 \pm 0.0408	0.094
Prasugrel 10 vs. Clopidogrel 75 mg/d	-0.0683 \pm 0.0480	0.155	-0.0134 \pm 0.0504	0.790
Bare-metal stent vs. drug-eluting stent	0.0435 \pm 0.0310	0.161	-0.0100 \pm 0.0302	0.741
VASP PRI (%)	0.0018 \pm 0.0008	0.026	0.0001 \pm 0.0009	0.882
Glucose (mmol/L)	0.0371 \pm 0.0080	\leq 0.001	0.0216 \pm 0.0079	0.007
Triglyceride (g/L) [‡]	0.3018 \pm 0.0729	\leq 0.001	0.0489 \pm 0.0738	0.507
High density lipoprotein (g/L)	-0.6456 \pm 0.1263	\leq 0.001	-0.5720 \pm 0.1326	\leq 0.001
Platelet count ($\times 10^3/\mu\text{L}$)	0.0016 \pm 0.0002	\leq 0.001	0.0017 \pm 0.0002	\leq 0.001
Creatinine ($\mu\text{mol/L}$)	0.0012 \pm 0.0003	\leq 0.001	0.0012 \pm 0.0003	\leq 0.001
IM vs. UM	0.0555 \pm 0.0360	0.124	0.0239 \pm 0.0336	0.477
PM vs. UM	0.0532 \pm 0.0437	0.223	0.0243 \pm 0.0413	0.555

[‡]Log10 transformed values; VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index; BMI: body mass index; MAP: mean arterial pressure; SE: standard error; PM: poor metabolizer (*2 carriers/*17 non-carriers); IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers); UM: ultrametabolizer (*2 allele non-carriers/*17 carriers)

Table 3. VASP PRI and CRP levels according to smoking status and CYP2C19 polymorphisms**A. Smoking and CYP2C19*2 polymorphism**

Genotype	GG		AG		AA		ANOVA		
	Yes	No	Yes	No	Yes	No	*2 allele	Smoking	Interaction
CS									
Number	320	512	103	164	9	20			
VASP PRI (%)	37.1 (18.6) [†]	37.2 (17.9)	43.4 (19.7)	44.2 (20.1)	58.2 (24.4)	51.0 (20.3)	≤0.001	0.159	0.429
CRP (mg/L) ^{‡, ¥}	2.19(0.69-6.92)	2.00(0.60-6.61)	3.16(0.93-10.72)	1.66(0.49-5.62)	4.27(1.51-12.02)	2.04(0.63-6.61)	0.312	≤0.001	0.004

B. Smoking and CYP2C19*17 polymorphism

Genotype	CC		CT		TT		ANOVA		
	Yes	No	Yes	No	Yes	No	*17 allele	Smoking	Interaction
CS									
Number	290	453	123	215	19	28			
VASP PRI (%)	39.7 (19.5)	40.1 (18.8)	38.2 (19.1)	38.6 (18.4)	34.3 (18.6)	31.7 (20.1)	0.022	0.331	0.560
CRP (mg/L) ^{‡, ¥}	2.63(0.79-8.71)	1.91(0.58-6.31)	2.00(0.66-6.03)	1.86(0.56-6.17)	2.00(0.65-6.17)	2.29(0.68-7.76)	0.450	0.055	0.196

C. Smoking and metabolizing status

MS	PM		IM		UM		ANOVA		
	Yes	No	Yes	No	Yes	No	MS	Smoking	Interaction
Current smoker									
Number	96	149	210	339	126	208			
VASP PRI (%)	37.1 (18.6)	37.2 (17.9)	43.4 (19.7)	44.3 (20.1)	58.2 (24.4)	51.0 (20.3)	≤0.001	0.466	0.720
CRP (mg/L) ^{‡, ¥}	3.39(0.98-11.75)	1.62(0.48-5.50)	2.34(0.76-7.24)	2.09(0.65-6.76)	1.95(0.63-6.03)	1.91(0.58-6.31)	0.412	≤0.001	0.001

[†] Arithmetic mean (SD)

[‡] ANOVA test performed using log₁₀ transformed values after adjustment for sex, age, AAP, type of stent, BMI, blood pressure, fasting glucose, HDL-cholesterol, creatinine, platelet count and VASP PRI

[¥] Geometric mean (range of 1 SD)

VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index; CRP: C-reactive protein; CS: current smoker; MS: metabolizing status; PM: poor metabolizer (*2 carriers/*17 non-carriers); IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers); UM: ultrametabolizer (*2 allele non-carriers/*17 carriers)

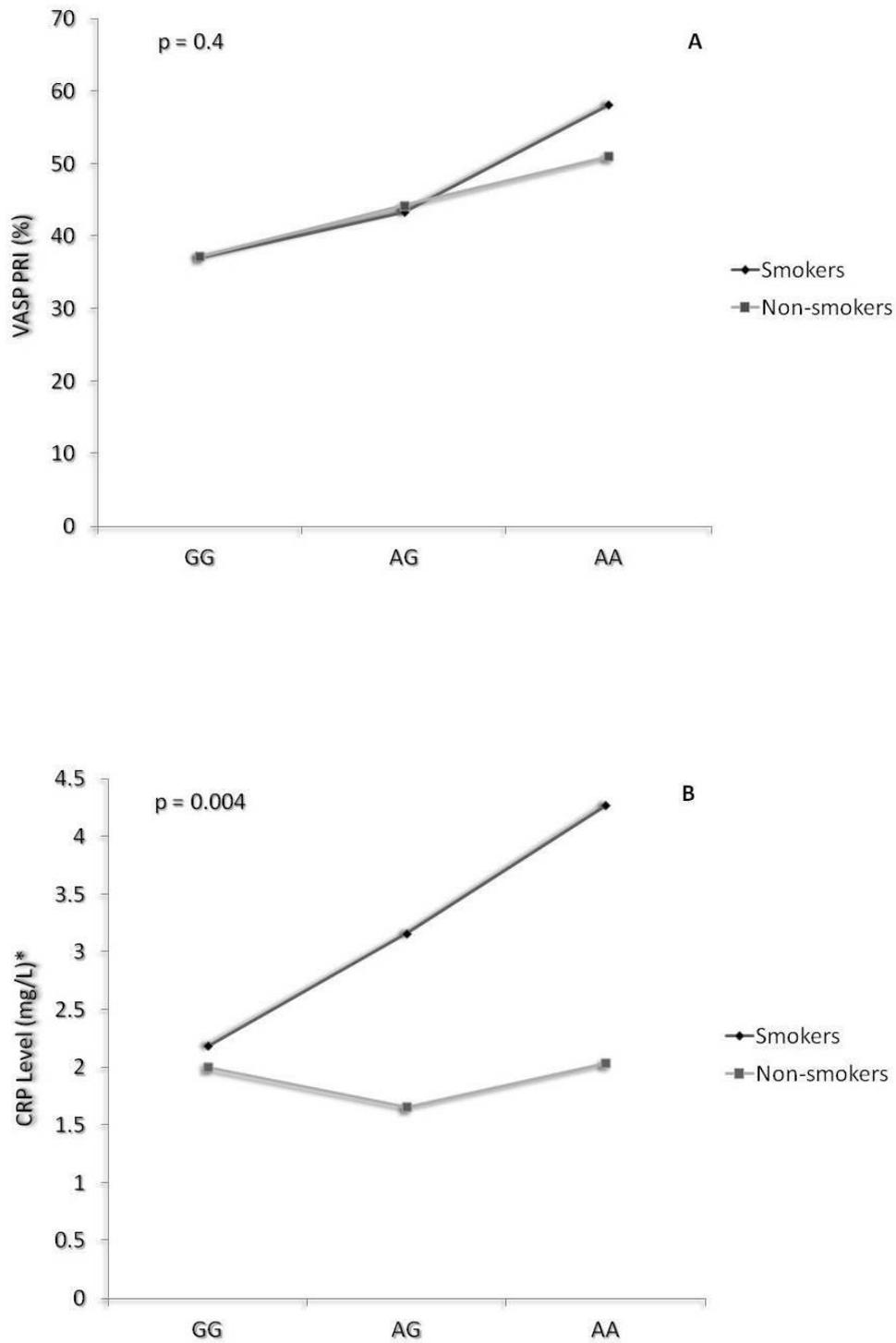


Figure 1. Interaction between CYP2C19 loss-of-function allele and smoking is associated with higher levels of CRP, but not with the level of platelet inhibition. VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index, CRP: C-reactive protein; *Log10 transformed values

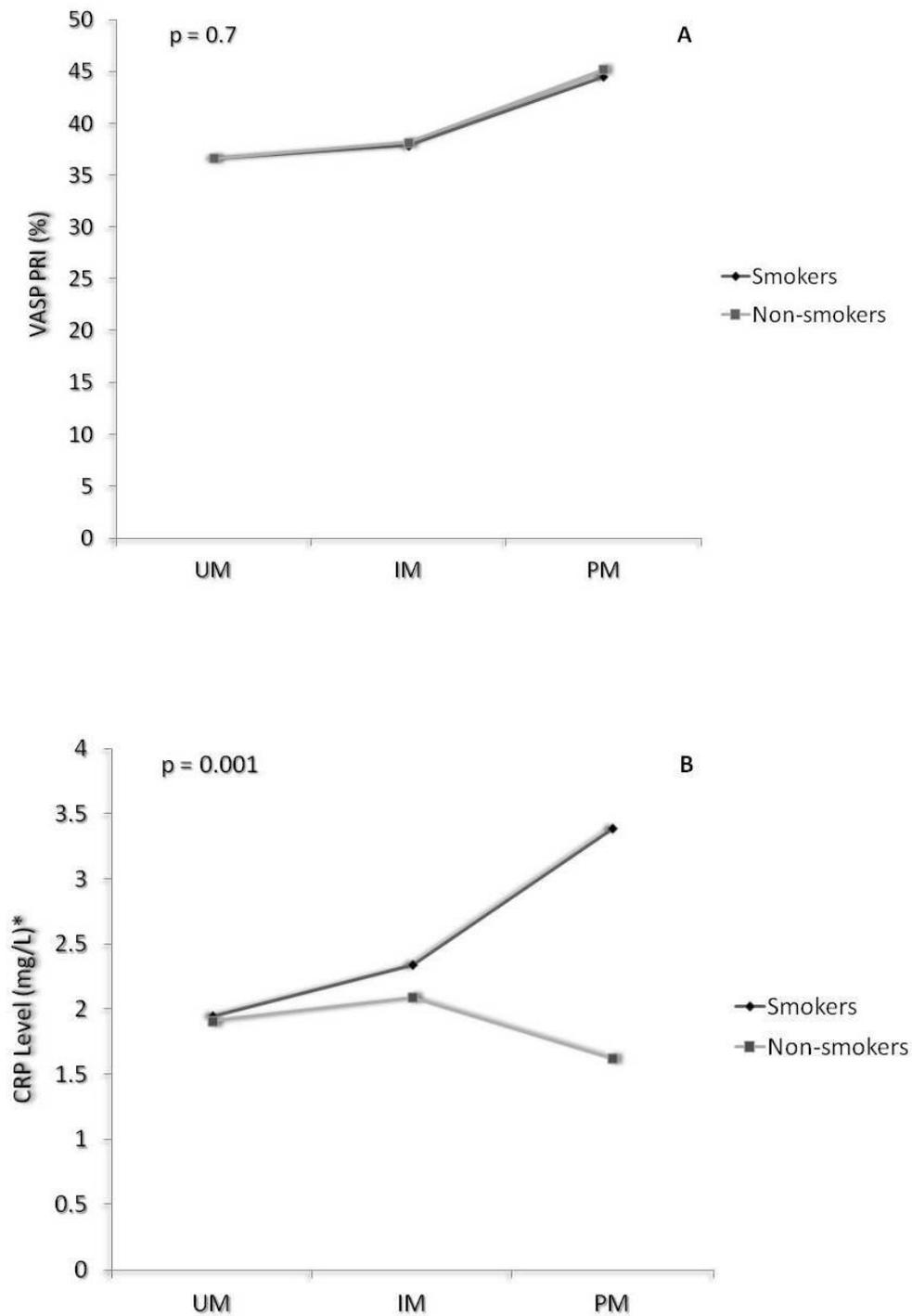


Figure 2. Interaction between MS and smoking is associated with levels of CRP, but not with the level of platelet inhibition. VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index, CRP: C-reactive protein, MS: metabolizing status, PM: poor metabolizer (*2 carriers/*17 non-carriers), IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers), UM: ultrametabolizer (*2 allele non-carriers/*17 carriers); *Log10 transformed values

Supplementary data

Considering the fact that, similar to CRP, Hp and orosomuroid acid are categorized as the acute phase proteins and that IL-6 and TNF- α have been demonstrated to induce the production of all these markers (see more in the “*Discussion*”), in this thesis, the plasma levels of Hp and orosomuroid acid were determined like CRP, at one month follow-up after PCI. We then examined whether interactions between CYP2C19 polymorphisms/MS and smoking is potentially associated with the levels of Hp and orosomuroid acid in our study population.

S1. Protein quantification

The plasma levels of Hp and orosomuroid were immunonephelometrically determined, using BN II autoanalyzer (Siemens Healthcare Diagnostics, Germany).

Note: The study design including treatment groups, number of recruited patients, genotyping,... were the same as described in paper III for CRP.

S2. Results

S2.1. Predictors of Hp and orosomuroid acid levels

S2.1.1. Hp. Based on the multiple regression analysis, the Hp level was positively associated with age ($p = 0.024$), blood glucose ($p = 0.029$), triglyceride ($p \leq 0.001$) and platelet count ($p \leq 0.001$), and was also negatively associated with male gender ($p = 0.028$) and high density lipoprotein ($p \leq 0.001$). Furthermore, the univariate regression analysis revealed that current smoking (CS) significantly affects the level of CRP ($p = 0.003$) and this impact remained statistically significant after multivariate analysis and adjustment for other covariates ($p = 0.010$).

In the general population, in the univariate regression analysis, the level of platelet inhibition, assessed by VASP PRI, associated with Hp levels ($p = 0.015$). This correlation, however, did not reach a statistical significance in the multivariate regression analysis ($p = 0.186$). Likewise, neither

*CYP2C19*2* nor *CYP2C19*17* polymorphisms were found to be associated with the Hp level ($p = 0.657$ and $p = 0.105$, respectively) and no difference was observed in Hp levels between the three metabolizer groups (PM vs. IM vs. UM) ($p = 0.473$).

Table 4. Predictors of Hp level using univariate and multivariate regression analysis

Parameter	Univariate		Multivariate	
	Regression coefficient \pm SE	<i>p</i>	Regression coefficient \pm SE	<i>p</i>
Age (years)	0.0002 \pm 0.0017	0.891	0.0042 \pm 0.0019	0.024
BMI (kg/m ²)	0.0109 \pm 0.0048	0.024	-0.0109 \pm 0.0071	0.127
Male vs. female	-0.1463 \pm 0.0529	0.006	-0.1176 \pm 0.0536	0.028
MAP (mm Hg)	0.0061 \pm 0.0017	0.001	0.0048 \pm 0.0026	0.067
Current smoker vs. non smoker	0.1253 \pm 0.0425	0.003	0.1100 \pm 0.0428	0.010
Clopidogrel 150 vs. Clopidogrel 75 mg/d	-0.0104 \pm 0.0585	0.858	0.0303 \pm 0.0546	0.579
Prasugrel 10 vs. Clopidogrel 75 mg/d	0.0099 \pm 0.0649	0.878	0.1268 \pm 0.0671	0.059
Bare-metal stent vs. drug-eluting stent	0.1027 \pm 0.0416	0.014	0.0743 \pm 0.0400	0.064
VASP PRI (%)	0.0026 \pm 0.0011	0.015	0.0016 \pm 0.0012	0.186
Glucose (mmol/L)	0.0395 \pm 0.0109	<0.001	0.0232 \pm 0.0106	0.029
Triglyceride (g/L) [‡]	0.8005 \pm 0.0957	<0.001	0.5486 \pm 0.0978	<0.001
High density lipoprotein (g/L)	-0.8554 \pm 0.1694	<0.001	-0.6814 \pm 0.1753	<0.001
Platelet count ($\times 10^3/\mu\text{L}$)	0.0031 \pm 0.0003	<0.001	0.0029 \pm 0.0003	<0.001
Creatinine ($\mu\text{mol/L}$)	0.0001 \pm 0.0004	0.870	0.0001 \pm 0.0004	0.839
IM vs. UM	0.0094 \pm 0.0484	0.847	-0.0355 \pm 0.0445	0.426
PM vs. UM	0.0357 \pm 0.0589	0.545	-0.0008 \pm 0.0548	0.988

VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index; BMI: body mass index; MAP: mean arterial pressure; SE: standard error; PM: poor metabolizer (*2 carriers/*17 non-carriers); IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers); UM: ultrametabolizer (*2 allele non-carriers/*17 carriers)

Additionally, Hp levels did not differ significantly between patients treated with bare metal stent versus those treated with drug eluting stent ($p = 0.064$); statin therapy and treatment with other cardiovascular medications did not influence Hp levels; and no significant difference was found in the Hp level between the three treatment groups (Clo75 vs. Clo150 vs. Pra) (Table 4). Since the variation of Hp level between the groups was trivial, further analyses were carried out on the whole population.

S2.1.2.Orosomuroid. Based on the multiple regression analysis, the orosomuroid level was positively associated with age ($p = 0.032$), MAP ($p = 0.037$), triglyceride ($p \leq 0.001$), platelet count ($p \leq 0.001$) and creatinine level ($p \leq 0.001$), and was also negatively associated with BMI ($p = 0.005$), male gender ($p = 0.028$) and high density lipoprotein ($p = 0.003$). Furthermore, the univariate regression analysis revealed that current smoking (CS) significantly affects the level of orosomuroid ($p = 0.010$) and this impact remained statistically significant after multivariate analysis and adjustment for other covariates ($p = 0.011$).

Table 5. Predictors of orosomuroid level using univariate and multivariate regression analysis

Parameter	Univariate		Multivariate	
	Regression coefficient \pm SE	<i>p</i>	Regression coefficient \pm SE	<i>p</i>
Age (years)	0.0008 \pm 0.0007	0.241	0.0015 \pm 0.0007	0.032
BMI (kg/m ²)	-0.0013 \pm 0.0019	0.495	-0.0077 \pm 0.0027	0.005
Male vs. female	-0.0638 \pm 0.0205	0.002	-0.0699 \pm 0.0205	0.001
MAP (mm Hg)	0.0008 \pm 0.0007	0.252	0.0021 \pm 0.0010	0.037
Current smoker vs. non smoker	0.0427 \pm 0.0165	0.010	0.0419 \pm 0.0164	0.011
Clopidogrel 150 vs. Clopidogrel 75 mg/d	-0.0576 \pm 0.0226	0.011	-0.0460 \pm 0.0208	0.028
Prasugrel 10 vs. Clopidogrel 75 mg/d	-0.0352 \pm 0.0250	0.160	-0.0090 \pm 0.0256	0.726
Bare-metal stent vs. drug-eluting stent	0.0585 \pm 0.0160	<0.001	0.0382 \pm 0.0153	0.013
VASP PRI (%)	-0.0002 \pm 0.0004	0.612	-0.0006 \pm 0.0004	0.166
Glucose (mmol/L)	0.0010 \pm 0.0042	0.808	-0.0016 \pm 0.0041	0.700
Triglyceride (g/L) [‡]	0.3494 \pm 0.0367	<0.001	0.3019 \pm 0.0373	<0.001
High density lipoprotein (g/L)	-0.2537 \pm 0.0659	<0.001	-0.2027 \pm 0.0669	0.003
Platelet count (x 10 ³ /μL)	0.0010 \pm 0.0001	<0.001	0.0008 \pm 0.0001	<0.001
Creatinine (μmol/L)	0.0011 \pm 0.0002	<0.001	0.0010 \pm 0.0002	<0.001
IM vs. UM	0.0101 \pm 0.0187	0.590	-0.0079 \pm 0.0170	0.641
PM vs. UM	0.0069 \pm 0.0228	0.764	0.0041 \pm 0.0209	0.846

VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index; BMI: body mass index; MAP: mean arterial pressure; SE: standard error; PM: poor metabolizer (*2 carriers/*17 non-carriers); IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers); UM: ultrametabolizer (*2 allele non-carriers/*17 carriers)

There was no significant association between the plasma levels of orosomucoid and the level of platelet reactivity determined by VASP PRI ($p = 0.166$). Likewise, neither *CYP2C19*2* nor *CYP2C19*17* polymorphisms were found to be associated with the CRP level ($p = 0.808$ and $p = 0.947$, respectively) and no difference was observed in orosomucoid levels between the three metabolizer groups (PM vs. IM vs. UM) ($p = 0.485$).

On the other hand, after multivariate regression analysis, orosomucoid levels were found to be higher in patients treated with BMS vs. those treated with DES ($p = 0.013$). Likewise, between the three treatment groups, orosomucoid level was significantly higher in patients of Clo75 group compared with those in the Clo150 group ($p = 0.028$); however, no significant difference was found between Pra group and Clo groups. Also, statin therapy and treatment with other cardiovascular medications did not influence Hp levels (Table 5).

S2.2. Association of smoking and CYP2C19 polymorphisms with Hp and orosomucoid levels

The associations of smoking and *CYP2C19*2*; smoking and *CYP2C19*17*; and smoking and MS with on-treatment Hp and orosomucoid levels are represented in Tables 5A, 5B and 5C, respectively.

The interaction of *CYP2C19*2* and smoking was not significant for Hp and orosomucoid levels in the allele model ($p = 0.183$ and $p = 0.119$ for Hp and orosomucoid, respectively). Also, in the allele model, no significant interaction was observed between the **17* and smoking on the Hp level ($p = 0.476$) nor orosomucoid level ($p = 0.250$).

Interestingly, in regard to the MS, the MS-smoking interaction was significant for both Hp and orosomucoid levels in the population ($p = 0.020$, $p = 0.022$, respectively) (Table 5 and Figure 3 and Figure 4).

Table 5. VASP PRI and CRP levels according to smoking status and CYP2C19 polymorphisms**A. Smoking and CYP2C19*2 polymorphism**

Genotype	GG		AG		AA		ANOVA‡		
	Yes	No	Yes	No	Yes	No	*2 allele	Smoking	Interaction
CS									
Number	320	512	103	164	9	20			
Orosomuroid (g/L)†	0.88±0.23	0.87±0.29	0.95±0.26	0.84±0.28	0.93±0.24	0.82±0.22	0.808	0.017	0.119
Haptoglobin (g/L)†	1.74±0.68	1.67±0.67	1.92±0.77	1.62±0.70	1.70±0.93	1.66±0.65	0.657	0.078	0.183

B. Smoking and CYP2C19*17 polymorphism

Genotype	CC		CT		TT		ANOVA‡		
	Yes	No	Yes	No	Yes	No	*17 allele	Smoking	Interaction
CS									
Number	290	453	123	215	19	28			
Orosomuroid (g/L)†	0.91±0.24	0.85±0.27	0.87±0.23	0.87±0.32	0.89±0.23	0.90±0.26	0.947	0.270	0.250
Haptoglobin (g/L)†	1.81±0.73	1.65±0.69	1.77±0.67	1.69±0.65	1.54±0.58	1.53±0.64	0.105	0.234	0.476

C. Smoking and metabolizing status

MS	PM		IM		UM		ANOVA‡		
	Yes	No	Yes	No	Yes	No	MS	Smoking	Interaction
CS									
Number	96	149	210	339	126	208			
Orosomuroid (g/L)†	1.20±0.27	0.82±0.26	0.95±0.22	0.87±0.28	0.87±0.23	0.87±0.31	0.793	≤0.001	0.021
Haptoglobin (g/L)†	2.15±0.82	1.60±0.67	1.85±0.66	1.68±0.71	1.74±0.69	1.67±0.63	0.473	≤0.001	0.025

† Arithmetic mean ± SD

‡ ANOVA test performed after adjustment for sex, age, AAP, type of stent, BMI, blood pressure, fasting glucose, HDL-cholesterol, creatinine, platelet count and VASP PRI

VASP PRI: vasodilator-stimulated phosphoprotein - platelet reactivity index; CRP: C-reactive protein; CS: current smoker; MS: metabolizing status; PM: poor metabolizer (*2 carriers/*17 non-carriers); IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers); UM: ultrametabolizer (*2 allele non-carriers/*17 carriers)

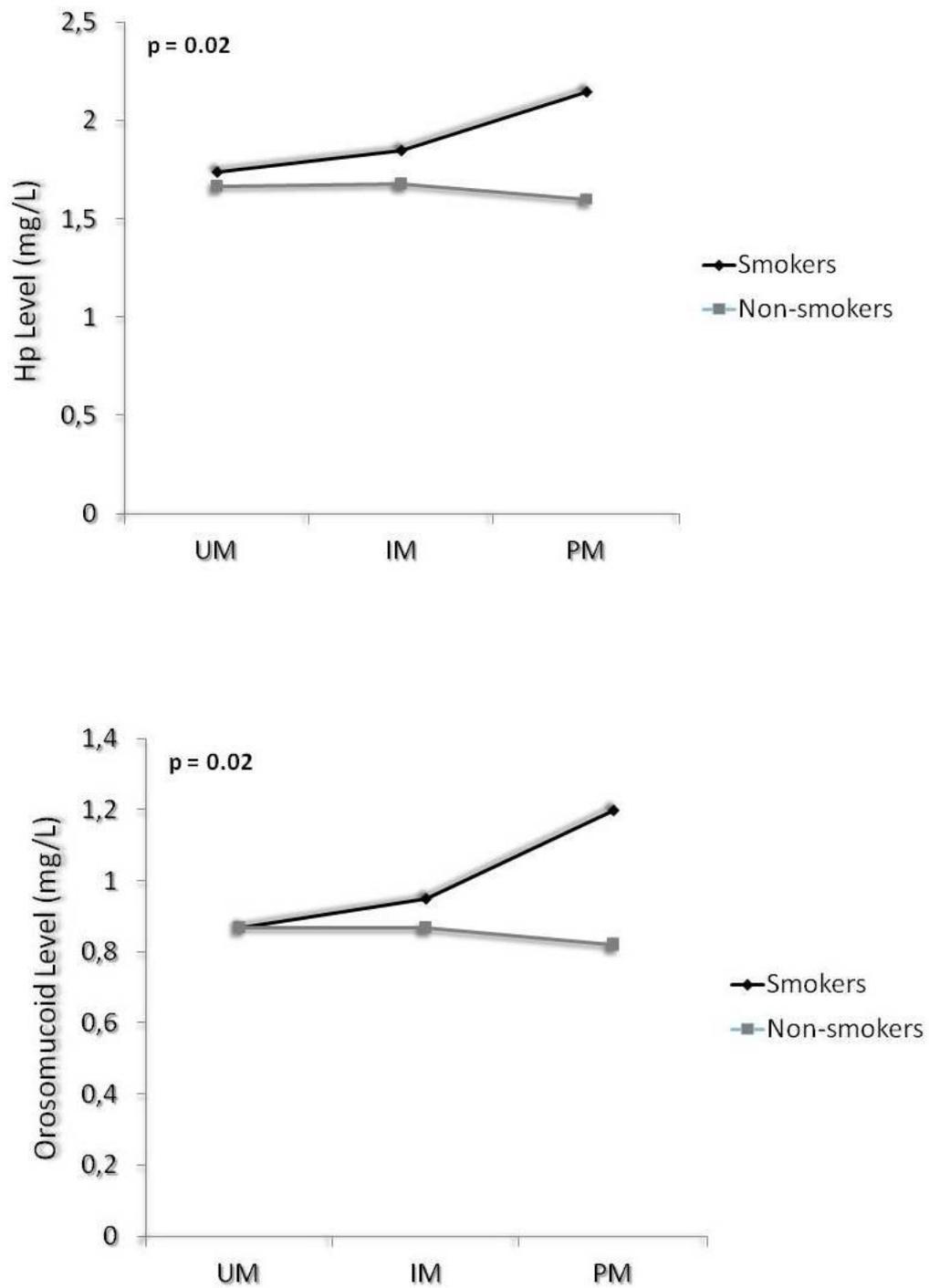


Figure 3, 4. Interaction between MS and smoking is associated with levels of Hp and orosomucoid acid. MS: metabolizing status, PM: poor metabolizer (*2 carriers/*17 non-carriers), IM: intermediate metabolizer (*2 carriers/*17 carriers or *2 non-carriers/*17 non-carriers), UM: ultrametabolizer (*2 allele non-carriers/*17 carriers)

IV. Association between CRP Levels and High On-treatment Platelet Reactivity in Post-PCI on-Thienopyridine Patients

Submitted in the International Journal of Cardiology

Association entre taux de PCR et forte réactivité plaquettaire sous traitement chez les patients en post-ICP sous thiénoopyridines

La bithérapie antiagrégante plaquettaire par aspirine et une thiénoopyridine, dont le clopidogrel et le prasugrel, réduit d'une façon spectaculaire l'incidence des complications thrombotiques après syndrome coronarien aigu et intervention coronarienne percutanée (ICP). Toutefois, de multiples études ont mis en évidence une nette variation interindividuelle dans la réponse tant au clopidogrel qu'au prasugrel et établi que les patients à forte réactivité plaquettaire malgré un traitement par thiénoopyridines sont exposés à un plus grand risque d'échec thérapeutique se manifestant sous la forme d'événements cardiovasculaires ischémiques et de thrombose à l'intérieur du stent. La littérature, néanmoins, manque de cohérence au sujet de la définition optimale de forte réactivité plaquettaire en post-ICP sous thiénoopyridines. Si les modifications absolues ou relatives de l'agrégation plaquettaire par rapport à l'état initial semblent constituer le plus fiable indicateur d'efficacité antiagrégante, il a été démontré que ce n'était peut-être pas le meilleur moyen d'identifier les patients à haut risque. A la place, l'idée a été émise que le niveau absolu de réactivité plaquettaire au cours du traitement (c'est-à-dire, la réactivité plaquettaire sous traitement) serait une méthode plus exacte de dépistage des patients à haut risque d'événements thrombotiques. Sur la base d'une récente définition consensuelle, la forte réactivité plaquettaire sous traitement en post-ICP peut être déterminée à l'aide de quatre critères, dont l'un est un indice de réactivité plaquettaire (IRP) supérieur à 50 % par analyse de la phosphoprotéine stimulée par vasodilatateur (VASP).

Il est désormais bien admis que les plaquettes activées jouent, au-delà de leur contribution à la formation de thrombi, un rôle crucial dans le déclenchement et l'entretien de l'inflammation et que la réponse inflammatoire à l'acte interventionnel est considérée comme le mécanisme clé des complications post-ICP. En conséquence, il a été démontré que les taux de marqueurs inflammatoires et notamment de protéine C réactive (PCR) chez les patients subissant une ICP sont d'utiles prédicteurs de problèmes cliniques en post-ICP. Collectivement, il serait logique de supposer que des niveaux accrus d'activation plaquettaire sont potentiellement associés à des taux plus élevés de marqueurs inflammatoires et, de ce fait, à une incidence supérieure d'événements cardiovasculaires après ICP et pose de stent.

Aussi avons-nous recherché dans une importante population de patients de race blanche en post-ICP (n = 1128) qui ont quitté l'hôpital avec soit du clopidogrel à raison de 75 mg/jour (Clo75 ; n = 118), soit du clopidogrel à raison de 150 mg/jour (Clo150 ; n = 631), soit du prasugrel à raison de 10 mg/jour (Pra10 ; n = 309), si une forte réactivité plaquettaire sous traitement (IRP VASP > 50%) à l'issue d'un mois de bithérapie antiagrégante plaquettaire était associée à des taux plus élevés de PCR.

Une analyse de régression multivariée a montré l'absence de toute corrélation entre une forte réactivité plaquettaire sous traitement et des taux plus élevés de PCR au sein des groupes de traitement « Clo » ($p = 0,213$ et $p = 0,615$ pour les groupes respectifs Clo75 et Clo150). Dans le groupe Pra10, toutefois, on a pu constater que les patients à forte réactivité plaquettaire sous traitement présentaient des taux de PCR plus élevés que ceux chez qui les niveaux d'activation plaquettaire étaient faibles ($p = 0,012$). A notre connaissance, il s'agit là de la première étude à avoir exploré l'association d'une forte réactivité plaquettaire sous traitement aux taux de PCR dans une importante population de patients en post-ICP sous thiénoopyridines.

Association between CRP Levels and High on-treatment Platelet Reactivity in Post-PCI on-Thienopyridine Patients

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Dual anti-platelet therapy with aspirin and a thienopyridine, including clopidogrel and prasugrel, dramatically reduces the incidence of thrombotic complications after acute coronary syndrome, and percutaneous coronary intervention (PCI). However, multiple studies have demonstrated that there is a marked inter-individual variation in response to both clopidogrel and prasugrel (1), and that the patients with high platelet reactivity despite treatment with thienopyridines, are more likely to suffer therapeutic failure manifested by ischemic cardiovascular events and stent thrombosis (2). Nevertheless, there is inconsistency in the literature regarding the optimal definition of high platelet activity in on-thienopyridine post-PCI patients (3). While absolute or relative changes in platelet aggregation from baseline, seems to be the most reliable indicator of anti-platelet efficacy, it has been shown that it may not be the optimal method to identify high risk patients (3). Instead, the absolute level of platelet reactivity during treatment (i.e., on-treatment platelet reactivity) has been suggested to be a more accurate way of detecting the patients who are at high risk of thrombotic events (3). Based on a recent consensus definition, the post-PCI high on-treatment platelet reactivity can be determined by four criteria, one of which is platelet reactivity index (PRI) > 50% by vasodilator-stimulated phosphoprotein (VASP) analysis (3).

It is now well accepted that, beyond their contribution to the thrombus formation, activated platelets play a crucial role in the initiation and maintenance of inflammation and that inflammatory response to the interventional procedure has been attributed as the key mechanism of post-PCI complications (4). Accordingly, the levels of inflammatory markers, particularly C-reactive protein (CRP), in patients undergoing PCI have been demonstrated to be useful predictors of post-PCI clinical problems (5). Collectively, it would be logical to speculate that higher levels of platelet activation are potentially associated with higher levels of inflammatory markers and consequently with an increased incident of

cardiovascular events after PCI and stenting. Therefore, in a large post-PCI white patients (n = 1128) who were discharged with either clopidogrel 75 mg/d (Clo75; n = 118), clopidogrel 150 mg/d (Clo150; n = 631) or prasugrel 10 mg/d (Pra10; n = 309), we investigated whether high on-treatment platelet reactivity (VASP PRI > 50%) after one-month treatment with dual anti-platelet therapy is associated with higher CRP levels. Also, the association of on-treatment VASP PRI with the plasma levels of two additional acute phase proteins (Hp and orosomucoid acid) was evaluated.

The level of phosphorylated VASP was determined using a standardized flow cytometric assay (platelet VASP, Diagnostica Stago/BioCytex, Asnières, France) and the VASP PRI was calculated from the median fluorescence intensity (MFI) of samples incubated with prostaglandin E1 (PGE1) or PGE1 and adenosine diphosphate (ADP) according to the formula: $VASP\ PRI = [MFI_{(PGE1)} - MFI_{(PGE1 + ADP)}] / MFI_{(PGE1)} \times 100$. Also, CRP levels were determined using enzyme-linked immunosorbent assays and the plasma levels of Hp and orosomucoid were determined immunonephelometrically, using BN II autoanalyzer (Siemens Healthcare Diagnostics, Germany).

For those variables of which the distribution exhibited a long-tailed positive skewness and kurtosis, \log_{10} transformed values were used for analysis. Correlation and univariate regression analyses were used to evaluate the relationship between the CRP/Hp/orosomucoid levels and other variables and multivariate regression analysis was used to identify the variables that are independently related to the levels of these markers. Statistical analyses were performed using SAS software package version 9.3 (SAS Institute Inc., Cary, NC) and for all analyses, statistical significance was set at $p \leq 0.05$.

Multivariate regression analysis showed that in Clo treatment groups, the CRP levels were non-significantly higher in those patients who had high on-treatment platelet reactivity ($p =$

0.213 and $p = 0.615$ for Clo75 and Clo150 groups, respectively). In Pra10 group, however, patients with high on-treatment platelet reactivity were found to have significantly higher CRP levels than those with low levels of platelet activation ($p = 0.012$) (Figure 1). It should be noted that neither stent type used (i.e. bare metal stents vs. drug-eluting stents), nor concomitant treatment with other drugs, including statins, were associated with the CRP levels in our population. Likewise, after multivariate regression analysis, the high on-treatment platelet reactivity was significantly associated with higher level of orosomucoid in Pra10 group ($p = 0.002$); in Clo groups, however, the difference was not statistically significant ($p = 0.863$ for Clo75 and $p = 0.271$ for Clo150) (Figure 2). In addition, the Hp levels were significantly higher in those patients of Clo75 and Pra10 groups who had high on-treatment platelet reactivity ($p = 0.037$ and $p < 0.001$, respectively). In Clo150 group, Hp level was non-significantly higher in patients with high on-treatment platelet reactivity (Figure 3).

To our knowledge, this is the first study investigating the association of high on-treatment platelet reactivity with the plasma acute phase protein levels in a large post-PCI, on-thienopyridine patient population. Our data show that compared with those patients with low levels of on-treatment platelet activation, there is a positive and marked increase in the plasma levels of acute phase proteins in post-PCI patients who have high on-treatment platelet reactivity after one month treatment with prasugrel. Also, there was a general trend toward increased acute phase proteins in patients treated with clopidogrel with high on-treatment platelet reactivity. Accordingly, the plasma levels of these markers, particularly CRP, might be potentially a reliable and independent indicator of and a surrogate for the levels of platelet activation, particularly in on-prasugrel patients. Further studies are needed to confirm our findings and also to determine optimal threshold values for CRP and other

markers correlating reliably with high on-treatment platelet reactivity. Optimistically speaking, in this case CRP measurement, as a cheap and easily accessible biomarker, could be used as the substitute of platelet aggregometry, as a relatively expensive and not-easily available test, for prognostic stratification of post-PCI patients in daily medical practice.

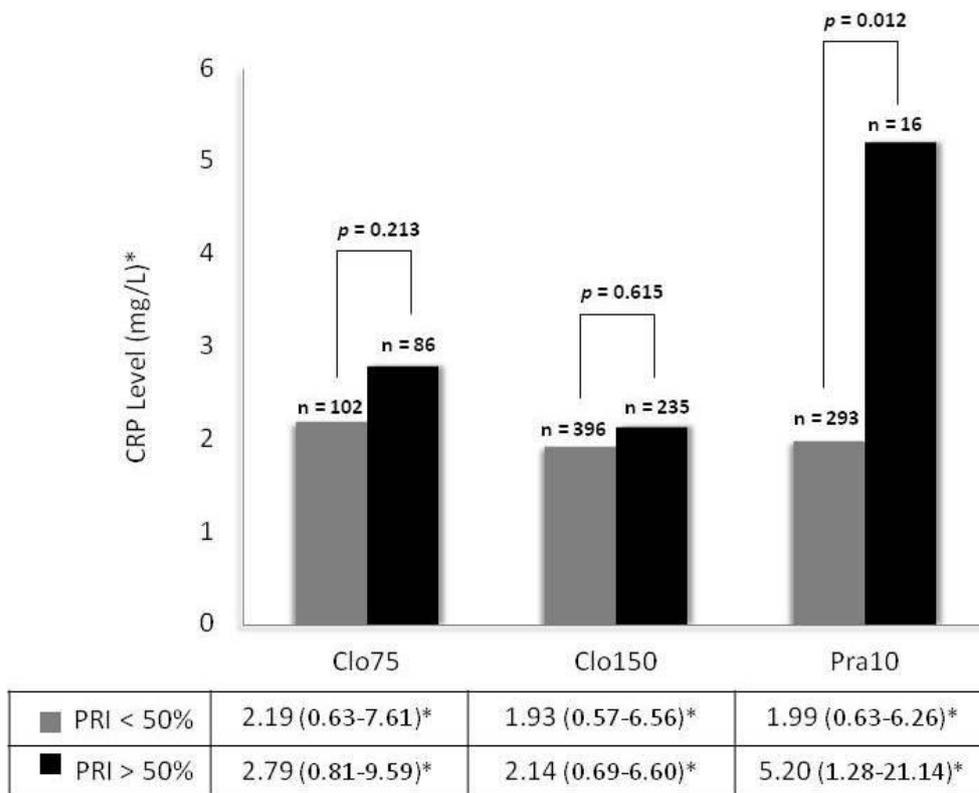


Figure 1. Association of CRP levels with high on-treatment platelet reactivity in the three treatment groups. *Log10 transformed values; PRI: platelet reactivity index

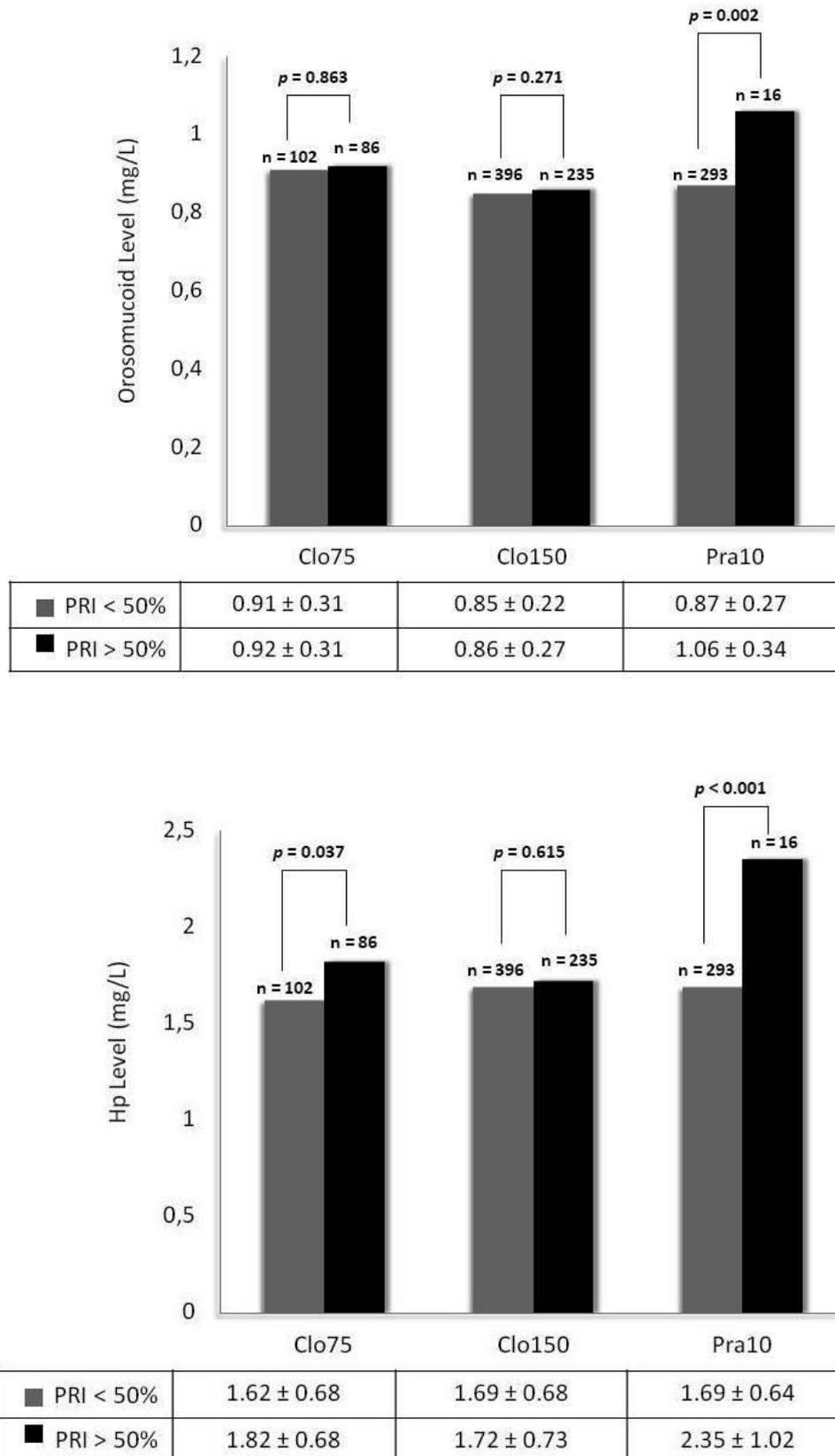


Figure 2, 3. Association of orosomucoic acid and Hsp levels with high on-treatment platelet reactivity in the three treatment groups

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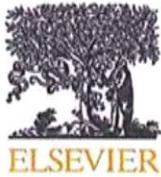
V. Newly Identified Synergy Between Clopidogrel and Calcium Channel Blockers for Blood Regulation Possibly Involves CYP2C19rs4244285

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Il est possible que la synergie nouvellement identifiée entre le clopidogrel et les inhibiteurs calciques pour la régulation sanguine implique le CYP2C19 rs4244285

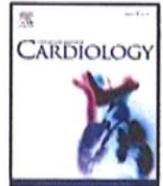
Outre son action antiagrégante plaquettaire, il a été démontré que le clopidogrel exerçait un effet protecteur sur la fonction endothéliale et la biodisponibilité du monoxyde d'azote en réduisant le stress oxydatif et l'inflammation, ce qui a permis de formuler l'hypothèse d'un lien avec la régulation de la pression artérielle (PA). Par ailleurs, des interactions existent entre le clopidogrel et un certain nombre de médicaments prescrits sur une grande échelle, dont les inhibiteurs calciques (IC, couramment utilisés dans le traitement de l'hypertension). Il a été démontré que ces interactions retentissent sur la pharmacocinétique / pharmacodynamie du clopidogrel. Des travaux sur les interactions entre clopidogrel et IC ont fait état d'une moindre inhibition de la fonction plaquettaire chez les patients traités par IC, ceci étant susceptible de réduire l'efficacité clinique du clopidogrel, alors que d'autres ont conclu à la possible absence d'importance clinique de cette interaction chez différents types de patients. L'effet conjoint de ces médicaments sur les niveaux de PA n'a cependant pas été étudié. De plus, ces interactions pourraient aussi être influencées par le génotype CYP2C19, hypothèse que nul n'avait encore fait valoir auparavant.

Nous avons constaté un effet synergique du clopidogrel et des IC sur la réduction de la PA. La concurrence métabolique entre ces médicaments, qui aboutit à une plus faible bioactivation du clopidogrel, pourrait également se traduire par une moindre inactivation des IC, conduisant à un renforcement de l'effet hypotenseur. Une association originale entre un polymorphisme CYP2C19 et les niveaux de PA a en outre pu être observée. Il a récemment été démontré que le CYP2C19 et le CYP3A4 améliorent la 21-hydroxylation de la progestérone en cas de déficit en 21-hydroxylase. Cet effet était modifié par un allèle de gain de fonction du CYP2C19. Cette étude a conclu que le CYP2C19 et le CYP3A4 pouvaient, par cette action, moduler les taux de minéralocorticoïdes tels que l'aldostérone et que les polymorphismes CYP2C19 jouaient peut-être un rôle. Ceci nous a permis de formuler l'hypothèse de la possibilité de l'implication d'un polymorphisme de perte de fonction comme le rs4244285 dans la régulation de l'aldostérone et, donc, dans les niveaux de PA.



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Letter to the Editor

Newly identified synergy between clopidogrel and calcium-channel blockers for blood pressure regulation possibly involves CYP2C19 rs4244285[☆]

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Apart from its antiplatelet action, clopidogrel has been shown to exert a protective effect on endothelial function and nitric oxide bioavailability by reducing oxidative stress and inflammation [1,2], thus a link with blood pressure (BP) regulation could be hypothesized. Additionally, there are interactions between clopidogrel and a number of widely prescribed medications including calcium-channel blockers (CCB, commonly used for hypertension treatment). These interactions have been shown to affect the pharmacokinetics/pharmacodynamics of clopidogrel [3]. Studies on the interactions between clopidogrel and CCBs have reported a decreased platelet inhibition in patients treated with CCBs [4,5], which may decrease the clinical efficacy of clopidogrel [6], while others have concluded that this interaction may not have a clinical importance in different types of patients [7,8]. However, the joint effect of these medications on BP levels has not been studied. Furthermore, these interactions could also be influenced by CYP2C19 genotype [3]; a hypothesis not previously assessed.

We aimed to investigate 1) the effect of clopidogrel use on BP levels in a large sample of cardiovascular disease patients, 2) whether the co-treatment with CCB may modify BP levels, and 3) the contribution of a common CYP2C19 polymorphism (rs4244285) on the possible relationship between clopidogrel-related metabolism and BP.

This investigation was conducted according to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Hospital and University Center of Coimbra, Coimbra, Portugal and the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine", Nancy, France. All individuals gave written informed consent.

Hospitalized patients in the Coimbra University Hospital, admitted between May 2004 and July 2011, for acute myocardial infarction or unstable angina, were recruited. Based on complete availability of data, a sample of 2322 patients participated in the study (Table 1). Data collected included age, gender, weight, height and body mass index (BMI). Systolic (SBP) and diastolic (DBP) blood pressure were measured at rest under constant temperature (19 °C–21 °C) and standardized conditions (supine position) using a manual sphygmomanometer (Colonne à mercure, Mercurius) by expert nurses. The recorded values were the means of three readings on 20 min intervals. An adjustable BP cuff was used to correct errors due to variations in arm circumference. During the procedure of medical history assessment by medical doctors (blinded to BP levels), data for pharmacological therapy prior to admission were recorded. Analyses were performed using the SPSS® software version 16.0 (SPSS, Inc, Chicago, Illinois) and significance was assessed at a two-tailed $P = 0.05$ level. Multiple linear regression models adjusted for age, gender, BMI, acetylsalicylic acid (ASA) use, treatment with any anti-hypertensive medication and/or statins were applied to assess the effect of clopidogrel use on BP. Clopidogrel was shown to be significantly associated with a lower DBP ($P = 0.034$, $\beta = -1.47$ mmHg), however, when adjusting for statins use, this association was no longer significant ($P = 0.216$). No significant association was observed between clopidogrel and SBP ($P = 0.233$).

Due to the observational nature of the study, interactions between clopidogrel and CCB could not be directly assessed. Thus, the sample of patients was split in CCB ($n = 535$) and non-CCB ($n = 1787$) groups. Similar multiple regression models were applied separately

[☆] All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Table 1
Cardiovascular disease patients and healthy individuals' characteristics.

	Cardiovascular disease patients (n = 2322)			Healthy individuals (n = 2155)		
	%	Mean	Standard deviation	%	Mean	Standard deviation
Age (years)		68.6	11.6		42.7	8.9
Body mass index (kg/m ²)		27.6	4.0		25.9	4.1
Systolic blood pressure (mmHg)		137.7	24.1		127.3	16.9
Diastolic blood pressure (mmHg)		73.5	13.8		79.24	11.2
Male gender	68.4			63.0		
Clopidogrel	23.5					
Acetylsalicylic acid	48.2					
Angiotensin-converting-enzyme inhibitor	48.0					
Beta blockers	35.0					
Calcium-channel blockers	23.0					
Diuretics	27.1					
Statins	48.4					

in the two groups. Clopidogrel was significantly associated with low DBP only in the CCB group (adjusted for age, gender, BMI, use of ASA and antihypertensive medication) ($P = 0.030$, $\beta = -3.07$ mmHg) and no association was detected in the non-CCB group ($P = 0.193$). These results remained unchanged after adjustment for statin use. Therefore, clopidogrel seems to improve the DBP lowering effect of CCB. The analysis for SBP did not give significant results.

Additionally, the physiological contribution of CYP2C19 polymorphism to the regulation of BP was investigated on 2155 unrelated supposed healthy adults of European origin, from populations of the Biological Resources Center "Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire" in Nancy, France (Table 1). Inclusion criteria were: no antihypertensive therapy at recruitment and complete clinical and genotypic data. All individuals underwent complete medical examination including anthropometric and biochemical measurements [9]. BP levels were measured as previously described. Genomic DNA was extracted from peripheral blood samples using the salting out method. Polymorphism rs4244285 was genotyped using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system (<http://www.kbioscience.co.uk/reagents/KASP/KASP.html>). Call rate was >90% and minor allele frequency (A allele) was 0.14. Statistical analyses were performed using Plink v01.7 (additive model, minor allele as reference). Hardy-Weinberg equilibrium was tested using the chi-square test ($P = 0.164$). The assessment of the effect of rs4244285 on BP was performed with multiple linear regression models adjusted for age, gender, and BMI at a two-tailed $P = 0.05$ level. The variant rs4244285 was significantly associated with low values of SBP and DBP (SBP: $P = 0.008$, $\beta = -1.69$ mmHg and DBP: $P = 0.003$, $\beta = -1.28$ mmHg).

In conclusion, we found a synergic effect of clopidogrel and CCB on the reduction of BP. The metabolic competition between these medications, which results in decreased bioactivation of clopidogrel [5], could also result in decreased inactivation of CCB, leading to enhance BP lowering effect. Furthermore, a novel association between a CYP2C19 polymorphism and BP levels was observed. Recently, CYP2C19 and CYP3A4 were shown to improve 21-hydroxylation of progesterone in

21-hydroxylase deficiency patients. This effect was modified by a gain-of-function allele of CYP2C19. This study concluded that both CYP2C19 and CYP3A4 could through this action modulate the levels of mineralocorticoid such as aldosterone and that CYP2C19 polymorphisms might play a role [10]. We could therefore hypothesize that a loss-of-function polymorphism like rs4244285 could be implicated in aldosterone regulation and thus in BP levels.

Further studies are warranted to confirm and extend these findings. As these two medications are commonly co-prescribed in cardiovascular patients, the understanding of their exact simultaneous clinical effects and the identification of the underlying mechanisms are of utmost importance for the outcome and safety of patients. Our study provides a baseline and important insight for subsequent studies to elucidate the pharmacogenomic mechanisms involved in the clopidogrel \times CCB interactions in BP regulation and their clinical implications in daily medical practice.

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The authors would like to thank the study participants for helping in the creation of the present study. They would like to thank also all the field investigators for the recruitment and examinations of the populations involved in this study.

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CHAPTER 5 – GENERAL DISCUSSION AND PERSPECTIVES

I. Thesis main findings

This thesis was performed based on the following scientific evidences: 1) inflammation has a great contribution to the pathogenesis of a large number of disorders including CVDs; 2) platelets are important for primary hemostasis, but they also play an important pro-inflammatory role; 3) CYP enzymes play a central role in the metabolism and overall disposition of the endobiotics (e.g. EETs) and xenobiotics (e.g. thienopyridines) that can potentially influence on the inflammatory responses, in the human body; 4) the common SNPs in CYP and inflammatory marker genes are associated with altered activity and level of the corresponding enzymes and marker of inflammation, respectively; and 5) the great variability among different individuals in response to antiplatelet effects of thienopyridines is attributed mainly to the CYP2C19 polymorphisms and also, in some cases, to smoking (“smoker’s paradox”). Accordingly, the main results of the thesis are summarized as follow:

A): Our findings on a small population of healthy males, failed to support the hypothesis that the genetic variations of CYP epoxygenases are associated with the level of markers of systemic inflammation. **B):** Our observations in this population also could that demonstrate that *CYP2C19** and *P2Y12 H* polymorphisms are associated with the variability in response to the anti-inflammatory properties of clopidogrel.

C): In a large population of post-PCI patients who were on clopidogrel or prasugrel, we found that CYP2C19 loss- and gain-of-function polymorphisms (**2* and **17*) were associated with the level of on-treatment platelet reactivity; however, these SNPs were not, independently, associated with the level of inflammatory markers in this population. Nevertheless, the

interaction of thienopyridine poor metabolizing status and smoking has been found to be a strong regulator of systemic inflammation; this effect was seemingly independent to the platelet activity, since the CYP2C19 SNPs – smoking interaction was not associated with levels of on-treatment platelet reactivity determined by VASP PRI. Also, although our findings did not provide supporting evidence for that aspect of “smoker’s paradox” phenomenon in which smoking may positively modify the beneficial anti-platelet effects of thienopyridines, they may present a new aspect of the phenomenon suggesting that smoking is associated with an increase in the anti-inflammatory effects of thienopyridines.

D): In this population, we also observed that the patients who were on prasugrel, but not clopidogrel, and had high on-treatment levels of platelet reactivity, defined as VASP PRI > 50%, had higher levels of systemic inflammatory markers.

E): Additionally, to demonstrate the clinical necessity of reference range and the importance of genetics in the partitioning of inflammatory markers reference values, we determined the reference intervals of Hp in the function of its main sources of biological variation, including gender, age, smoking, plasma hemoglobin levels and the newly-discovered Hp polymorphism, rs2000999. We found that the median Hp level is >150% and >200% higher, respectively in males and females who were homozygous for wild type allele compared with those who were homozygous for mutated allele.

II. Inflammation, CVD and post-PCI ISR

The incidence and prevalence of CVD has increased remarkably in recent years. Currently, CVD remains the leading cause of death in the developed countries and the second common cause worldwide (1). According to the World Health Organization, by the year 2020, CVDs will surpass the infectious diseases and become responsible of nearly 40% of all death throughout the world, more than twice the percentage of deaths from cancer (2,3).

Accumulating lines of evidence have supported the concept that inflammation contributes to the initiation, progression and clinical manifestations of CVDs (4-8). Inflammation participates centrally in all stages of atherosclerosis from the initial lesion to the thrombotic complications. As such, atherosclerosis is recognized currently as a chronic inflammatory of large arteries (9,10).

Vascular inflammation is characterized by a complex interaction network consisting of activated ECs, inflammatory leukocytes, SMCs and activated platelets – as an important source of inflammatory mediators. These interactions trigger autocrine and paracrine activation cascades that results in leukocyte recruitment in the vascular wall (11,12).

1. Platelets: key players in vascular inflammation

Platelets are anucleated cells of 1–2 μm in length and pinched-off multinuclear megakaryocytes in the bone marrow (13) that provides an important linkage between

inflammation, thrombosis and atherosclerosis (11). Here, we briefly discuss about the key role of platelets in the inflammatory responses.

It has been demonstrated that activated platelets express a number of surface molecules (Figure 1), and also a multitude of immune receptors which enables the platelets to interact with ECs and leukocyte which facilitates cell activation and cell migration/recruitment to sites of vascular injury and inflammation (13).

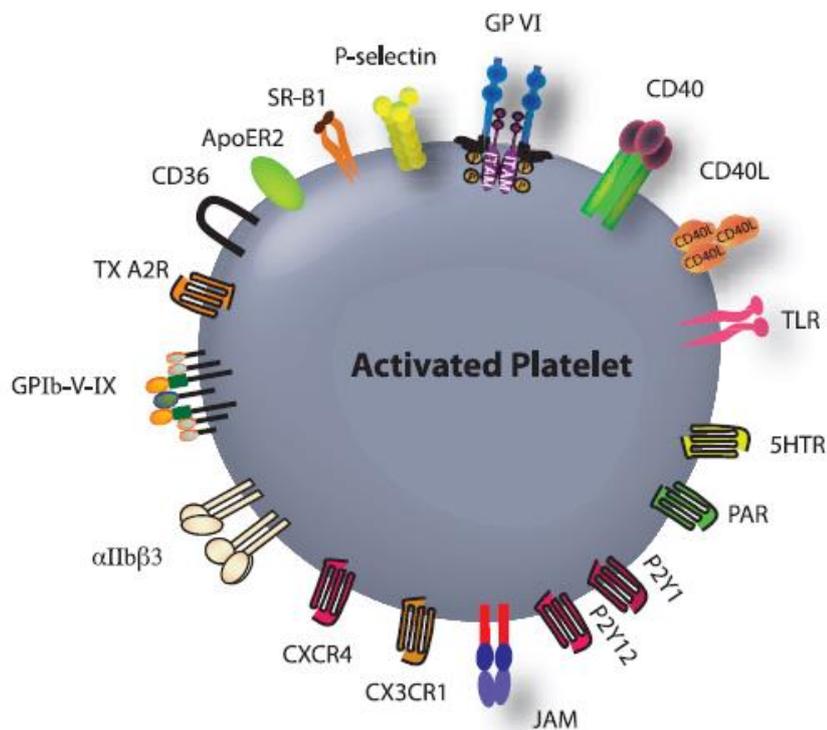


Figure 1. Surface molecules expressed by activated platelets that facilitate platelet-EC, platelet-leukocyte crosstalk

1.1. Platelet interaction with ECs

Under physiological conditions, the intact, non-activated endothelium normally prevents platelet adhesion to the ECs through inhibitory and modulating mechanisms involving COX-2, PGI₂, or prostanoid synthetic systems (11). However, through “transient” and “stable” interactions with activated ECs, platelets can induce and propagate a chronic inflammatory

response in the vessel wall, resulting in the development and progression of inflammation (11,12) (Figure 2A).

The initial loose contact between circulating platelets and vascular endothelium (“platelet rolling”) occurs through P-selectin presented on the ECs (14,15). P-selectin is an integral membrane GP expressed by platelets, ECs, and macrophages of atherosclerotic plaques (16). Normally, P-selectin is stored in the inactivated ECs, but is rapidly transported to the vessel wall after endothelial activation, allowing leukocytes and also platelets to interact quickly with the endothelium during inflammation (11). Also, activated platelets are able to express functional PSGL-1, which assists in the platelet rolling in stimulated mesenteric venules (15). Activated platelets also express the GPIb-IX-V complex as an alternative receptor for rolling over endothelial P-selectin (17). The interaction between P-selectin and PSGL-1 or GPIb/IX/V, is rapidly reversible and not enough strong for stable adhesion. Conversion to stable adhesion needs additional contacts between the platelet and the ECs (11) (Figure 2A).

Stable or firm adhesion of platelets to the activated endothelium is mainly mediated by β_3 integrins ($\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$) (18,19). In fact, the interaction occurs between GPIb with vWF bound to the ECs via GPIb or the vitronectin receptor ($\alpha_v\beta_3$) (18). Also, ICAM-1 on activated endothelium may bind to fibrinogen, which in turn, binds to $\alpha_{IIb}\beta_3$ on platelets (19). Fibronectin can also make such a bridge between endothelial $\alpha_v\beta_3$ and platelet $\alpha_{IIb}\beta_3$ similarly (18) (Figure 2).

The platelet-EC adhesion seems to be the first step in the initiation and progression of vascular inflammation. Indeed, the adhesion process activates platelets and the activated platelets are capable to release a large number of potent inflammatory substances into the local microenvironment which alter chemotactic, adhesive, and proteolytic properties of ECs. These platelet-induced alterations of the endothelial phenotype lead eventually to

chemotaxis, adhesion and transmigration of monocytes to the site of inflammation (11,13)

(Figure 2B).

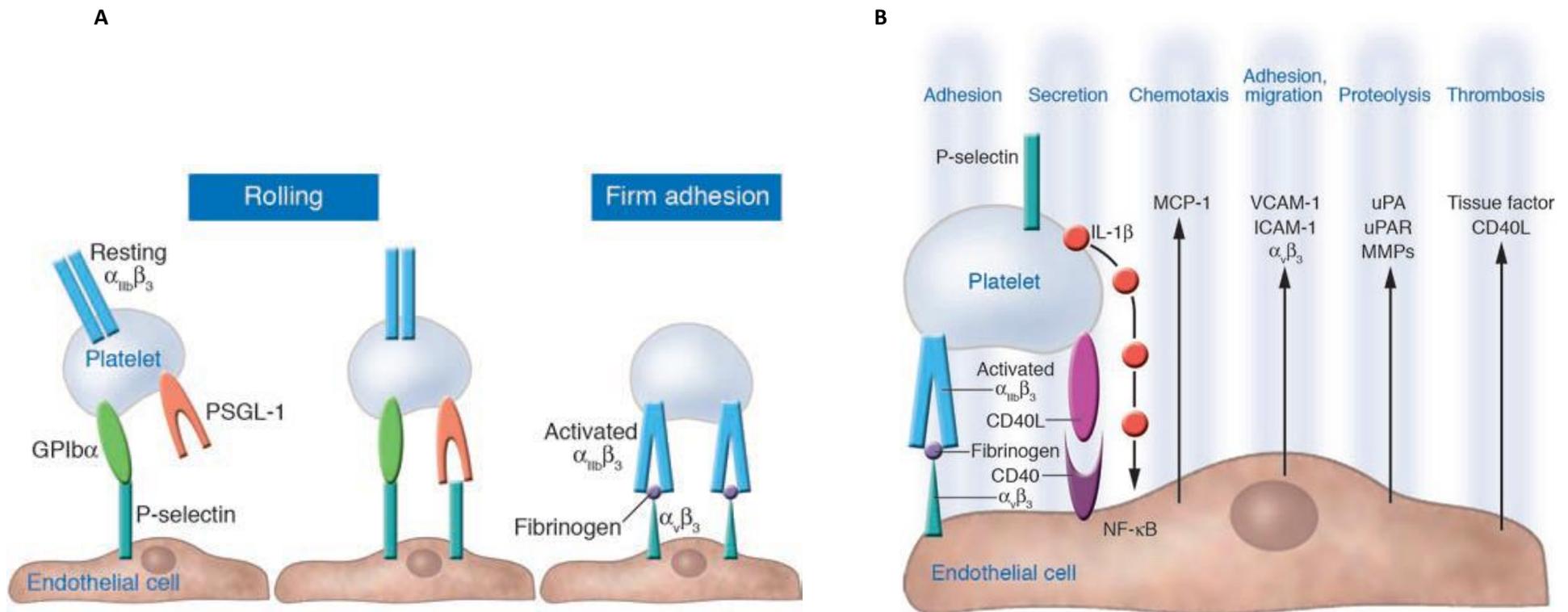
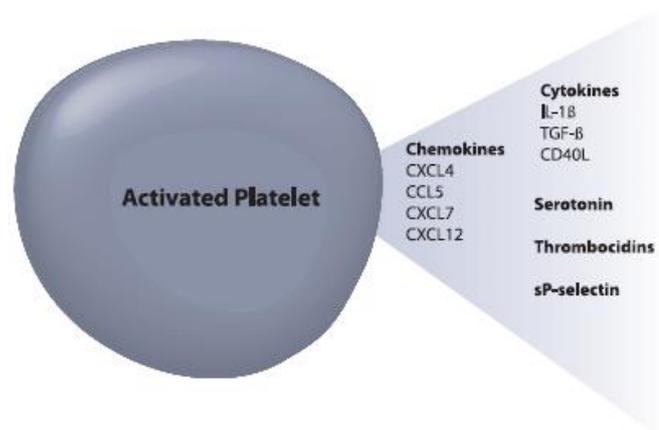


Figure 2. A. Platelet-endothelium adhesion. Activated endothelium surface expresses P-selectin. Platelet surface receptors GPIIb α and PSGL-1 interact with endothelial P-selectin and mediate platelet rolling. Subsequent firm adhesion is mediated through β_3 integrins. **B.** Adherent platelets inflame ECs. Firm platelet adhesion involving $\alpha_{IIb}\beta_3$ induces platelet surface exposure of P-selectin (CD62P) and release of CD40L and IL-1 β , which stimulate ECs to provide an inflammatory milieu that supports pro-atherogenic alterations of endothelium.

In general, the activated platelets secrete adhesion proteins (e.g., fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GPIIb/IIIa), growth factors (e.g., PDGF, TGF- β , EGF, bFGF), chemokines (e.g., RANTES, platelet factor 4 [CXC chemokine ligand 4], epithelial neutrophil-activating protein 78 [CXC chemokine ligand 5]), cytokine-like factors (e.g., IL-1 β , CD40 ligand, β -thromboglobulin), and coagulation factors (e.g., factor V, factor XI, PAI-1, plasminogen, protein S) (11,13) (Figure 3). These proteins act in a concerted to regulate finely different biological functions such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis, all of which accelerate inflammatory processes and cell recruitment. For instance, IL-1 β has been discovered to be a major mediator of platelet-induced activation of ECs promoting neutrophil and monocytes adhesion to the endothelium (20,21). It has been shown that through activation of NF- κ B in the ECs, IL-1 β modifies endothelial release of chemotactic protein (e.g. MCP-1) and also the adhesion molecules (e.g. ICAM-1) (22,23).

Figure 3. Upon activation, platelets release chemokines and cytokines which, respectively, attract other cell types towards the site of inflammation and promote the activation of ECs/leukocytes.



1.1. Platelet interaction with leukocytes

Once activated, platelets show robust interaction with circulating leukocytes (24). Also, the adhered platelets to the ECs provide a sticky surface to recruit leukocytes to the vessel wall (11,25).

Leukocytes initially adhere to platelets via PSGL-1–P-selectin interactions (26,27) and, subsequently, firmly adhere via binding of Mac-1 (CD11b/CD18, $\alpha\text{M}\beta 2$) to GPIIb α (28) and/or other receptors of the platelet membrane, including JAM-3 (29) and ICAM-2 (30), or bridging proteins such as fibrinogen (bound to GPIIb/IIIa) (31,32) or high-molecular weight kininogen (bound to GPIIb α) (33). In this way, the activated platelets induce the inflammatory cascades in monocytes. The inflamed monocytes release chemokines, cytokines and pro-coagulatory TF, upregulate and activate adhesion receptors and proteases, and induce monocyte differentiation into macrophages (11). Unsurprisingly, the platelet-induced secretion of inflammatory mediators and adhesive molecules is also mediated by NF- κ B (11) (Figure 4).

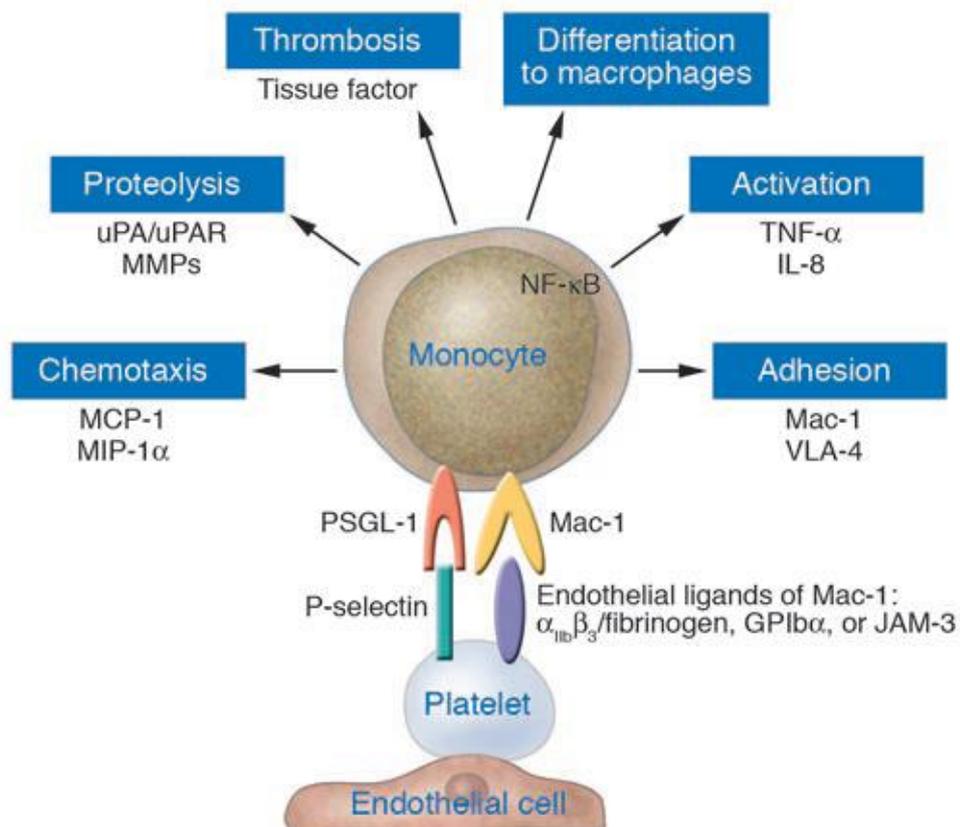


Figure 4. Adherent platelets recruit and inflame monocytes

1.2. *Activated platelets and CRP, Hp and orosomucoid acid*

CRP is an acute-phase protein which has been recognized to be potentially an important etiological factor in inflammation and atherosclerosis (34). In addition, there exists robust evidence that CRP has a clinically-significant prognostic value and among patients presenting with ACS, elevated CRP levels predict future cardiovascular events (35,36).

It has been shown that CRP is synthesized in the liver in response to inflammatory cytokines, mainly IL-6, IL-1 β and TNF- α (4). As mentioned earlier, the activated platelets and also the platelet-induced activated ECs and inflamed monocytes secrete a large amount of various cytokines, particularly IL-1 β . It is, therefore, logical to speculate and makes sense that the activated platelets can potentially provide a bridge between local and systemic inflammatory responses.

Likewise, Hp and orosomucoid acid are classified under the category of acute-phase proteins (37-40). Although in compared to CRP, the role of Hp and orosomucoid acid, as markers of systemic inflammation, have been less studied, they have been demonstrated in several investigations to be reliable indicators of systemic inflammatory response (37-43). Similar to CRP, Hp and orosomucoid acid are overproduced in response to inflammatory stimulators mainly IL-6, IL-1 and TNF- α and (37,41,44,45), thereby, under inflammatory conditions, platelets may indirectly cause an increase in the hepatic production of these inflammatory markers.

Considering all the mentioned points, in this thesis we assumed that the plasma levels of CRP, Hp and orosomucoid acid can indirectly, but reliably, represent the level of platelet activity. However, as we have already acknowledged, the platelet-specific markers, like soluble P-selectin and soluble CD-40, may be more proper indicators of the platelet-induced inflammatory responses. This point has been considered in our ongoing clinical trial (see “*Perspective*”).

2. Inflammation and post-PCI complications

Coronary atherosclerosis is a treatable, but not curable, disease (46) and the introduction of PCI marked a revolutionized therapeutic approach by reducing the incidence of IST after balloon angioplasty (47). However, despite technological advances, the main limitation of PCI, i.e. ISR of the treated vessel, remains a challenge and results in renewed symptoms and the need for target vessel revascularization (48,49).

Although the pathophysiology of ISR has not been fully elucidated, there is convincing evidence that a procedure-evoked inflammatory response is probably the main causative mechanism (48,49). Stent placement leads to platelet activation, mechanical injury and endothelial denudation that triggers the cascade of events including resulting in substantial local and systemic inflammatory responses, which stimulates vascular SMC proliferation and extracellular matrix deposition, neointimal thickening and restenosis (48-51) (Figure 5, 6).

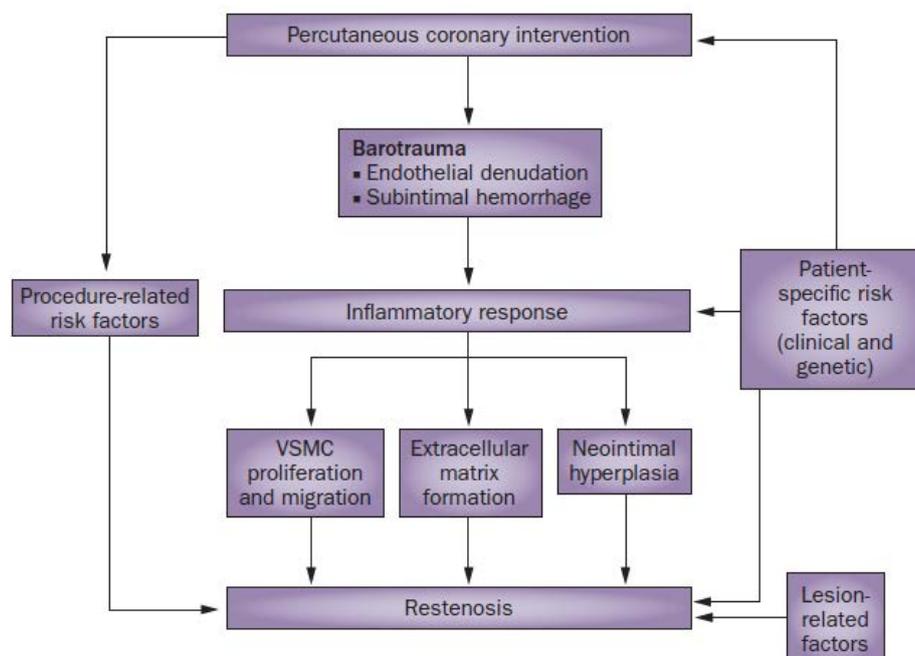


Figure 5. Factors involved in the development of restenosis. Flowchart showing the interactions between the main contributors to the development of restenosis. Abbreviation: VSMC, vascular smooth muscle cell.

Human studies showed that, coronary stents cause disruption of the coronary endothelial layer leading to an initial acute inflammatory response within 0 to 3 days after the procedure, centered at the stent struts (52,53).

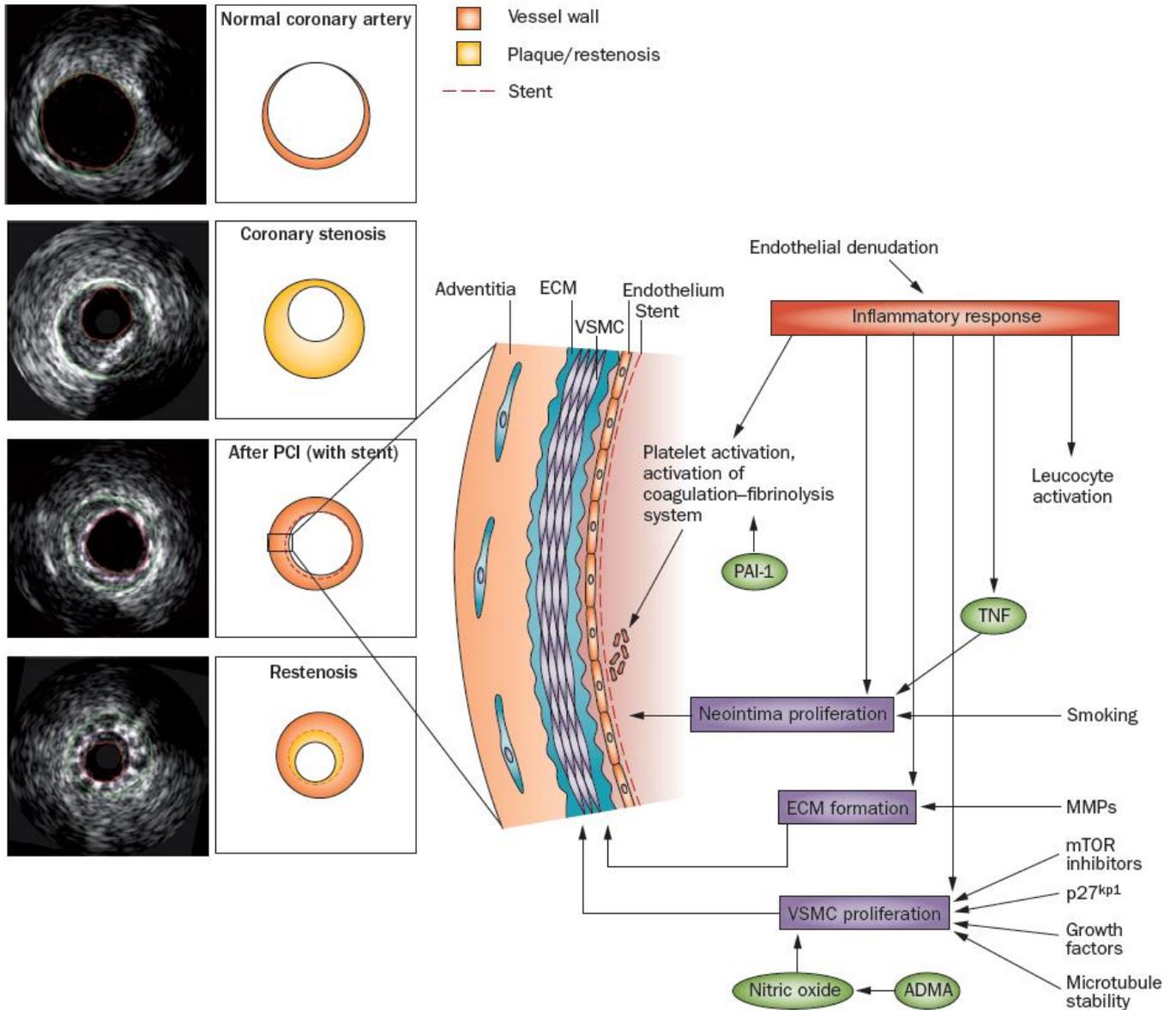


Figure 6. Mechanisms of restenosis. Intravascular ultrasound images of the various stages of coronary artery disease. Indicated are the outer border of the vessel (green), the lumen (red), and the coronary stent (pink). Schematic representations are shown to the right of these images. The processes occurring in the vessel immediately after PCI with stent placement are highlighted in detail. Abbreviations: ADMA, asymmetric dimethylarginine; ECM, extra cellular matrix; MMPs, matrix metalloproteinases; mTOR, mammalian target of rapamycin; PAI-1, plasminogen activator inhibitor 1; PCI, percutaneous coronary interventions; TNF, tumor necrosis factor; VSMC, vascular smooth muscle cell.

By 2 to 4 weeks, acute inflammation subsides and is replaced by chronic inflammatory cells, along with proliferating SMCs associated with organizing thrombus and a thin provisional extracellular matrix. Beyond 30 days, fibrin and chronic inflammation persist, and SMCs and extracellular matrix further enrich the expanding neointimal (53,54).

Considering the fact that inflammation plays a significant role in the development of ISR and that the post-PCI acute inflammatory response is substituted by one month with a chronic inflammation, in this thesis, we studied the potential anti-inflammatory effects of thienopyridines in post-PCI patients after 4 weeks treatment with clopidogrel or prasugrel.

The PCI-induced systemic inflammatory response is evidenced by a rise in the plasma levels of acute-phase proteins like CRP, cytokines, chemokines and soluble forms of adhesion molecules (48,49,53,55). Multiple studies have demonstrated that the incidence of cardiovascular events and post-procedural ISR are linked to the magnitude of the systemic inflammation. As such, post-PCI patients with higher levels of inflammatory markers have an increased incidence of ISR (46,52,55,56), and patients undergoing PCI may be risk stratified according to an increase in their concentrations after PCI (56).

Several markers of inflammation have been reported to increase risk of ISR and could be used as predictors of post-PCI complications and adverse events. In addition to CRP, a number of these are as below: white blood cell count (particularly a rise of the cytotoxic T lymphocytes CD3+/CD56+ and activated monocytes CD11b), IL-1, IL-6, TNF- α , lipoprotein (a), serum amyloid A (SAA), MCP-1, certain complement components (C3a and C5a), asymmetric dimethylarginine (ADMA), PAI-1, NO, soluble CD-40L, glycosylphosphatidyl-inositol-80,

MMP-2, MMP-9 and pregnancy-associated plasma protein A (PAPPA). However, since most of these markers are biochemically unstable, expensive or not easily accessible, the predictive value of CRP has been most widely explored. As such the plasma levels of CRP is used for the prediction of post-PCI ISR in the clinical settings (48,49,52,53,57).

Considering the fact that CRP is currently applied in daily clinical practice to assess the risk of ISR, and also due to the technical problems for the measurements of other inflammatory markers, in this thesis, we tested our hypothesis using the plasma levels of CRP. Also, we investigated whether Hp or orosomucoid acid, as two other acute phase proteins, can be used as alternatives of CRP.

In this thesis, unfortunately, we did not have the opportunity to follow the patients and to evaluate if our findings are associated with the clinical outcomes; however, this drawback has been overcome in our ongoing clinical trial (see "*Perspectives*").

2.1. ISR after DES vs. BMS

BMS has been reported, by several clinical and angiographic studies, to be associated with up to 30% restenosis (57,58). The introduction of DES reduced substantially the incidence of ISR (by 70% across broad patient and lesion subsets); however, depending on lesions and procedural lesions, ISR still occurs by 7% to 13% after DES (57-59).

Considering the fact that the incidence of ISR after DES has been reported to be significantly lower than that of after BMS, it should be noted that, in this thesis, we explored to see if there is any difference in our findings between patients treated with DES vs. those who treated with BMS.

III. CYP and Metabolism of EETs and Thienopyridines

The contribution of CYP enzymes to the metabolism of both EETs, as AA-derived endogenously produced substances with potent anti-inflammatory effects, and clopidogrel/prasugrel, the thienopyridines derivatives with potential anti-inflammatory properties, have been comprehensively reviewed in the first chapter, *“Introduction”*. In addition, thienopyridines pharmacogenetics and also the potential influences of CYP epoxygenase SNPs on the biosynthesis of EETs were discussed in detail.

It is easy to note that certain CYPs, specifically CYP2C isoforms, are involved in the metabolism of both thienopyridines and EETs. This fact may provide a basis for studying a real world interaction between xenobiotics and endonbiotics. The potential EET-thienopyridine interaction and its clinical/pharmacological applications warrant further investigations in future.

In this thesis, we made a great effort to measure the plasma levels of EETs in our study populations and to test whether EETs interacts with thienopyridines. However, due to several difficulties in the pre-analytical and analytical phases, we did not succeed to evaluate the hypothesis. Nonetheless, we are trying to investigate, even in a small subset of patients, the hypothesis that there is a CYP-mediated interaction between EETs and thienopyridines.

IV. Thienopyridine Resistance, Non-responsiveness and High On-treatment Platelet Reactivity

As it mentioned in the chapter 1, several studies have shown that there is direct relationship between clopidogrel non-responsiveness and/or high on treatment platelet reactivity measured by multiple platelet assays and adverse clinical ischemic events. However, lack of consensus on the optimal methods to quantify high platelet reactivity and the cutoff values associated with clinical risk, the routine measurement of platelet reactivity has not been widely implemented in clinical practice nor recommended in the guidelines.

1. Resistance, hypo-, non-responsiveness to thienopyridines

The definition of resistance or non-responsiveness to thienopyridines is based on the changes in platelet response (reactivity) to ADP from baseline after thienopyridines intake (60,61). Accordingly, pharmacologic resistance to clopidogrel is defined as an absolute $\leq 10\%$ decrease in platelet aggregation in response to drug (baseline vs. on-treatment) (61,62). In addition, patients are categorized as “nonresponsive,” “semi-responsive,” and “responsive” using absolute platelet inhibition cut points of $<10\%$, 10% to 30% , and $>30\%$, respectively (60,62,63).

2. High on-treatment platelet reactivity

Controversy has surrounded the optimal method to quantify platelet reactivity as well as the threshold definition for high on-treatment platelet reactivity to ADP (60). However, currently, high on-treatment platelet reactivity in the setting of PCI has been defined using the following criteria: 1) PRI $>50\%$ by VASP analysis; 2) >235 to 240 P2Y₁₂ reaction units by

VerifyNow P2Y12 assay; 3) >46% maximal 5 $\mu\text{mol/l}$ ADP-induced aggregation; and 4) >468 arbitrary aggregation units/min in response to ADP by Multiplate analyzer (60).

3. Resistance vs. high on-treatment platelet reactivity

It has been suggested that although a measurement of responsiveness (absolute or relative changes in platelet aggregation from baseline) appears as the most reliable indicator of a treatment effect, it may not be the optimal method to identify patients at high risk (60). Considering the fact that there is inter-individual variability in baseline ADP-induced platelet aggregation, the measurement of thienopyridine responsiveness may overvalue ischemic risk in non-responders with low pre-treatment reactivity as well as underestimate risk in responders who remain with high platelet reactivity after treatment (60,64,65). Alternatively, it has been proposed that the absolute level of platelet reactivity during treatment (i.e., on-treatment platelet reactivity) is a better measure of thrombotic risk than responsiveness to thienopyridines (60).

Since on-treatment platelet reactivity has been proposed to be an acceptable indicator for the quantification of platelet reactivity, in this thesis, we employed on-treatment VASP PRI as a measure of platelet reactivity. It is noteworthy to mention that given the study design of our ongoing clinical trial (see “Perspectives”), we will have the opportunity to test our hypotheses using the measure of both responsiveness and on-treatment platelet reactivity.

V. Smoker's paradox

Cigarette smoking is a well-established risk factor for the development of atherosclerosis and thrombotic cardiovascular events. However, some studies have used multivariate analyses to report what may be termed a “smoker's paradox” whereby on-clopidogrel non-smokers seem to have either less or no CV-event reduction when compared to the substantial CV-event reduction in clopidogrel-treated smokers (66). The proposed mechanism to explain the new smoker's paradox in clopidogrel-treated patients is that cigarette smoking in a dose-related manner induces CYP 1A2 and 2B6 activity which are involved in the bioactivation of clopidogrel, resulting in greater clopidogrel active metabolite generation, an enhanced PD effect and less ischemic event occurrence in current smokers versus nonsmokers. In normal volunteers, CYP1A2 activity was increased relative to the number of cigarettes smoked per day, with a 1.22-, 1.47-, 1.66-, and 1.72-fold increase in activity observed with smoking 1 to 5, 6 to 10, 11 to 20, and >20 cigarettes a day, respectively (66). However, available evidence for an association of smoking with an altered PD response to clopidogrel is highly conflicting; and it is unknown if smoking can affect the PD response to prasugrel in patients undergoing PCI (66-70). While several observations suggest that nonsmokers undergoing PCI exhibit lower clopidogrel-induced antiplatelet effects than smokers and support a relation between smoking status and clopidogrel response (66-70), other investigators have reported no relation (66,67). According to the latter, the reported paradoxical findings could be explained by the presence of systematic errors (including publication bias), residual confounding or different pathogenesis (67). For more detailed review on the influence of cigarette smoking on the PD of clopidogrel, we refer to reviews by Bliden et al. (66), and Aune et al (67).

It should also be mentioned that in addition to the inter-study inconsistency regarding the reduced antiplatelet effect of clopidogrel in non-smokers as compared to smokers, the clinical relevance of this interaction has never been demonstrated in a prospective trial (66). Collectively, and considering the well established harmful effects of smoking, it would be wise to encourage smoking cessation rather than relying on the “positive effects” of the so-called paradox.

Although the influence of smoking on the anti-platelet effects of thienopyridines has been widely explored, this interaction on the potential anti-inflammatory properties of thienopyridines has not been evaluated.

Considering the importance of smoker’s paradox in the anti-platelet effects of clopidogrel, in this thesis, we investigated whether smoking has paradoxical effects on on-treatment platelet reactivity of patients treated with thienopyridines. Also, we aimed to evaluate the potential paradoxical effect of smoking on the plasma levels of inflammatory markers in on-thienopyridine patients.

VI. Necessity of experimental studies?

The main hypothesis of this thesis was to investigate the influences of CYP2C19 polymorphisms on the anti-inflammatory effects of thienopyridines. It should be mentioned that since CYP2C19 gene possesses both functional and non-functional alleles in human, it seems that the hypothesis can be evaluated on the human samples and pre-clinical experimental studies are not necessary.

VII. Perspectives

In general, the perspectives of this thesis are to assess if our findings in the Marseille Study are potentially associated with the clinical outcomes of the recruited patients and also to retest our hypotheses in a well-conducted ongoing project, the Liverpool Collaborative Study.

1. Marseille Study:

As mentioned before, our pharmacogenetic approach to the anti-inflammatory effects of thienopyridines in the Marseille Study was based on the well documented following backgrounds:

1. Activated platelets have a central role in the initiation and progression of inflammation.
2. CYP2C19 loss- and gain-of-function polymorphisms (*2 and *17) influence the response to the anti-platelet effects of both clopidogrel and prasugrel.

Accordingly, we hypothesized that *2 and *17 polymorphisms contribute to the inter-individual variability in response to the anti-platelet effects of thienopyridines and consequently to an inter-individual variability in the inflammation-reducing properties of these medications. Also, since some studies suggested that smoking positively modify response to the anti-platelet effects of clopidogrel, we investigated whether interaction of smoking and CYP2C19 metabolizing status are associated with levels of inflammatory markers.

However, there were two main drawbacks in this study which might limit our ability to test the hypotheses. Firstly, although the markers of systemic inflammation (CRP, Hp and orosomucoid acid) are produced in the liver mainly in response to inflammatory cytokines,

particularly IL-6, which is released from activated platelets, they would not appropriately reflect directly the real levels of platelet activity. Indeed, specific markers of platelet activation, such as soluble P-selectin and soluble CD-40, would be better choices to evaluate our hypothesis. Secondly, in this study the data regarding biochemical profile and platelet aggregation test of patients were available just at one time point, i.e. after, but not before, taking thienopyridines. These limitations have been removed in our ongoing Liverpool collaborative study in which, in addition to markers of systemic inflammation, platelet specific markers as well as a panel of inflammatory cytokines will be determined at two time points, i.e. before and after clopidogrel therapy.

In the Marseille Study, we found interestingly that the interaction of smoking and CYP2C19 poor metabolizing status is associated with higher levels of systemic inflammation. It is noteworthy to mention that, fortunately, the data regarding the clinical outcome of the patients recruited in this study will be available within the next few months and we will, then, have the opportunity to evaluate the potential influences of our findings on the patients' clinical outcomes.

2. Liverpool Study:

Our ongoing Liverpool Collaborative Study, is a prospective study with a before/after design in which we aim, as the main objective, to have a pharmacogenetic approach to the anti-inflammatory effects of clopidogrel. Accordingly, the plasma levels of markers of systemic inflammation as well as a complete panel of inflammatory markers including adhesion molecules and inflammatory cytokines before and at one month and 12 months time points

after taking clopidogrel will be compared in a 1350 patients who presented clinically with ACS.

In this study, in addition to assessment of the potential influences of polymorphisms of genes involved in the PK/PD of clopidogrel on its ability to reduce the levels of inflammatory markers, we have the opportunity to evaluate whether the interactions of genes encoding clopidogrel-metabolizing enzymes with those that encode markers of inflammation (ex. Hp and CRP), are associated with an inter-individual variability in the levels of inflammatory markers in on-clopidogrel patients. Accordingly, the newly-discovered Hp SNP (rs2000999) and a selected number of CRP SNPs will be determined in the enrolled subjects.

More importantly and more interestingly, in this study we have the chance to investigate whether the genetic-mediated inter-individual variability in response to the inflammation-reducing effect of clopidogrel is associated with primary and secondary clinical outcomes.

The outcomes defined as below:

- Primary outcome: Composite outcome measure of cardiovascular mortality, non-fatal myocardial infarction and non-fatal stroke
- Secondary outcome: All cause mortality, bleeding and left ventricular failure

2.1. *EET measurement:*

As mentioned previously, due to technical difficulties, in this thesis we failed to measure the plasma levels of EETs in our study population. In the Liverpool Study, we aim to determine the level of EETs in a subgroup of population. Accordingly, we will have the opportunity to assess if there exists an interaction between clopidogrel and EETs as a real model of endobiotics-xenobiotic interaction and if this potential interaction has clinical consequence.

VIII. References

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CHAPTER 6 - Résumé de Thèse En Français

Résumé de Thèse

A. Introduction

La contribution de l'inflammation à la pathogénèse de différents troubles, dont les maladies cardiovasculaires, est largement reconnue. De plus, les cytochromes hépatiques P450 (CYP) sont une superfamille d'enzymes membranaires jouant un rôle crucial dans le métabolisme global et le sort aussi bien de substances endogènes que d'un grand nombre de xénobiotiques doués de propriétés anti-inflammatoires. Dans cette thèse, nous nous intéressons essentiellement aux influences potentielles des polymorphismes CYP sur les effets réducteurs d'inflammation des acides époxy-eicosatriénoïques (EET) en tant qu'endobiotique connus pour leurs effets anti-inflammatoires puissants et des thiénopyridines (clopidogrel, prasugrel) en tant qu'anti-agrégants plaquettaires dont certaines études ont démontré qu'ils exerçaient des effets anti-inflammatoires pléiotropiques.

Dans l'introduction, nous donnons tout d'abord (partie I) un aperçu de la définition générale de la pharmacogénétique et de la médecine personnalisée, ainsi qu'une vue d'ensemble de la pharmacocinétique, la pharmacodynamie et la pharmacogénétique du clopidogrel et de prasugrel. Sont également discutées dans cette partie les propriétés anti-inflammatoires des thiénopyridines. Nous présentons ensuite les résumés des articles de synthèse (parties II et III) où nous avons essayé de parler des aspects mécanistiques de l'interaction potentielle entre les CYP et EET. Le premier de ces articles (partie II) met en lumière le type d'influence que la variabilité intertissulaire, interindividuelle et intraindividuelle de l'expression des CYP pourrait avoir sur la formation des EET et leurs effets anti-inflammatoires. Le deuxième (III) examine les aspects moléculaires des effets réducteurs d'inflammation des EET et montre comment l'inflammation peut potentiellement modifier la production d'EET. Dans la dernière partie de l'introduction (IV) et à travers un bref Editorial qui a été publié dans le "*Pharmacogenomics Journal*", nous nous sommes efforcés de démontrer comment les tests génétiques au chevet

du patient peuvent contribuer à encourager l'intégration par les cliniciens et les pharmaciens des tests pharmacogénétiques à leurs contextes cliniques.

I. Pharmacogénomique des effets anti-inflammatoires des thiénoypyridine

La dernière décennie, en particulier après l'achèvement du Projet sur le Génome Humain, a été témoin d'une explosion d'informations concernant la sensibilité génétique à des maladies complexes et la variabilité liée à la génétique de la réponse aux médicaments.

La pharmacogénétique ou pharmacogénomique (PGx) est l'étude des associations entre la génétique des individus et leurs réponses aux médicaments et traite la variation déterminée génétiquement de la manière dont les individus répondent aux traitements. La majorité des enzymes métabolisant les médicaments (EEM) de phases I et II est en fait polymorphe. Ces polymorphismes sont à l'origine de différences interindividuelles majeures dans l'exposition à un médicament et ses métabolites et peuvent influencer sur la réponse au traitement aussi bien que sur le risque de réactions indésirables.

Depuis quelques années, nous comprenons mieux l'importance clinique d'une telle variation et plusieurs bases de données renfermant des informations de PGx relatives aux EEM sont facilement disponibles. Avec l'usage des données de PGx, nous prévoyons que les soins aux patients passeront de l'approche "taille unique" à un traitement sur mesure pour chacun.

1. Pharmacogénétique-génomique et médecine personnalisée

L'adénosine diphosphate (ADP) joue un rôle important à la fois dans la genèse de bouchons hémostatiques riches en plaquettes physiologiques et dans la formation de thrombi artériels pathologiques. L'ADP active les plaquettes en se liant, sur la surface de ces dernières, à des récepteurs nucléotidiques membranaires du nom de récepteurs P2. Les plaquettes humaines possèdent deux principaux récepteurs de l'ADP couplés aux protéines G : P2Y₁ et P2Y₁₂. Les métabolites actifs des prodrogues thiénoypyridiniques (clopidogrel et prasugrel) forment des liaisons covalentes avec le récepteur P2Y₁₂ et sont des anti-agrégants plaquettaires indirects irréversibles.

1.1. Clopidogrel

Actuellement, une bithérapie antiplaquettaire à base d'aspirine et de clopidogrel constitue la norme de soins chez les patients atteints de syndrome coronarien aigu (SCA) et ceux traités par l'intervention coronarienne percutanée (ICP). Le clopidogrel est une thiénoypyridine de seconde génération qui a remplacé la ticlopidine en raison de sa plus faible toxicité hématologique pour

absorption intestinale et une part majoritaire (85 %) de la prodrogue clopidogrel absorbée se transforme en métabolites inactifs sous l'action des estérases ubiquitaires. Le reliquat (15 %) subit une activation au niveau du foie par la voie enzymatique du cytochrome hépatique P-450 (CYP). L'activation du clopidogrel nécessite une conversion par un processus oxydatif à deux étapes, tout d'abord en oxo-2-clopidogrel, un métabolite intermédiaire qui est encore inactif, puis en son métabolite actif. Aux deux étapes interviennent plusieurs isoenzymes CYP hépatiques, dont CYP2C19, CYP3A4/5, CYP2C9, CYP1A2 et CYP2B6. Plus récemment, il a été démontré que l'anneau gamma-thiobutyrolactone de l'oxo-2-clopidogrel pouvait être ouvert au métabolite thiol actif par clivage hydrolytique catalysé par l'enzyme paraoxonase-1 (PON-1) et non par oxydation catalysée par le CYP2C19. En outre, l'idée a été émise que la PON-1 serait une enzyme clé pour l'inhibition des fonctions plaquettaires induite par le clopidogrel et pour l'efficacité clinique du médicament, bien que d'autres études n'aient pas étayé cette suggestion.

On a observé une forte variabilité interindividuelle de l'inhibition des fonctions plaquettaires par le clopidogrel, imputée pour l'essentiel à des différences dans le degré de métabolisation de la prodrogue clopidogrel. A cette variabilité s'attache une importance clinique puisque de nombreuses études ont mis en évidence une protection insuffisante contre les événements indésirables cardiaques majeurs chez les patients à forte réactivité plaquettaire sous traitement (résistance ou hyposensibilité au clopidogrel). D'après les estimations, environ un tiers des patients traités par clopidogrel présente une diminution de la réponse plaquettaire *ex vivo* au clopidogrel. Des facteurs cliniques, cellulaires et génétiques ont été associés à la résistance au clopidogrel. A cet égard, le polymorphisme mononucléotidique *ABCB1 C3435T* réduit de manière significative l'absorption du clopidogrel sous sa forme tant homozygote qu'hétérozygote. Les patients de génotype *ABCB1 T3435T* et *C3435T* ont une évolution clinique plus défavorable que ceux de génotype *C3435C*. En outre, la perte des allèles fonctionnels *CYP2C19* (*2, *3, *4, *5, *6, *7 et *8), surtout de l'allèle *CYP2C19*2*, peut atténuer l'effet pharmacodynamique et l'efficacité clinique du clopidogrel. Les polymorphismes génétiques dans le *CYP2C19* et l'*ABCB1*, qui se trouvent impliqués dans le métabolisme et le sort du clopidogrel, n'expliquent toutefois que partiellement la corrélation avec une réponse suboptimale au clopidogrel. L'hypothèse d'une mauvaise observance du traitement a été avancée comme cause importante de la résistance au clopidogrel. Les patients obèses ou diabétiques ont une incidence augmentée de réponse diminuée au clopidogrel et, de façon correspondante, une sensibilité accrue à l'adhésion et à l'agrégation

plaquettaires induites par l'ADP. De plus, il peut aussi y avoir chez les mauvais répondeurs au clopidogrel une régulation à la hausse des voies intracellulaires dépendantes et indépendantes du P2Y₁₂.

1.1.1. *Effets anti-inflammatoires du clopidogrel*

Bien que ce soit avant tout un anti-agrégant plaquettaire, il a également été démontré que le clopidogrel exerçait des effets anti-inflammatoires pléiotropiques chez les patients atteints de pathologies vasculaires ischémiques et aussi en post-ICP. L'aptitude du clopidogrel et du prasugrel à réduire l'inflammation présenterait une importance particulière dans le contexte clinique puisqu'il a été démontré que la réponse inflammatoire post-ICP était le mécanisme causatif principal de la resténose intra-stent (RIS) et qu'une élévation des marqueurs inflammatoires était associée à une plus forte incidence d'événements cardiaques indésirables en post-ICP. Toutefois, les propriétés anti-inflammatoires du clopidogrel ne se retrouvent pas systématiquement dans tous les essais et il arrive que différentes études dans ce domaine aboutissent à des résultats contradictoires. L'analyse de la variation de la protéine C-réactive montre cependant que : 1) les nombres de patients recrutés sont souvent restreints ; 2) la durée de traitement diffère, tout en étant souvent d'un mois ; 3) le clopidogrel est donné habituellement à la dose quotidienne unique de 75 mg. Sur la base de tout ce qui est mentionné ci-dessus, il serait logique et pertinent de supposer que, potentiellement et théoriquement, les mêmes variations génétiques au niveau des gènes impliqués dans la pharmacocinétique (PC)/ pharmacodynamique (PD) du clopidogrel sont responsables aussi de la divergence observée à l'occasion de différentes études dans les effets anti-inflammatoires pléiotropiques du clopidogrel ; cette hypothèse n'a jamais été évaluée.

1.2. *Prasugrel*

Le prasugrel est une nouvelle thiényridine à laquelle son activation métabolique plus rapide confère, par rapport au clopidogrel, un délai raccourci d'action anti-agrégante plaquettaire *ex vivo*. Comme le clopidogrel, le prasugrel est une prodrogue qui subit en premier lieu une rapide désestérification en thiolactone intermédiaire, souvent convertie alors dans le foie en le métabolite actif lors d'une seule étape dépendante du CYP impliquant les CYP3A, CYP2B6, CYP2C9 et CYP2C19. Le métabolite actif du prasugrel apparaît dans la circulation moins de 15 min. après une dose d'attaque de 60 mg et atteint sa concentration plasmatique maximale à 30 min. En fait, chez les patients subissant un cathétérisme cardiaque avec ICP programmée,

la mise en charge avec 60 mg de prasugrel a entraîné une plus forte inhibition des fonctions plaquettaires qu'une dose d'attaque de 600 mg. En outre, une dose d'entretien de prasugrel de 10 mg/jour inhibe l'activation plaquettaire d'une manière plus puissante et plus constante que les doses d'entretien standard de clopidogrel de 75 ou 150 mg/jour.

1.2.1. Effets anti-inflammatoires du prasugrel

On a également démontré dans quelques études que le prasugrel avait des effets inhibiteurs plus puissants sur le processus inflammatoire ; en comparaison du clopidogrel, toutefois, ses propriétés anti-inflammatoires n'ont pas fait l'objet d'explorations aussi poussées. Qui plus est, toute éventuelle association entre la variabilité génétique des gènes impliqués dans la PC/PD du prasugrel et la fonction anti-inflammatoire de ce dernier n'a pas été évaluée.

B. Hypothèses et objectifs

I. Hypothèses

1. Récemment et par le biais d'une étude d'association pangénomique (EAP), nous avons pu identifier dans le gène haptoglobine un polymorphisme mononucléotidique courant, rs2000999, qui s'est révélé être un solide prédicteur des taux plasmatiques de haptoglobine ($P = 8,1 \times 10^{-59}$) et dont on a démontré aussi qu'il expliquait 45,4 % de sa variabilité génétique. Eu égard au rôle important des valeurs de référence en chimie clinique et en médecine biologique, nous avons supposé (hypothèse (H) 1) que le polymorphisme mononucléotidique de haptoglobine tout juste découvert, rs2000999, pourrait servir au cloisonnement des intervalles de référence du haptoglobine plasmatique humain.

2. Un nombre croissant d'études a fourni des éléments à l'appui du concept selon lequel les EET dérivés des époxygénases du CYP possèdent un puissant effet anti-inflammatoire. De même, les SNP d'époxygénases du CYP les plus courants, à savoir *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C8*2* et *CYP2J2*7*, sont associés à une perte d'activité partielle ou totale de l'enzyme correspondante. Nous avons, en conséquence, posé l'hypothèse (H2) que ces polymorphismes seraient susceptibles d'entraîner une diminution de la formation d'EET et du même coup, une différence de taux de marqueurs inflammatoires à médiation par les EET.

3. Il y a dans la réponse aux effets anti-agrégants plaquettaires des dérivés de thiénopyridine clopidogrel et prasugrel une nette variabilité interindividuelle principalement imputée aux polymorphismes de perte et de gain de fonction du CYP2C19 (*CYP2C19*2* et *CYP2C19*17*). Qui plus est, certains travaux ont fait état d'un phénomène que l'on a baptisé "paradoxe du fumeur" et en vertu duquel les fumeurs traités par clopidogrel semblent présenter, par rapport aux non-fumeurs, davantage d'inhibition et moins d'agrégation plaquettaires. En outre, plusieurs études ont proposé l'éventualité de l'exercice d'effets anti-inflammatoires pléiotropiques par les thiénopyridines ; cependant, une énorme controverse inter-études entoure les effets d'antiagrégants plaquettaires sur le taux de marqueurs inflammatoires. Dans la mesure où les plaquettes activées jouent un rôle central dans l'inflammation, nous

avons posé l'hypothèse (H3) que l'allèle *CYP2C19*2* était associé à des niveaux supérieurs de réactivité plaquettaire et donc à des taux plus élevés de marqueurs inflammatoires en post-ICP sous thiénoxyridines.

4. Nous avons aussi avancé une hypothèse (H4) selon laquelle le phénomène "paradoxe du fumeur" pourrait être associé à une variabilité interindividuelle dans la réponse aux effets anti-inflammatoires des thiénoxyridines.

5. Il a été démontré que les patients à "forte réactivité plaquettaire" malgré un traitement par thiénoxyridines courent davantage de risques d'échec thérapeutique se manifestant sous la forme d'événements cardiovasculaires ischémiques et d'une RIS. La littérature, néanmoins, manque de cohérence au sujet de la définition optimale de "forte réactivité plaquettaire" chez les patients sous thiénoxyridines. Alors que les *modifications* absolues ou relatives de l'agrégation plaquettaire par rapport à l'état initial semblent constituer le plus fiable indicateur d'efficacité antiagrégante, l'idée a été émise que le niveau absolu de réactivité plaquettaire au cours du traitement (c'est-à-dire, la réactivité plaquettaire *sous traitement*) serait une méthode plus exacte de dépistage des patients à haut risque d'événements thrombotiques. Sur la base d'une récente définition consensuelle, la forte réactivité plaquettaire sous traitement peut être déterminée à l'aide de quatre critères, dont l'un est un indice de réactivité plaquettaire (IRP) > 50 % par analyse de la phosphoprotéine stimulée par vasodilatateur (PSPV). Là encore, puisque les plaquettes activées jouent un rôle crucial dans le déclenchement et l'entretien de l'inflammation, on pourrait logiquement poser l'hypothèse (H5) que les patients à forte réactivité plaquettaire sous traitement (IRP PSPV > 50 %) ont des taux plus élevés de marqueurs inflammatoires.

II. Objectifs

1. Déterminer les valeurs de référence de haptoglobine sur la base de ses principales sources de variation biologique, y compris le rs2000999, chez des sujets sains.
 2. Evaluer l'association entre les polymorphismes les plus courants des époxygénases du CYP sur les taux plasmatiques de marqueurs inflammatoires chez des sujets sains.
 3. Evaluer si les polymorphismes de perte et de gain de fonction du CYP2C19 (*CYP2C19*2* et *CYP2C19*17*) sont associés aux taux de marqueurs inflammatoires en post-ICP sous thiénoxyridines.
 4. Rechercher si les taux de marqueurs inflammatoires sont associés à l'usage du tabac chez les patients en post-ICP traités par thiénoxyridines (autre aspect du phénomène de "paradoxe du fumeur").
 5. Rechercher si une forte réactivité plaquettaire sous traitement (IRP PSPV > 50 %) chez les patients sous thiénoxyridines est associée à une élévation des taux de marqueurs inflammatoires.
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C. Populations

D'une façon générale, nous poursuivions dans la présente thèse trois objectifs principaux : A) cloisonnement des valeurs de référence de haptoglobine dans la fonction du polymorphisme mononucléotidique de haptoglobine nouvellement découvert, rs2000999 ; B) approche pharmacogénétique des effets anti-inflammatoires des thiénoxydines ; et C) approche génétique des propriétés réductrices d'inflammation des EET. La fourchette de référence de haptoglobine a été déterminée sur la population de la cohorte STANISLAS (CS) et, pour atteindre les autres objectifs, nous avons mené deux études collaboratives, avec deux centres de recherche réputés à Paris et à Marseille. Les hypothèses ont été contrôlées au sein de chaque population à l'aide de tests biochimiques, hématologiques et génétiques. Nous présentons ici une brève description des populations ; les méthodes de laboratoire employées ont été décrites en détail dans les articles correspondants (voir Résultats).

1. CS

La SFS est une étude de cohorte longitudinale sur 10 ans avec la participation de 1.006 familles de volontaires d'ascendance européenne dont les membres étaient exempts de pathologies chroniques (maladie cardiovasculaire ou cancer), le recrutement ayant eu lieu au cours de la période 1993–1995. Les échantillons et données CS sont rattachés au Centre de Ressources Biologiques (CRB) "Interactions Gène - Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) à Nancy, France.

Dans notre étude, nous avons tout d'abord identifié les variables biologiques principales de haptoglobine chez 3.129 sujets de la CS apparemment exempts de pathologies. La fourchette de référence de haptoglobine a été établie ultérieurement dans un sous-groupe de 2.923 sujets sains sélectionnés, en qualité de population de référence, par la prise en compte du polymorphisme nouvellement découvert de haptoglobine, rs2000999.

2. Populations sous clopidogrel et prasugrel

2.1. Etude de Paris

Cette étude portait sur quarante-neuf jeunes volontaires sains de sexe masculin et de race caucasienne (âgés de 18 à 35 ans) répondant aux critères d'inclusion suivants : non-fumeurs, résultats normaux à l'examen clinique et aux explorations biologiques, absence d'anamnèse de traitement médicamenteux et numération plaquettaire normale, agrégation plaquettaire maximale normale.

2.2. Etude de Marseille

Cette étude a recruté 1128 patients atteints de SCA ayant subi avec succès une ICP et quitté l'hôpital entre avril 2007 et décembre 2012 avec une prescription soit de clopidogrel 75 mg/j (n = 118), soit de clopidogrel 150 mg/j (n = 631), soit de prasugrel 10 mg/j (n = 309).

D. Résultats

Résultats sont présentés à la suite sous forme de publications.

E. Discussions

I. Principaux résultats

Le présent travail s'est appuyé sur les arguments scientifiques suivants : 1) l'inflammation a une large part dans la pathogénèse de nombreux troubles, dont les maladies cardiovasculaires (MCV) ; 2) importantes pour l'hémostase primaire, les plaquettes jouent aussi un rôle pro-inflammatoire non négligeable ; 3) les enzymes du CYP jouent, dans le métabolisme et le sort général des endobiotiques (par ex. les EET) et xénobiotiques (par ex. les thiényridines), un rôle central potentiellement en mesure d'influencer les réponses inflammatoires de l'organisme humain ; 4) les polymorphismes mononucléotidiques courants dans le CYP et les gènes marqueurs inflammatoires sont associés à une modification de l'activité

et du taux, respectivement, des enzymes et des marqueurs d'inflammation correspondants ; et 5) la grande variabilité entre individus différents dans la réponse aux effets antiagrégants plaquettaires des thiénoxyridines est principalement attribuée aux polymorphismes CYP2C19 et aussi, dans certains cas, au tabac ("paradoxe du fumeur"). En conséquence, les principaux résultats de cette thèse sont résumés comme suit :

- A)** : Nos constatations sur une population restreinte de sujets sains de sexe masculin n'ont pas corroboré l'hypothèse selon laquelle les variations génétiques des époxygénases du CYP sont associées aux taux de marqueurs d'inflammation systémique.

- B)** : Nos observations dans cette population n'ont pas non plus été à même de démontrer que les polymorphismes *CYP2C19*2* et *P2Y12 H* soient associés à la variabilité dans la réponse aux propriétés anti-inflammatoires du clopidogrel.

- C)** : Au sein d'une large population de patients en post-ICP sous clopidogrel ou prasugrel, nous avons constaté que les polymorphismes de perte et de gain de fonction CYP2C19 (**2* et **17*) étaient associés au niveau de réactivité plaquettaire sous traitement ; mais indépendamment, ces polymorphisme mononucléotidique n'étaient cependant pas associés aux taux de marqueurs inflammatoires dans cette population. Néanmoins, l'interaction d'un mauvais statut de métabolisme des thiénoxyridines et du tabac est apparue comme étant un puissant régulateur de l'inflammation systémique ; cet effet était apparemment indépendant de l'activité plaquettaire, puisque l'interaction polymorphisme mononucléotidique du CYP2C19 – tabac n'était pas associée aux niveaux de réactivité plaquettaire sous traitement déterminés par l'IRP PSPV. Et aussi, bien que nous n'ayons rien constaté qui vienne étayer cet aspect du phénomène de "paradoxe du fumeur" dans lequel il se peut que le tabac modifie les effets antiagrégants plaquettaires des thiénoxyridines d'une manière positive, peut-être nos résultats présentent-ils un nouvel aspect du phénomène suggérant que le tabac est associé à une augmentation des effets antiagrégants plaquettaires des thiénoxyridines.

- D)** : Dans cette population, nous avons également observé que les patients qui étaient sous prasugrel, mais non sous clopidogrel, et avaient des niveaux élevés de réactivité plaquettaire sous traitement, définis comme IRP PSPV > 50 %, présentaient de plus forts taux de marqueurs inflammatoires systémiques.
- E)** : Afin de démontrer la nécessité clinique d'une fourchette de référence et l'importance de la génétique dans le cloisonnement des valeurs de référence des marqueurs inflammatoires, nous avons de plus déterminé les intervalles de référence de haptoglobine en fonction de ses principales sources de variation biologique, comprenant le sexe, l'âge, le tabac, les taux d'hémoglobine plasmatiques et le polymorphisme de haptoglobine qui venait d'être découvert, rs2000999. Nous avons trouvé que le taux médian de Hp est respectivement supérieur de > 150 % et > 200% chez les hommes et les femmes homozygotes pour l'allèle de type sauvage par rapport à ceux ou celles qui étaient homozygotes pour l'allèle muté.

Nous essayons ici de compléter de quelques éléments la partie discussion des articles publiés / soumis :

II. Inflammation, MCV et RIS post-ICP

L'incidence et la prévalence des MCV ont considérablement augmenté ces dernières années. Actuellement, les MCV viennent toujours au premier rang des causes de décès dans les pays développés et occupent la seconde place à l'échelle mondiale. Selon l'organisation mondiale de la santé, à l'horizon 2020, les MCV prendront le pas sur les maladies infectieuses et deviendront responsables de près de 40 % de tous les décès dans le monde entier, plus du double du pourcentage de décès par cancer.

Les preuves ne cessent de s'accumuler à l'appui du concept que l'inflammation contribue au déclenchement, à l'évolution et aux manifestations cliniques des MCV. L'inflammation est au centre de tous les stades de l'athérosclérose, depuis la lésion initiale jusqu'aux complications thrombotiques. A ce titre, l'athérosclérose est aujourd'hui reconnue comme une pathologie inflammatoire chronique des grosses artères. L'inflammation vasculaire se caractérise par un réseau complexe d'interactions à base de cellules endothéliales (CE) activées, de leucocytes

inflammatoires, de cellule musculaire lisse (CML) et de plaquettes activées – comme importante source de médiateurs inflammatoires. De ces interactions résultent des cascades d'activation autocrine et paracrine qui se traduisent par le recrutement de leucocytes dans la paroi vasculaire.

1. Plaquettes : acteurs clés de l'inflammation vasculaire

La plaquette fournit un lien important entre l'inflammation, la thrombose et l'athérosclérose. Il a été démontré que les plaquettes activées expriment un certain nombre de molécules de surface et aussi une multitude de récepteurs immuns, ce qui permet aux plaquettes d'interagir avec les CE et les leucocytes, ceci facilitant à son tour l'activation cellulaire et la migration / le recrutement cellulaire vers les sites de lésion vasculaire et d'inflammation.

1.1. Plaquettes activées et protéine C-réactive, haptoglobine et acide orosomucoïde

La PCR est une protéine de phase aiguë qui a été reconnue comme étant potentiellement un facteur étiologique important dans l'inflammation et l'athérosclérose. Il existe en outre des preuves robustes que la protéine C-réactive possède une valeur pronostique cliniquement significative et que chez les patients souffrant d'un SCA, des taux élevés de protéine C-réactive prédisent de futurs événements cardiovasculaires.

Il a été établi que la protéine C-réactive est synthétisée dans le foie en réponse aux cytokines inflammatoires, principalement interleukine (IL)-6, IL-1 β et facteur nécrosant des tumeurs- α . Comme nous l'avons mentionné plus tôt, les plaquettes activées et aussi les CE activées et monocytes inflammés induits par les plaquettes secrètent une grande quantité de diverses cytokines, en particulier IL-1 β . Il est par conséquent logique et pertinent de supposer que les plaquettes activées sont potentiellement en mesure de jeter une passerelle entre les réponses inflammatoires locale et systémique.

De même, le haptoglobine et l'acide orosomucoïde se classent dans la catégorie des protéines de phase aiguë. Bien qu'en comparaison de la protéine C-réactive, le rôle du haptoglobine et de l'acide orosomucoïde en tant que marqueurs d'inflammation systémique ait été moins étudié, plusieurs travaux ont montré qu'il s'agissait là d'indicateurs fiables d'une réponse inflammatoire systémique. Tout comme pour la protéine C-réactive, il existe une surproduction de haptoglobine et d'acide orosomucoïde en réponse aux stimulants

inflammatoires, principalement IL-6, IL-1 et facteur nécrosant des tumeurs- α , et il n'est dès lors pas impossible que dans des conditions inflammatoires, les plaquettes provoquent de façon indirecte un accroissement de la production de ces marqueurs inflammatoires.

- **Au vu de tous les points mentionnés, nous sommes partis dans cette thèse du principe que les taux plasmatiques de protéine C-réactive, de haptoglobine et d'acide orosomucoïde peuvent, indirectement mais fiablement, représenter le niveau d'activité plaquettaire. Toutefois, ainsi que nous l'avons déjà admis, les marqueurs spécifiques de plaquettes comme la P-sélectine soluble et le CD-40 soluble sont peut-être des indicateurs plus appropriés des réponses inflammatoires induites par les plaquettes. Cet élément a été pris en compte dans notre essai clinique en cours (voir "*Perspective*").**

2. Inflammation et complications post-ICP

L'athérosclérose coronaire est une maladie traitable mais non curable et l'introduction de l'ICP a marqué une approche thérapeutique révolutionnaire en réduisant l'incidence de la thrombose intra-stent après angioplastie par ballonnet. En dépit des avancées technologiques, la principale limitation de l'ICP, à savoir la RIS du vaisseau traité, demeure cependant un défi et entraîne un retour des symptômes et la nécessité d'une revascularisation du vaisseau cible.

Bien que la physiopathologie de la RIS n'ait pas été totalement élucidée, il existe des preuves convaincantes de la probabilité qu'une réponse inflammatoire suscitée par l'intervention soit le mécanisme causatif numéro un. La pose de stents entraîne une activation plaquettaire, une lésion mécanique et une dénudation endothéliale qui déclenchent la cascade d'événements, y compris d'importantes réponses inflammatoires au niveau local et systémique, ce qui stimule la prolifération de CML vasculaires et un dépôt de matrice extracellulaire, un épaississement néointimal et une resténose. Des études chez l'homme ont montré que les stents coronaires provoquent une rupture de la couche endothéliale coronaire entraînant de 0 à 3 jours après l'intervention une réponse inflammatoire aiguë initiale centrée sur les supports du stent. Au bout de 2 à 4 semaines, l'inflammation aiguë cède, remplacée par des cellules inflammatoires chroniques ainsi que des CML proliférantes associées à un thrombus en cours d'organisation et une fine matrice extracellulaire provisoire.

- **En considération du fait que l'inflammation joue un rôle significatif dans l'apparition de RIS et qu'à la réponse inflammatoire aiguë post-ICP vient se substituer dans le délai d'un mois une inflammation chronique, nous avons, dans cette thèse, étudié les effets inflammatoires potentiels des thiénopyridines en post-ICP après un traitement de 4 semaines par clopidogrel ou prasugrel.**

La réponse inflammatoire systémique induite par l'ICP est attestée par une élévation des taux plasmatiques de protéines de phase aiguë telles que la protéine C-réactive, les cytokines, les chimiokines et les formes solubles de molécules d'adhésion. De multiples études ont mis en évidence un lien entre l'incidence des événements cardiovasculaires et de la RIS post-interventionnelle, d'une part, et l'importance de l'inflammation systémique d'autre part. Dès lors, les patients en post-ICP qui présentent des taux plus élevés de marqueurs inflammatoires ont une incidence de RIS supérieure et il est possible de stratifier par risque les patients subissant une ICP en fonction de la hausse de leurs concentrations après ICP.

Plusieurs marqueurs d'inflammation, signalés comme étant associés à un risque accru de RIS, pourraient servir de prédicteurs de complications post-ICP et d'événements indésirables. En plus de la protéine C-réactive, voici un certain nombre d'entre eux : numération leucocytaire (notamment une augmentation des lymphocytes T cytotoxiques CD3+/CD56+ et des monocytes activés CD11b), IL-1, IL-6, facteur nécrosant des tumeurs- α , lipoprotéine (a), amyloïde sérique A, la protéine chimiotactique monocyttaire-1, certains composants du complément (C3a et C5a), diméthylarginine asymétrique, l'inhibiteur I de l'activateur du plasminogène-1, l'oxyde nitrique, CD-40L soluble, glycosylphosphatidyl-inositol-80, métalloprotéase de matrice -2, -9 et protéine plasmatique A associée à la grossesse. Eu égard toutefois à l'instabilité biochimique, au coût élevé ou à la faible accessibilité de la plupart de ces marqueurs, les explorations se sont avant tout intéressées à la valeur prédictive de la protéine C-réactive. Aussi, dans le contexte clinique, la prédiction de la RIS post-ICP fait-elle intervenir les taux plasmatiques de protéine C-réactive.

- **Compte tenu du fait que la protéine C-réactive est aujourd'hui appliquée en pratique clinique quotidienne à des fins d'évaluation du risque de RIS, et en raison également des problèmes techniques que soulèvent les dosages d'autres marqueurs inflammatoires, nous avons, dans cette thèse, mis à l'épreuve notre hypothèse à l'aide des taux plasmatiques de protéine C-réactive. Nous avons par ailleurs recherché si le Hp ou l'acide orosomucoïde, deux**

autres protéines de phase aiguë, peuvent à ce titre être utilisées comme alternatives à la protéine C-réactive. Dans cette thèse, malheureusement, nous n'avons pas eu la possibilité de suivre les patients et d'évaluer si nos résultats sont associés à l'évolution clinique ; cet inconvénient a toutefois été surmonté dans notre essai clinique en cours (see "*Perspectives*").

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2.1 RIS après stent à élution médicamenteuse (SEM) vs. stent métallique (SM)

Plusieurs études cliniques et angiographiques ont fait état de l'association des SM à un pourcentage de resténose allant jusqu'à 30 %. L'introduction du SEM a considérablement réduit l'incidence de la RIS (de 70 % sur de larges sous-groupes de patients et de lésions) ; cependant, en fonction des lésions et des lésions d'origine interventionnelle, la survenue d'une RIS s'observe encore dans 7 % à 13 % des cas après SEM.

- **Au vu du fait que l'incidence de la RIS après SEM a été signalée comme significativement plus faible qu'après SM, on notera que dans cette thèse, nous avons soumis nos constatations à une recherche d'éventuelles différences entre les patients traités par SEM et ceux traités par SM.**

III. CYP et métabolisme des EET et des thiénoxyridines

La contribution des enzymes du CYP au métabolisme à la fois des EET, en tant que substances à production endogène issues de l'acide arachidonique et douées de puissants effets anti-inflammatoires, et du clopidogrel/prasugrel, les dérivés de thiénoxyridine ayant des propriétés anti-inflammatoires potentielles, a fait l'objet d'un bilan exhaustif dans le premier chapitre "*Introduction*". De plus, la pharmacogénétique des thiénoxyridines a été discutée en détail, tout comme les influences potentielles des époxycgénases SNP du CYP sur la biosynthèse des EET.

Il est facile de remarquer l'implication de certains CYP et spécifiquement des isoformes CYP2C dans le métabolisme aussi bien des thiénoxyridines que des EET. Ce fait pourrait servir de base à l'étude d'une interaction réelle entre les xénobiotiques et les endobiotiques. L'interaction potentielle EET-thiénoxyridine et ses applications clinico-pharmacologiques justifient de futurs travaux complémentaires.

- **Nous nous sommes sérieusement efforcés dans cette thèse de mesurer les taux plasmatiques d'EET au sein de nos populations d'étude et de rechercher une éventuelle interaction des EET avec les thiénoxyridines. En raison de plusieurs difficultés rencontrées au cours des phases pré-analytique et analytique, nous n'avons cependant pas réussi à évaluer cette hypothèse. Néanmoins, nous essayons d'étudier, ne fût-ce que dans un petit sous-groupe de patients, l'hypothèse de l'existence d'une interaction médiée par le CYP entre EET et thiénoxyridines.**

IV. Résistance aux thiénoxyridines, absence de réponse et forte réactivité plaquettaire sous traitement

Ainsi que le rappelle le chapitre 1, plusieurs études ont montré qu'il existe une relation directe entre l'absence de réponse au clopidogrel et une forte réactivité plaquettaire sous traitement, mesurée par des dosages plaquettaires multiples et par les événements ischémiques cliniques indésirables. Un défaut de consensus au sujet des méthodes optimales de quantification de la forte réactivité plaquettaire et des valeurs seuils associées au risque clinique a toutefois pour conséquence que la mesure systématique de la réactivité plaquettaire n'est ni mise en œuvre à grande échelle en pratique clinique, ni préconisée dans les directives.

1. Résistance, hyposensibilité, absence de réponse aux thiénoxyridines

La définition de la résistance ou de l'absence de réponse aux thiénoxyridines est basée sur les modifications de la réponse (réactivité) plaquettaire à l'ADP par rapport à l'état initial après la prise de thiénoxyridines. La résistance pharmacologique au clopidogrel est définie, de manière correspondante, comme une diminution absolue de l'agrégation plaquettaire en réponse au médicament $\leq 10\%$ (état initial vs. sous traitement). De plus, les patients se voient rattacher à l'une des catégories "non-répondeurs", "semi-répondeurs" et "répondeurs" à l'aide de valeurs limites absolues d'inhibition de la fonction plaquettaire de respectivement $< 10\%$, 10% à 30% et $> 30\%$.

2. Forte réactivité plaquettaire sous traitement

Une controverse existe tant autour de la méthode optimale de quantification de la réactivité plaquettaire qu'autour de la définition d'un seuil de forte réactivité plaquettaire à l'ADP sous traitement. Actuellement, toutefois, on définit la forte réactivité plaquettaire sous traitement dans un contexte d'ICP à l'aide des critères suivants : 1) IRP $> 50\%$ par analyse de la PSPV ; 2) $>$

235 à 240 unités de réaction du P2Y₁₂ par dosage VerifyNow P2Y₁₂ ; 3) > 46 % d'agrégation maximale induite par 5 µmol/l d'ADP ; 4) > 468 unités arbitraires d'agrégation/min en réponse à l'ADP par analyseur Multiplate.

3. Résistance vs. forte réactivité plaquettaire sous traitement

L'idée a été émise qu'alors qu'une mesure de la réponse (modifications absolues ou relatives de l'agrégation plaquettaire par rapport à l'état initial) paraît l'indicateur le plus fiable d'un effet thérapeutique, ce n'est peut-être pas le meilleur moyen d'identifier les patients à haut risque. Compte tenu de l'existence d'une variabilité interindividuelle des valeurs initiales d'agrégation plaquettaire induite par l'ADP, il est possible que la mesure de la réponse aux thiénoopyridines surestime le risque ischémique chez les non-répondeurs présentant une faible réactivité avant traitement et le sous-estime chez les répondeurs conservant une forte réactivité plaquettaire après traitement. Par ailleurs, il a été avancé que le niveau absolu de réactivité plaquettaire au cours du traitement (c'est-à-dire, la réactivité plaquettaire sous traitement) constituerait une meilleure mesure du risque thrombotique que la réponse aux thiénoopyridines.

- **La réactivité plaquettaire sous traitement ayant été proposée comme indicateur acceptable pour la quantification de la réactivité plaquettaire, nous avons, dans cette thèse, employé l'IRP VASP en tant que mesure de la réactivité plaquettaire. Il convient de mentionner que le schéma d'étude retenu dans notre essai clinique en cours (voir "Perspectives") nous fournira l'occasion de tester nos hypothèses en utilisant à la fois la mesure de la réponse et celle de la réaction plaquettaire sous traitement.**

F. Perspectives

Nous participons en ce moment même à une étude collaborative avec le "Wolfson Centre for Personalized Medicine of the Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine in University of Liverpool" portant sur les effets anti-inflammatoires du clopidogrel. Dans cette EAP, l'exploration des propriétés réductrices d'inflammation du clopidogrel se déroulera chez environ 1350 patients présentant de signes cliniques de SCA. Pour cela, un profil complet des taux plasmatiques de marqueurs inflammatoires sera mesuré

avant, puis 1 mois et 12 mois après traitement par clopidogrel. En outre, les critères cliniques suivants seront évalués aux temps 1 mois et 12 mois après la prise de clopidogrel :

1. Mortalité cardiovasculaire
2. Infarctus du myocarde non fatal
3. Accident vasculaire non fatal
4. Hémorragie
5. Insuffisance ventriculaire gauche

Il y a lieu de mentionner que dans le cadre de cette approche pharmacogénétique des effets anti-inflammatoires du clopidogrel, l'occasion s'offrira aussi à nous d'évaluer si l'interaction des gènes impliqués dans la pharmacocinétique/ pharmacodynamie du clopidogrel avec ceux qui codent pour les marqueurs d'inflammation est associée aux effets anti-inflammatoires du clopidogrel. Et, chose encore plus intéressante, nous aurons la possibilité grâce à ce projet de vérifier l'influence de nos constatations sur l'évolution clinique des patients étudiés.

RÉSUMÉ

Cette thèse est dédiée à l'approche pharmacogénétique des effets anti-inflammatoires de la thérapie par les thiénoxyridines. Prenant en compte que les plaquettes activées jouent un rôle central dans les états inflammatoires et que des polymorphismes du cytochrome P450 (CYP) 2C19 ont été montrés responsables de différences inter-individuelles dans la réponse de l'effet antiplaquettaire de thiénoxyridines, nous avons émis l'hypothèse que CYP2C19 *2 ou *17 sont également associés à la variabilité interindividuelle du potentiel anti-inflammatoire des thiénoxyridines. Les marqueurs d'inflammation utilisés pour suivre l'effet des thiénoxyridines sont : la CRP, l'haptoglobine et l'orosomucoïde.

Nous avons démontré que pour interpréter les valeurs de l'haptoglobine il était nécessaire de tenir compte du statut génétique et obtenir des valeurs de référence stratifiées.

D'abord dans une population saine, nous n'avons pas trouvé d'association entre les valeurs de base des marqueurs inflammatoires et les polymorphismes fréquents de CYP époxygénases.

Dans une population après intervention coronarienne percutanée qui était composée de 1128 sujets traités par clopidogrel ou prasugrel, le niveau de CRP observé a montré une interaction significative entre le tabac et le polymorphisme de CYP 2C19 ; cet effet est indépendant du niveau d'agrégation plaquettaire.

Dans une 3^{ème} population, sur plus de 1000 sujets hospitalisés à Coimbra, nous avons identifié une interaction entre le clopidogrel CYP2C19 et les médicaments bloqueurs des canaux calciques.

En résumé, tous ces résultats obtenus sur plusieurs populations laissent envisager que les marqueurs d'inflammation pourraient être un moyen intéressant de suivi des patients lors de la thérapie par les thiénoxyridines.

Mots Cles: Thiénoxyridines ; Clopidogrel ; Prasugrel ; Cytochrome P450 ; Époxygénases ; Inflammation

ABSTRACT

The main part of the thesis is devoted to pharmacogenetic approach to the anti-inflammatory effects of thienopyridine therapy. Taking into the account that activated platelets play a central role in the inflammatory responses and that CYP2C19 gain- and loss-of-function polymorphisms (*2 and *17) are sources of inter-individual difference in response to the anti-platelet effects of thienopyridines, we hypothesized that *2 and/or *17 alleles are also associated with inter-individual variability in the potential inflammation-reducing effects of thienopyridines. The following markers were used to test the hypothesis: CRP, haptoglobin and orosomucoïd acid. To be reliably interpretable in daily medical practice, genetic status should be considered for partitioning the reference values of haptoglobin.

In a small healthy population, no significant association was observed between *2 allele and changes in levels of inflammatory markers from baseline to 7 days after administration of clopidogrel and our findings did not support the notion that the genetic variations of CYP epoxygénases are associated with the level of inflammatory markers. Also, in post-PCI population consisting of 1128 on-clopidogrel or on-prasugrel patients, CRP levels were observed to be regulated with a significant interaction between smoking and CYP2C19 polymorphisms; this effect was independent to the level of platelet aggregation.

Additionally, in a large population of 1000 on-clopidogrel patients, whether there is a potential interaction between clopidogrel and calcium channel blockers.

Collectively, we demonstrated in this thesis that inflammatory markers might be alternative tools for the prediction of response to thienopyridines.

Key Words: Thienopyridines; Clopidogrel; Prasugrel; Cytochrome P450; Epoxygénases; Inflammation
