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Influence du diabète de type 2 sur l'activité et l'expression des cytochromes P450.

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Résumé

Mon projet de doctorat a pour objet l'étude des facteurs pouvant influencer le métabolisme des médicaments et la variabilité interindividuelle dans la réponse aux médicaments. Mon projet cible plus précisément les Cytochromes P450 (CYP450), le système enzymatique majeur impliqué dans la biotransformation des médicaments. Mes travaux de recherche évaluent l'impact d'une condition pathologique, le diabète de type 2 (DT2), sur l'activité métabolique des CYP450s. Mes études comprennent un volet de métabolisme systémique chez le patient et un volet de métabolisme *in vitro*. Dans cette thèse, les résultats de mes recherches sont rapportés sous forme de présentation par articles.

Le volet *in vivo* consistait en une étude de pharmacocinétique qui visait à évaluer l'impact du diabète sur l'activité métabolique de différentes isoformes des CYP450s en utilisant un *cocktail* de substrats-marqueurs. Des patients avec le DT2 et des sujets non diabétiques ont reçu une dose orale de notre *cocktail* VM/JT de substrats-marqueurs composé de caféine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), oméprazole (CYP2C19), dextrométhorphane (CYP2D6) et midazolam (CYP3A4/5) suivi d'une administration de chlorzoxazone (CYP2E1). Le protocole pour cette étude est détaillé dans l'article disponible à la section 2.1; manuscrit 1. Les concentrations plasmatiques et urinaires des médicaments marqueurs et de leurs métabolites spécifiques ont été quantifiées par LC-MS/MS suivant la méthode publiée dont l'article est disponible à l'annexe 1. Cette étude m'a permis de montrer que les patients avec le DT2 présenteraient une clairance systémique réduite via les isoformes CYP2B6, CYP2C19 et CYP3A. L'article présentant ces résultats se trouve à la section 2.1; manuscrit 2. Au cours de cette étude clinique, nous avons aussi évalué l'utilisation du 4β-

hydroxycholestérol comme biomarqueur endogène de l'activité du CYP3A dans une population avec le DT2 (objectif secondaire). Les conclusions démontrant la validité de ce biomarqueur sont disponibles dans l'article qui se trouve à la section 2.2; manuscrit 3.

Le volet *in vitro* de mes travaux a permis d'évaluer au niveau du duodénum l'influence du DT2 sur l'expression de plusieurs CYP450s et transporteurs, ainsi que sur l'activité des CYP2B6, CYP2C9, CYP2J2 et CYP3A. Aucun impact significatif du DT2 n'a été mesuré sur l'expression d'ARNm des CYP450s et transporteurs testés exprimés dans des biopsies duodénales. Les niveaux d'activité mesurés à l'aide d'incubations avec des substrats-marqueurs des CYP450s dans des fractions S9 de biopsies duodénales étaient semblables chez des sujets avec le DT2 et des non diabétiques. L'article sur les résultats de ce volet *in vitro* est disponible à la section 2.3; manuscrit 4. Ces résultats suggèrent que les effets du diabète sur le métabolisme des substrat-marqueurs observés dans l'étude clinique peuvent s'expliquer par une modulation au niveau hépatique ou dans différentes sections de l'intestin. En accord avec ces résultats chez l'humain, notre groupe avait déjà rapporté que l'effet du diabète sur les CYP450s était isoforme et tissu spécifique chez la souris (annexe 2).

L'objectif de ma thèse était de mieux comprendre les mécanismes sous-jacents à la variabilité dans la réponse aux médicaments observés chez les patients diabétiques, lesquels nécessitent fréquemment une polypharmacie. Les résultats de ces travaux permettront éventuellement d'optimiser la pharmacothérapie chez ces patients.

Mots-clés : Cytochrome P450, diabète de type 2, substrat-marqueur, *cocktail*, biomarqueur endogène, métabolisme des médicaments, pharmacocinétique, intestin, duodénum, variabilité interindividuelle.

Abstract

My PhD project evaluates factors that can influence drug metabolism and interindividual variability in drug response. More precisely, my thesis focuses on the major drug metabolizing enzymes, the cytochromes P450 (CYP450). My researches evaluated the impact of a pathological condition, namely type 2 diabetes (T2D), on CYP450 metabolic activities in two parts. First, the effect of diabetes on systemic metabolism was evaluated in patients. Then, *in vitro* experiments enabled us to measure the impact of T2D on organ-specific or metabolism. In this thesis, my research results are presented in 4 scientific papers.

The *in vivo* part of my PhD research consisted of a pharmacokinetic study assessing metabolic activity of different isoforms of the CYP450s using a *cocktail* of probe drugs in T2D patients and non-diabetic subjects. All participants of both study groups received a dose of our oral VM/JT probe drugs *cocktail* consisting of caffeine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4/5) followed by a dose of chlorzoxazone (CYP2E1), alone. Study procedures are detailed in the protocol article (*manuscrit* 1) presented in section 2.1. Plasma and urine concentrations for all probe drugs and specific metabolites were quantified using a published LC-MS/MS method that is available in annexe 1. This study showed that patients with T2D exhibited reduced systemic clearances for the isoforms CYP2B6, CYP2C19 and CYP3A. Results of this pharmacokinetic research are presented in *manuscrit* 2 of section 2.1. As a secondary objective, this *in vivo* part of my PhD project enabled us to verify the validity of 4β-hydroxycholesterol as an endogenous biomarker of CYP3A activity in a population with T2D.

Conclusions showing its validity as an endogenous biomarker in such population are presented in section 2.2 (*manuscrit* 3).

The *in vitro* part of my doctoral project evaluated in the intestines the influence of T2D on the mRNA expression of numerous CYP450 isozymes and drug transporters, as well as on metabolic activity of CYP2B6, CYP2C9, CYP2J2 and CYP3A. Using duodenal biopsies, no significant impact of T2D was detected on the mRNA expression levels of all tested CYP450s and transporters. Activity levels measured following incubations of probe-substrates in S9 fractions of duodenal biopsies obtained from patients with T2D and non-diabetic patients were similar. Results from this *in vitro* study are reported in section 2.3 (*manuscrit* 4) of this thesis. These results obtained in human subjects are in agreement with our previously published results showing isoform- and tissue-specific effects of T2D on CYP450s in mice (annexe 2).

Overall, the central theme of this thesis is to better understand the underlying mechanisms of drug response variability observed in diabetic patients, whom often require polypharmacy, in order to eventually optimize drug therapy in those patients.

Keywords : Cytochrome P450, type 2 diabetes, probe drug, *cocktail*, endogenous biomarker, drug metabolism, pharmacokinetics, intestines, duodenum, interindividual variability.

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Liste des abréviations

4β-OHC :   4β-hydroxycholestérol

ABC :   *ATP-binding casette*

ADME :   Absorption, distribution, métabolisme et excrétion

AhR :   *Aryl hydrocarbon Receptor*

ATP :   Adénosine-tri-phosphate

BCRP :   *Breast-cancer resistance protein*

BSEP :   Transporteurs des sels biliaires

CAR :   *Constitutive androstane receptor*

CEDR :   *Center for Drug Evaluation and Research*

CES :   Carboxylestérases

CHO :   Cholestérol

CRP :   Protéines C-réactives

CY :   Cytochromes

CYP450 :   Cytochrome P450

DT2 :   Diabète de type 2

Fraction S9 :   Fraction sous-cellulaire contenant le cytosol et les microsomes

GR :   *Glucocorticoïd receptor*

HNF4α :   *Hepatocyte nuclear factor 4 alpha*

IFN-γ :   Interféron gamma

IL-1β :   Interleukines 1 bêta

IL-6 :   Interleukines 6

JNK :   *JUN N-terminal Kinase*

MRP :   *Multidrug resistance protein*

NADPH :   Nicotinamide adénine dinucléotide phosphate

NO :   Oxyde nitrique

NOS :   Synthase de l'oxyde nitrique

OAT :   *Organic anion transporters*

OATP :	*Organic anion transporting polypeptides*
OCT :	*Organic cation transporters*
P :	Pigment
PD :	Pharmacodynamique
PEPT :	*Peptide transport proteins*
P-gp :	Glycoprotéine-P
PK :	Pharmacocinétique
PXR :	*Pregnane X receptor*
RH :	Dénomination du médicament ou substrat dans le contexte du cycle catalytique
ROH :	Dénomination du métabolite oxydé dans le contexte du cycle catalytique
SLC :	*Solute carrier*
TNF-α :	*Tumor Necrosis factor alpha*
VDR :	*Vitamin D receptor*
VIH :	Virus d'immunodéficience humaine

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Section 1 : Introduction

1.1   Pharmacocinétique; principe de l'ADME

La réponse aux médicaments dépend en premier lieu du devenir de la molécule administrée dans l'organisme, appelée la pharmacocinétique du médicament. Les facteurs déterminant la pharmacocinétique ou devenir d'un médicament peuvent être synthétisés sous quatre phases soit l'absorption, la distribution, le métabolisme et l'excrétion (ADME).[1] En effet, pour effectuer son action, le médicament doit avoir accès au tissu ou composante biologique ciblée. Une fois que le médicament sous sa forme active se retrouve à son site d'action, l'interaction avec sa cible thérapeutique déterminera son effet pharmacologique. Cette relation entre la concentration du médicament et son effet est appelée pharmacodynamie.[1] Bien que plusieurs facteurs touchant la pharmacodynamie puissent influencer la réponse aux médicaments, nos études se concentrent sur la variabilité au niveau des phases ADME de la pharmacocinétique, avec une attention majeure sur le métabolisme par les cytochromes P450 **(CYP450s)**. Bien que de plus en plus de produits biologiques soient commercialisés pour traiter une variété de pathologies, les CYP450s ne sont pas directement impliqués dans leur métabolisme. Conséquemment, les principes de l'ADME présentés dans cette thèse sont principalement applicables aux molécules de plus ou moins petites tailles et ne peuvent être généralisés aux produits biologiques.

1.1.1. Absorption

L'absorption est le processus durant lequel un médicament passe du site d'administration à la circulation systémique.[2] La route d'administration est une composante importante qui détermine les différents obstacles à prendre en compte concernant l'absorption d'un médicament vers la circulation systémique. La voie d'administration privilégiée, à plus de 80%, est la voie

orale qui est moins invasive, plus acceptée et pratique en milieu ambulatoire comparativement aux autres voies dites parentérales comme les voies rectales, vaginales, intrathécales, intravasculaires, sublinguales et topiques.[3] Par voie orale, l'absorption dépendra de plusieurs facteurs tout au long du tractus gastro-intestinal; la solubilité du composé administré, le temps de vidange gastrique et de transit intestinal, la stabilité chimique du composé aux différents pH du tractus, ainsi que sa perméabilité ou sa capacité à être transporté pour traverser les parois intestinales. Parmi les différentes sections du tractus gastro-intestinal, l'intestin grêle est le principal site d'absorption permettant au médicament de rejoindre la circulation sanguine. À partir de l'administration orale, l'œsophage est la première partie du tractus gastro-intestinal par où passe rapidement le médicament pour se retrouver dans l'estomac. L'estomac est une étape de dégradation des composés plutôt que d'absorption. Son milieu est maintenu acide, de pH 1 à 3.5 à jeun, grâce à la production d'acide hydrochlorique par les cellules pariétale. La production de gastrine par les cellules de l'estomac augmente la production de pepsinogène qui est le précurseur de la pepsine responsable de digérer les protéines ingérées en peptides. L'étape limitante à l'absorption à ce niveau du tractus est le temps de vidange gastrique vers l'intestin grêle. Le petit intestin est divisé en trois sections à partir de l'estomac vers le gros intestin : le duodénum, le jéjunum puis l'iléon. En plus de compléter la digestion enzymatique commencée à l'estomac, le petit intestin est majoritairement responsable du processus d'absorption. Sa structure particulière grâce aux valvules de Kerckring ou valvules conniventes, aux villosités et microvillosités augmente la surface d'absorption de cet organe représentant environ 200 m^2 chez un adulte.[3] De plus, le petit intestin est hautement vascularisé. Chaque villosité a son réseau de capillaires intestinaux qui rejoignent les veines mésentériques pour se déverser dans la veine porte hépatique. C'est un processus important qui apporte 75% du flot sanguin hépatique total.[4]

Un autre aspect régissant l'absorption au niveau de l'intestin grêle est la présence de protéines de transport d'influx et d'efflux des médicaments exprimées au niveau de la paroi intestinale. Dépendamment de leurs propriétés physicochimiques, certains principes actifs ne peuvent pas traverser les membranes par simple diffusion et nécessitent l'intermédiaire de transporteurs membranaires. Les entérocytes expriment différents transporteurs au niveau des membranes basolatérales et apicales lesquels pourront réguler les sorties et entrées de médicaments dans l'organisme lors du processus d'absorption suivant leur administration par la voie orale. Les principaux transporteurs de médicaments peuvent être catégorisés en deux superfamilles, soit les *ATP-binding casette* (ABC) et les *solute carrier* (SLC).[5,6] Ces familles de transporteurs et leur rôle dans le transport des médicaments seront décrits brièvement dans la section 1.3. En plus de la présence des transporteurs, l'absorption au niveau des cellules de la paroi intestinale peut être affectée par la présence d'enzymes du métabolisme des médicaments de phase I et II.[7] Une fois traversés les parois de l'intestin, les médicaments se retrouvent dans le système circulatoire de la veine porte qui mène au foie, soit l'organe d'extraction majeur. Par sa structure en lobules et sa haute concentration d'enzymes du métabolisme et de transporteurs des médicaments, le foie est un obstacle majeur supplémentaire à l'apparition du médicament dans la circulation systémique.[3]

1.1.2. Distribution

La distribution du composé dans l'organisme réfère à la répartition du médicament à partir du sang vers les différents tissus ou organes et vice-versa.[2] Ce processus de distribution réversible dépend grandement des caractéristiques physico-chimiques du composé d'intérêt. Les déterminants majeurs de la distribution d'un médicament sont tout d'abord sa fixation protéique et sa diffusion tissulaire. L'affinité d'un médicament à se fixer aux protéines plasmatiques est

un facteur limitant de sa distribution, car seule la portion libre d'un médicament dans le sang aura la capacité de se distribuer vers des tissus périphériques. La distribution d'un médicament au travers les membranes peut se faire par 2 principaux mécanismes de transport, soit la diffusion passive et le transport actif ou facilité.[8] Selon la lipophilicité d'une molécule, sa diffusion tissulaire sera affectée. En général, un médicament liposoluble ou une petite molécule non chargée tendra à se diffuser plus facilement au travers des membranes et à résider davantage dans les tissus adipeux. Tandis qu'une molécule plus hydrosoluble aura tendance à rester dans le sang ou à nécessiter un transport transmembranaire, ce qui régira sa capacité à se distribuer dans certains tissus. En effet, la présence et quantité d'un médicament nécessitant un transport actif dans un tissu donné dépendront de l'expression tissulaire des transporteurs des médicaments. Les niveaux d'expression des transporteurs ABC et SLC varient entre les différents organes et même d'un type cellulaire à un autre.[9,10] Bien que certains facteurs tels l'âge et la composition corporelle influencent la distribution, celle-ci est aussi hautement tributaire des caractéristiques physico-chimiques du médicament.[11]

1.1.3. Métabolisme

Le métabolisme est le processus d'élimination des médicaments le plus commun. Il représente la transformation par réactions enzymatiques du médicament en métabolite(s). Mes travaux de doctorat portent d'ailleurs sur l'importance du métabolisme des médicaments comme facteur régissant la variabilité interindividuelle dans la réponse clinique. Les médicaments administrés par les voies parentérales se retrouvent directement dans la circulation systémique et évitent donc une première phase d'extraction ou métabolisme au niveau de l'intestin et du foie appelé le premier passage intestinale-hépatique.[2] Pour les molécules administrées via la voie entérale, soit par voie orale, un obstacle majeur avant de rejoindre la circulation systémique

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est la présence importante d'enzymes du métabolisme au niveau de la paroi intestinale. Une fois ce premier obstacle passé, la molécule ne se retrouve pas directement dans la circulation systémique. Tel que mentionné précédemment, les médicaments absorbés à l'intestin se retrouvent dans le système circulatoire de la veine porte qui achemine le sang directement au foie.[3] Par sa structure et sa composition, le foie est connu comme l'organe majeur du métabolisme des médicaments.[2] Les hépatocytes sont riches en enzymes capables de métaboliser les xénobiotiques. Tout comme au niveau des entérocytes, ces systèmes enzymatiques sont responsables des réactions de phase I (oxydation, hydrolyse et réduction) et de phase II (glucuronidation, sulfonation, acétylation, méthylation, conjugaison). Les réactions de phase I sont généralement caractérisées par l'ajout d'un groupement fonctionnel polaire et/ou réactif à la molécule. Le système enzymatique majoritairement responsable des réactions de phase I, et intérêt central de cette thèse, sont les CYP450s. Cette superfamille du métabolisme des médicaments sera détaillée à la section 1.2. Les réactions de phase II sont pour leur part réversibles et consistent en la conjugaison d'un groupement fonctionnel résultant d'une réaction de phase I ou déjà présent sur la molécule à un composé endogène par glucuronidation, sulfonation, acétylation, méthylation ou conjugaison à des acides aminés ou glutathion. Le but de ces réactions métaboliques par les enzymes de phase I et II est de faciliter l'élimination d'un composé en rendant la molécule plus polaire, moins réactive et moins toxique pour l'organisme.[12] Suite à cette étape de métabolisme hépatique, les médicaments inchangés et leurs métabolites peuvent rejoindre la circulation systémique ou être excrétés vers l'intestin via les canaux biliaires.[2]

1.1.4. Excrétion

L'excrétion du médicament ou de ses métabolites est l'élimination définitive du composé de l'organisme. L'organe majoritairement responsable de l'excrétion des médicaments est le rein. En effet, une fois dans la circulation systémique, le médicament sera filtré au niveau des reins qui reçoivent environ 20 à 25% du sang éjecté par le cœur chaque minute. Environ 10% de ce volume sera filtré par les glomérules pour se retrouver au niveau des tubules rénaux, lieu de sécrétion tubulaire et réabsorption. La sécrétion tubulaire est le déplacement des médicaments des capillaires péritubulaires vers les tubules rénaux. Limitant l'excrétion, la réabsorption est le mouvement de la molécule du filtrat dans le tubule vers la circulation sanguine.[2] Bien que certaines enzymes du métabolisme des médicaments se retrouvent au niveau des tubules rénales, l'élimination du médicament au niveau rénal est surtout régie par une forte concentration de transporteurs d'influx et d'efflux des médicaments. Tout comme au niveau des entérocytes et des hépatocytes, les transporteurs de la famille des ABC et SLC détermineront le niveau d'excrétion rénale du médicament et de ses métabolites.[13] En outre, la quantité du médicament ou de ses métabolites qui seront excrétés représente la somme du médicament filtré et sécrété moins la portion réabsorbée.

Les médicaments peuvent aussi être excrété dans les fèces directement pour la portion non absorbée ou via la bile à la suite d'un premier passage entéro-hépatique lorsqu'on considère une molécule administrée par voie entérique.[2] Les autres voies mineures d'excrétion sont via nos poumons, soit dans l'air exhalé pour les substances volatiles, via les glandes sudoripares par sudation, par voie salivaire ou encore dans le lait maternel chez les femmes enceintes.[2]

Cette section présentait brièvement les grandes phases régissant la pharmacocinétique d'un médicament suivant son administration par voie orale. La prochaine section s'intéressera

principalement aux cytochromes P450 largement impliqués dans la phase du métabolisme des

médicaments.

1.2. Les cytochromes P450

La superfamille des CYP450s est majoritairement responsable du métabolisme des médicaments. Ces enzymes oxydatives seraient impliquées dans la biotransformation d'environ 75% de tous les médicaments prescrits en clinique.[14] Il est toutefois à noter que les CYP450s remplissent aussi un rôle important dans le métabolisme de substances endogènes, notamment en biotransformant des acides gras et le cholestérol (CHO).[15] Les CYP450s effectuent des réactions de phase I qui, tel qu'indiqué précédemment, consistent en l'ajout d'un groupement polaire aux médicaments afin de les rendre plus hydrosolubles et ainsi faciliter leur excrétion. Majoritairement, la localisation cellulaire des CYP450s est au niveau du réticulum endoplasmique (microsomal), bien que sa présence au niveau mitochondrial fût aussi démontrée.[16] Les niveaux d'expression des différentes isoformes des CYP450s varient selon le type cellulaire, mais surtout selon les tissus. Au niveau des hépatocytes et entérocytes, par exemple, l'expression des CYP450s est élevée. Une multitude de tissus extrahépatiques expriment des profils d'expression variables des différentes isoformes des CYP450s. Le foie présente une importante quantité de CYP450s et est connu comme étant l'organe majoritairement responsable du métabolisme des médicaments.[17] Toutefois, l'expression différentielle et distincte des différentes isoformes des CYP450s au niveau des tissus extrahépatiques pourrait jouer un rôle dans le métabolisme au niveau local et ainsi influencer la réponse aux médicaments.

1.2.1. Cycle catalytique

Les CYP450s sont une famille d'enzymes du métabolisme structurellement composés d'une apoprotéine avec en son centre une molécule d'hème. Au site actif, tous les CYP450s présentent cette portion héminique puisque le fer est essentielle à la réaction d'oxydation du substrat.[18]

L'apoprotéine présente aussi des séquences hautement conservées en lien avec son site actif telles qu'une séquence contenant une cystéine dont son souffre est responsable de la liaison avec le fer de l'hème. Une queue hydrophobique en N-terminal est aussi un élément constitutif conservé de la protéine qui permet la liaison des CYP450s à la membrane du réticulum endoplasmique.[15] Les séquences d'acides aminés variables des CYP450s, surtout au niveau périphérique de la structure de l'apoprotéine, généreront des différences de conformation. Ces différences structurelles entre les isoformes des CYP450s régiront l'affinité et l'efficacité à métaboliser certains substrats en limitant leur liaison ou l'accès au site d'action.[19] Les CYP450s microsomaux nécessitent l'aide du cofacteur NADPH (nicotinamide adénine dinucléotide phosphate). Au cours du cycle catalytique général, tel que décrit à la figure 1, le cofacteur NADPH fournira à deux reprises un électron aux CYP450s lorsqu'oxydé par un NADPH-P450 réductase.[15,18] Le deuxième électron lors du cycle catalytique peut aussi provenir d'un deuxième partenaire redox, soit la voie du NADH-cytochrome b_5 réductase/cytochrome b_5.[20] Tel qu'illustré à la figure 1, (1) le substrat ou médicament (RH) se lie au complexe CYP450, puis (2) l'oxyde ferrique (Fe^{3+}) de l'hème accepte un électron (e^-) provenant de l'oxydation du NADPH par le NADPH-P450 réductase. (3) Le fer ainsi réduit (Fe^{2+}) se lie à une molécule d'oxygène (O_2) qui se présentera sous sa forme réduite suite (4) à la réorientation d'un électron du Fe^{2+}. (5) L'O_2 sera ensuite activé par un deuxième électron provenant de la voie du NADPH ou celle du NADH-cytochrome b_5 réductase/cytochrome b_5. Le cytochrome b_5 est une protéine présente au niveau de la membrane du réticulum endoplasmique qui joue le rôle de donneur d'électron pour plusieurs réactions comme la biosynthèse des lipides ou la désaturation des acides gras à longues chaînes. Des résultats provenant d'expériences *in vitro* et à partir de modèles de souris montrent que le cytochrome b_5 stimulerait l'activité des isoformes CYP450s

de manière isoforme-dépendante.[21-23] (6) Une molécule d'eau (H_2O) sera ensuite libérée par la liaison de deux hydrogènes (H^+) à l'oxygène activé, laissant l'hème sous une forme très instable d'oxoferryle. Ainsi, (7) un hydrogène du substrat se transposera à l'oxoferryle pour former un groupe hydroxyle qui de suite (8) se recombinera avec les carbones du médicament pour former le métabolite (ROH). (9) Celui-ci se dissociera du Fe^{3+} de l'hème qui sera prêt pour un nouveau cycle.[15,18]
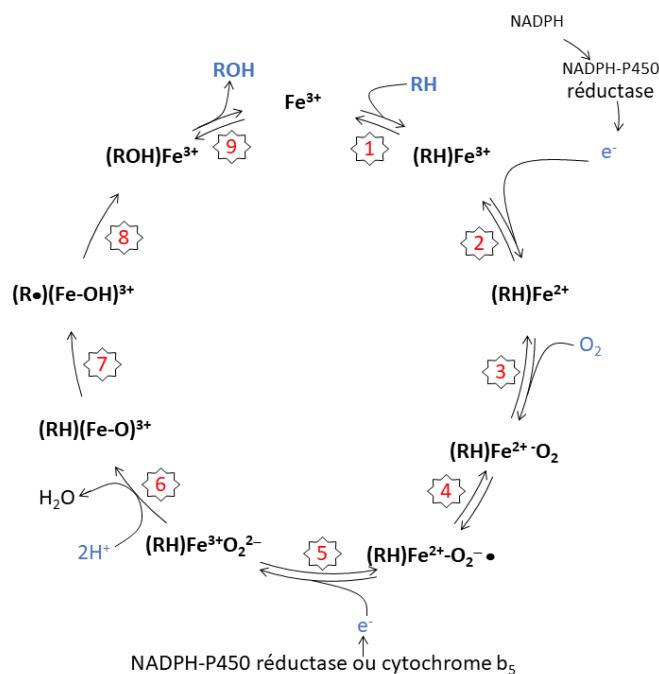
Figure 1.   Cycle catalytique général des cytochromes P450.
Figure adaptée de Danielson PB. (2002)[15] et Guengerich FP. (2013)[18]

1.2.2. Les isoformes

Les CYP450s tiennent leur dénomination du fait que ces cytochromes (CY) comportent en leur centre un pigment (P) rouge qui, lorsque lié à un groupement monoxyde de carbone, absorbe la lumière à une longueur d'onde de 450 nm. Les différentes isoformes des CYP450s, apoprotéines composées d'environ 480 et 560 acides aminé liées à un centre hème, présentent des séquences hautement variables. Certaines isoformes partageraient moins de 20% de similitude, alors que d'autres auraient une séquence d'acides aminés identique à 85%. De manière générale, les isoformes des CYP450s ont ainsi été classées en familles, puis sous-familles partageant plus de 40% et 55% d'homologie respectivement.[15] Suivant l'abréviation CYP, pour cytochrome et pigment, un premier chiffre désigne la famille (ex : CYP1, CYP2, CYP3 ou CYP4), puis une lettre représente la sous-famille (ex : CYP2B, CYP2C, CYP2D ou CYP2E). Finalement, sous une même sous-famille, un dernier chiffre désignera précisément une isoforme, par exemple CYP2C9 et CYP2C19. À ce jour, 57 gènes des CYP450s divisés en 18 familles et 43 sous-familles ont été identifiés chez l'humain.[14] De cette superfamille d'enzymes, les familles CYP1, CYP2 et CYP3 sont celles impliquées de façon importante dans le métabolisme des médicaments. En effet, les plus impliqués dans le métabolisme des médicaments (% des réactions métaboliques mineures et majeures effectuées par l'isoforme pour les médicaments sur le marché et en développement) sont les CYP3A4/5 (33%), 2D6 (13%), 2C9 (10%), 2C19 (9%), 1A2 (9%), 1A1 (5%), 2C8 (5%), 2B6 (4%) et 2E1 (3 %).[24]

1.2.2.1. CYP1

La famille CYP1 est constituée de trois isoformes divisées en 2 sous-familles, soit CYP1A (CYP1A1 et CYP1A2) et CYP1B (CYP1B1). Bien que les séquences d'acides aminés pour CYP1A1 et CYP1A2 soient homologues à plus de 70%, leur expression tissulaire est

distincte.[15] Le CYP1A1 est surtout exprimé au niveau des tissus extrahépatiques tels les poumons, le cœur, le placenta, la peau et les lymphocytes.[25-30] À l'inverse, le CYP1A2 est un cytochrome exprimé de manière constitutive au niveau du foie, mais très peu ou pas dans les divers tissus extrahépatiques.[16,30] Tout comme le CYP1A1, le CYP1B1 est surtout un cytochrome extrahépatique. De bas niveaux de CYP1B1 sont présents dans presque tous les tissus comme, entres autres, la peau, les reins, le système nerveux central, les glandes mammaires, la prostate et l'utérus.[15,30] Contrairement au CYP1A2, les isoformes CYP1A1 et CYP1B1 ne jouent qu'un rôle mineur dans le métabolisme des médicaments et sont surtout étudiées pour leur lien dans l'étiologie du cancer et la bioactivation de carcinogènes.[31-34]

1.2.2.1.1. CYP1A2

Le CYP1A2 est l'isoforme de la famille CYP1 qui est le plus impliqué dans le métabolisme des médicaments. Cette isoforme représente environ 13% de tous les CYP450s au niveau du foie.[35] Le CYP1A2 serait impliqué dans le métabolisme d'environ 9% des médicaments, par exemple, la caféine, l'imipramine, l'olanzapine, la clozapine, l'acétaminophène et la propafénone. La caféine est souvent utilisée comme substrat-marqueur de l'activité métabolique du CYP1A2. En effet, 70-80% de la caféine est transformée en paraxanthine par N-déméthylation via le CYP1A2.[36,37] Certains médicaments comme les antibiotiques quinolones (ciprofloxacine) et l'antidépresseur fluvoxamine sont rapportés comme étant des inhibiteurs puissants du CYP1A2.[38] Le CYP1A2 est aussi sujet à une induction par certains composés tels les légumes crucifères et les hydrocarbures aromatiques comme la fumée de tabac par exemple.[38] Les mécanismes d'induction identifiés au niveau transcriptionnel pour le CYP1A2 sont majoritairement régulés par la voie des AhR (*Aryl hydrocarbon Receptor*).[39]

1.2.2.2. CYP2

Chez l'humain, la famille CYP2 est la plus grande famille des CYP450s avec 16 gènes fonctionnels identifiés répartis en 13 sous-familles.[15] Cette famille est largement impliquée dans le métabolisme des médicaments et de certaines hormones stéroïdiennes.[15] Bien que le CYP2A6 joue un rôle dans le métabolisme de la coumarine ainsi que la nicotine et que le CYP2F métabolise différents composés chimiques, les sous-familles les plus largement étudiées dans un contexte pharmacologique sont les CYP2B, CYP2C, CYP2D, CYP2E et plus récemment le CYP2J.[15,24,38,40]

1.2.2.2.1 CYP2B

La sous-famille CYP2B n'est constituée que d'un seul gène, le CYP2B6. Cette isoforme représente entre 2 et 10% du contenu hépatique total des CYP450s. Toutefois, son expression est hautement variable due à sa régulation transcriptionnelle et à la présence de plusieurs polymorphismes génétiques dans la population. En effet, des variations interindividuelles de 20 à 250 fois ont été rapportées dans diverses études.[41] En plus de son expression hépatique, de bas niveaux du CYP2B6 ont été détectés dans plusieurs tissus périphériques tels les poumons, les reins, le cerveau, le cœur et la muqueuse nasale.[26,41-43] Le CYP2B6 serait impliqué dans le métabolisme de plusieurs médicaments prescrits en clinique comme le cyclophosphamide, l'ifosfamide, l'éfavirenz, le propofol, la kétamine, le bupropion et la méthadone.[41,44] Le CYP2B6 est le CYP450 le plus polymorphique chez l'humain avec plus de 100 variations et une trentaine d'allèles décrite à ce jour.[45]

L'haplotype *CYP2B6*6* (516G>T et 785A>G) constitué des allèles *CYP2B6*9* (516G>T) et le CYP2B6*4 (785A>G) est fréquemment exprimé chez les individus d'origine

caucasienne (14-27%) de même qu'à une fréquence allélique significative dans plusieurs autres populations majeures (16% chez les asiatiques du sud et 6% chez les africains).[46,47] Les individus homozygotes pour cet haplotype présenteraient une hydroxylation du bupropion significativement moindre par rapport à ceux avec deux allèles sauvages.[48-50] De façon moins marquée, les individus hétérozygotes pour l'haplotype *CYP2B6*6* présentaient aussi une diminution de l'hydroxylation du bupropion.[48-50] L'haplotype *CYP2B6*6* a été largement étudié dans le contexte du métabolisme de l'éfavirenz, un antirétroviral à fenêtre thérapeutique étroite. L'éfavirenz est désigné comme substrat-marqueur de l'activité du CYP2B6 par les agences règlementaires. Il a été rapporté que la présence de *CYP2B6*6* corrèlerait avec une concentration de l'éfavirenz supérieure à la fenêtre thérapeutique chez une population infectée avec le virus d'immunodéficience humaine (VIH).[51] D'autres études ont aussi montré que l'haplotype *CYP2B6*6* est lié à la survenue d'effets indésirables au système nerveux central et à la toxicité de l'éfavirenz.[52-54]

Bien que ce cas soit plutôt rare dans la population, le polymorphisme *CYP2B6*4* (785A>G) lorsque présent seul entrainerait une augmentation d'environ 65% de la clairance totale du bupropion qui est recommandé comme substrat-marqueur de l'activité du CYP2B6.[50,55] Un effet substrat dépendant du *CYP2B6*4* aurait été rapporté, mais les études sur ce polymorphisme sont limitées par la fréquence allélique faible chez les caucasiens et autres populations.[46,47] Plus fréquent dans diverses populations, le polymorphisme *CYP2B6*9* (516G>T) résulterait en une diminution de l'activité métabolique. Cependant, son effet a surtout été étudié dans le contexte de l'haplotype *CYP2B6*6*.[47,56-58]

Un autre polymorphisme du CYP2B6 identifié en 2001 et observé dans diverses populations est le *CYP2B6*5* (1459C>T) dont la fréquence serait d'environ 13% chez les européens, 8% chez les asiatiques du sud et 3% chez les africains.[47] Bien que certains aient rapporté une diminution d'expression du CYP2B6 au niveau du foie et du cerveau chez les porteurs de la mutation, la présence de ce polymorphisme ne semble pas affecter de façon significative la clairance du bupropion et de l'éfavirenz.[55,59-61]

En plus des nombreux polymorphismes, le CYP2B6 voit ses niveaux d'expression grandement influencés au niveau transcriptionnel par diverses cytokines. Plusieurs études ont montré que l'inflammation, par la voie des pro-cytokines interleukines 6 (IL-6) et interféron gamma (IFN-γ) entre autres, diminuait l'expression et l'activité du CYP2B6.[62-64] Aussi, en plus des polymorphismes génétiques, l'expression du CYP2B6 est modifiée par certains médicaments comme le dexaméthasone, la rifampicine et le phénobarbital qui montrent un fort potentiel inducteur sur l'expression et l'activité du CYP2B6.[41,56,65,66] Les voies des facteurs de transcription *pregnane X receptor* (PXR) et *constitutive androstane receptor* (CAR) seraient responsables de l'induction du CYP2B6 par ces médicaments chez l'humain.[67,68] De plus, le CYP2B6 est aussi sujet à l'auto-induction par certains de ses substrats, notamment par le cyclophosphamide, l'ifosfamide et l'éfavirenz.[69] Un autre facteur pouvant affecter le métabolisme via le CYP2B6 est que cette isoforme est susceptible à une inhibition enzymatique directe par différents médicaments dont les antiplaquettaires clopidogrel et ticlopidine.[70] Dans les dernières années, cette grande variabilité dans l'activité et l'expression du CYP2B6 a attiré de plus en plus l'attention sur cette isoforme du CYP450.

1.2.2.2.2. CYP2C

Les quatre gènes identifiés de la sous-famille CYP2C sont le CYP2C8, CYP2C9, CYP2C18 et CYP2C19. La séquence d'acides aminés de ces quatre isoformes partagent 80% d'homologie.[15] L'isoforme CYP2C18 n'est pas exprimée au niveau du foie et son rôle métabolique reste à déterminer. Outre l'isoforme CYP2C18, cette sous-famille représente environ 20% de tous les CYP450s hépatiques et serait impliquée dans le métabolisme du quart des médicaments utilisés en clinique en plus de participer à la transformation d'un peu moins de 20% de composés endogènes.[24] Dans des hépatocytes humains, il a été montré qu'une induction des CYP2Cs au niveau transcriptionnel par le rifampicine ou les barbituriques est possible et serait médiée par la voie CAR et PXR.[15,68,71]

1.2.2.2.2.1. CYP2C8

Le CYP2C8 est le deuxième cytochrome en importance au niveau hépatique après le CYP2C9. Il est impliqué dans le métabolisme d'environ 5% des médicaments en plus d'être responsable de la biotransformation de composés endogènes comme l'acide arachidonique au niveau du foie et du rein.[24,72] Le CYP2C8 est impliqué dans le métabolisme de plusieurs antidiabétiques comme la pioglitazone, la répaglinide et la rosiglitazone, en plus d'être souvent étudié pour son rôle dans le métabolisme d'anticancéreux comme le paclitaxel.[73] Les polymorphismes du CYP2C8 les plus communs sont le *CYP2C8*2* (805A>T), le *CYP2C8*3* (qui est une combinaison de deux mutations soit 416G>A [R139K] et 1196A>G [K399R]) et le *CYP2C8*4* (792C>G). La fréquence de ces allèles dans les différentes populations est très variable. Rare chez les autres populations, la fréquence allélique du *CYP2C8*2* est d'environ 16% chez les africains.[47] La présence de l'allèle *CYP2C8*2* entrainerait une réduction de l'activité enzymatique chez les porteurs.[47] Les allèles *CYP2C8*3* et *CYP2C8*4* sont surtout

exprimés chez les européens (11 et 6%) et les américains (7 et 2%), respectivement.[47] Des résultats d'expériences *in vitro* ont démontré que le *CYP2C8*4* résulterait en une clairance intrinsèque diminuée du paclitaxel et du répaglinide.[74,75] *In vivo*, bien qu'une diminution de l'activité de métabolisme du CYP2C8 ait été associée avec la présence du variant *CYP2C8*3*, un effet dose et substrat dépendent a été proposé.[73] En effet, différentes études ont rapporté des résultats contradictoires sur l'impact du polymorphisme *CYP2C8*3*. Par exemple, à des doses sous thérapeutiques chez l'humain, la présence du polymorphisme *CYP2C8*3* était associée à une clairance accrue du répaglinide, alors que dans d'autres études à dose plus élevée, les concentrations de répaglinide chez des individus porteurs de la mutation *CYP2C8*3* n'étaient pas différentes de celles chez les non-mutés.[76-79] Davantage d'études sur l'effet de ces polymorphismes sont nécessaires afin de bien comprendre leur impact sur la pharmacocinétique des différents substrats et leur potentiel impact clinique.

1.2.2.2.2.2. CYP2C9

En plus d'être la deuxième isoforme CYP450 hépatique en importance après le CYP3A, le CYP2C9 représente environ 14% des CYP450s au niveau des intestins, un organe important de métabolisme des médicaments lors du premier passage entéro-hépatique.[80] Le CYP2C9 joue un rôle important dans le métabolisme d'environ 10% des médicaments prescrits en clinique, dont des molécules à index thérapeutique étroit comme la S-warfarine, le tolbutamide et la phénytoïne.[24] L'activité du CYP2C9 peut être inhibée par plusieurs composés dont l'amiodarone, le sulfaphenazole ou le fluconazole.[81] Puisque le CYP2C9 est responsable de métaboliser des médicaments à fenêtre thérapeutique étroite pouvant donner lieu à des effets indésirables graves, cette isoforme est hautement étudiée et considérée en clinique. Un exemple d'interaction médicamenteuse impliquant le CYP2C9 est celui de l'amiodarone et de la S-

warfarine. L'utilisation de l'amiodarone chez des sujets sous warfarine entraine, par l'inhibition de CYP2C9, une augmentation de l'exposition à la warfarine, ce qui résulte en une augmentation du risque d'effets indésirables graves, soit de saignements importants.[82,83] Outre les interactions médicamenteuses engendrées par des inhibiteurs, les polymorphismes génétiques du CYP2C9 jouent aussi un rôle dans la variabilité de la réponse aux médicaments observée dans la population. Les deux polymorphismes les plus fréquents dans la population sont le *CYP2C9*2* (430C>T) et *CYP2C9*3* (1075A>C). La fréquence allélique chez les caucasiens pour *CYP2C9*2* est d'environ 15% comparativement à 10% pour *CYP2C9*3*.[84] Ces polymorphismes sont plus rares dans les autres populations, à l'exception du *CYP2C9*3* dont la fréquence allélique serait de 11% dans les populations du sud de l'asie.[47] L'hypoglycémiant tolbutamide est un substrat spécifique du CYP2C9 qui est considéré comme substrat-marqueur valide de l'activité de cette isoforme.[85] Les polymorphismes *CYP2C9*2* et *CYP2C9*3* ont été associés à une diminution de clairance du tolbutamide et dans certains cas à une augmentation des risques d'hypoglycémie chez les patients diabétiques.[84,86] Ces observations suggèrent que les polymorphismes *CYP2C9*2* et *CYP2C9*3* diminuent l'activité métabolique du CYP2C9. Il a donc été suggéré que les individus porteurs d'allèles mutés du CYP2C9, soit les métaboliseurs lents, nécessiteraient une plus petite dose de l'anticoagulant warfarine, un substrat du CYP2C9 qui possède un index à fenêtre thérapeutique étroite. En effet, aux mêmes doses que les individus non mutés pour CYP2C9, les porteurs des polymorphismes *CYP2C9*2* et *CYP2C9*3* sous warfarine sont exposés à un risque accru de présenter des saignements sévères.[84,86] Un autre exemple pour lequel la présence des polymorphismes *CYP2C9*2* et *CYP2C9*3* a un impact sur le métabolisme d'un médicament, et conséquemment sur la réponse au traitement, est celui de l'antiépileptique phénytoïne. En effet, le phénytoïne étant métabolisé à 90% par le CYP2C9, la

présence de ces polymorphismes entraine des concentrations du médicament plus élevées, ce qui semble lié à la survenue d'effets indésirables au système nerveux central.[84,86,87] L'influence des différents polymorphismes sur l'activité du CYP2C9, les nombreux inhibiteurs de l'enzyme, son rôle important dans le métabolisme d'une multitude de médicaments, dont certains à fenêtres thérapeutiques étroites, font de cette isoforme un des CYP450s les plus documentés dans un contexte clinique.

1.2.2.2.2.3. CYP2C19

Le CYP2C19 est la troisième isoforme en importance de la sous-famille CYP2C au niveau du foie après CYP2C9 et CYP2C8.[88] Son expression a aussi été rapportée au niveau de l'intestin grêle.[80] Bien que moins exprimé que le CYP2C9, le CYP2C19 est une isoforme importante dans un contexte clinique puisqu'il est impliqué dans le métabolisme d'environ 9% des médicaments.[24] Parmi les substrats du CYP2C19, l'anticonvulsivant S-méphénytoïne et l'inhibiteur de pompe à proton oméprazole ont été identifiés comme des substrats-marqueurs de l'activité de cette isoforme.[89] D'autres médicaments comme l'antiplaquettaire clopidogrel (un prémédicament), l'antifongique voriconazole et l'antidépresseur escitalopram sont des substrats connus du CYP2C19.[90] Une importante variation dans les niveaux d'activité du CYP2C19 a été rapportée et trois polymorphismes fonctionnels fréquents pour ce gène montrent une distribution hautement différentielle dans les différentes populations. Parmi ces polymorphismes, le *CYP2C19*2* (681G>A) et le *CYP2C19*3* (636G>A) résultent en une protéine tronquée et inactive, donnant ainsi lieu en un phénotype de métaboliseur intermédiaire chez les hétérozygotes et un phénotype de métaboliseur lent chez les individus homozygotes.[91] Le *CYP2C19*17* (-806C>T) résulte pour sa part en une augmentation de l'expression de la protéine, ce qui confère un statut de métaboliseur ultrarapide aux porteurs de cet allèle.[91] Alors que l'allèle

CYP2C19*17 est majoritairement exprimé chez les populations européennes, africaines et américaines (42-55%), le *CYP2C19*2* est surtout exprimé chez les populations asiatiques avec une fréquence allélique de plus de 30%.[47] Pratiquement absent dans plusieurs populations, l'allèle *CYP2C19*3* est exprimé surtout chez les populations de l'Asie de l'est avec un fréquence allélique inférieure à 7%.[47] Le CYP2C19 et ses polymorphismes est un gène important à considérer en pharmacologie, comme pour le cas du clopidogrel où les métaboliseurs lents ont été associés à un risque accru d'événements cardiovasculaires graves.[92]

1.2.2.2.3. CYP2D

Le seul gène fonctionnel identifié à ce jour de la sous-famille du CYP2D est le CYP2D6. Bien qu'il ne représente que 2 et 0,7% des CYP450s hépatiques et intestinales respectivement, cette isoforme serait impliquée dans le métabolisme d'environ 13% des médicaments provenant de classes thérapeutiques variées.[24,80] En effet, le CYP2D6 est entre autres impliqué dans le métabolisme d'agents β-bloqueurs, d'antihistaminiques de première génération, d'antidépresseurs tricycliques, d'inhibiteurs spécifiques du recaptage de la sérotonine, d'antiarythmiques de classe 1, d'analgésiques comme la codéine, le tramadol et l'oxycodone, d'antitussif comme le dextrométhorphane ou encore de l'antinéoplasique tamoxifène.[93] Contrairement aux autres CYP450s, le CYP2D6 ne semble pas inductible par des composés.[94] Par ailleurs, le gène du *CYP2D6* est hautement polymorphique et ces nombreuses mutations affectent la réponse à de nombreux médicaments.[93] En plus de la présence de nombreux polymorphismes génétiques, le CYP2D6 est sujet à une modulation de son activité par différents inhibiteurs dont notamment l'antiarythmique quinidine.[38] Les substrats-marqueurs de l'activité du CYP2D6 souvent utilisés sont le bufuralol et le dextrométhorphane.[95]

Bien que non inductible, la duplication des allèles à fonction normale *CYP2D6*1* (*CYP2D6*1xN*; 2850C>T) et *CYP2D6*2* (*CYP2D6*2xN*; 4180G>C) résulte en une augmentation de l'activité métabolique du CYP2D6, soit en des métaboliseurs dit ultrarapides. Une duplication du gène *CYP2D6* est rapportée pour 1,5 à 9,3% des allèles dans diverses populations. Au contraire, dépendamment des populations étudiées, entre 25,3 et 70,3% des allèles du CYP2D6 sont composés de variants donnant lieu à une diminution ou absence d'activité catalytique de l'enzyme. Le *CYP2D6*3* (1749A>G et 2549delA), détectable surtout chez les populations européennes avec une fréquence allélique de 4%, résulte en une absence d'activité. Un autre polymorphisme résultant en une absence d'activité est le *CYP2D6*4* (1846G>A) dont la prévalence est supérieure à 10% dans la majorité des groupes ethniques et atteindrait 16% chez les individus d'origine caucasienne. La nomenclature *CYP2D6*5* désigne une délétion complète du gène CYP2D6 et est identifié pour environ 4% des allèles. Surtout présent chez les caucasiens (< 2%), le polymorphisme *CYP2D6*6* (1707delT) est aussi associé à une absence d'activité métabolique. Finalement, deux variants du CYP2D6 majoritairement exprimés chez les populations d'Asie, le *CYP2D6*10* (100C>T) et *CYP2D6*41* (2988G>A), sont associés à une activité métabolique réduite. Alors que plutôt rare chez les pour les autres populations majeures, une fréquence allélique de 59% chez les populations de l'Asie de l'est a été rapportée pour le *CYP2D6*10*. Le variant *CYP2D6*41* est pour sa part plus fréquent chez les populations non asiatiques avec une fréquence allélique d'environ 3%.[47]

Les nombreuses combinaisons possibles des polymorphismes du CYP2D6 résultent en différents statuts de métaboliseurs basés sur les génotypes.[96-98] Pour notre étude, les génotypes ont été classés selon les variants identifiés tel que présenté dans la table I ci-dessous en se basant sur les études de pharmacogénétiques disponibles.[96,98-100] Les 4 catégories distinctes de

métaboliseurs sont réparties comme suit chez les caucasiens; 5-10% de métaboliseurs lents, 10-15% d'intermédiaires, 60-85% de métaboliseurs extensifs et 1-10% d'ultrarapides.[96] Conséquemment, ces différents phénotypes d'activité du CYP2D6 ont donné lieu à la création de plusieurs lignes directrices pour règlementer et monitorer l'utilisation de plusieurs de ses substrats afin d'éviter des échecs thérapeutiques et effets indésirables graves.[96-98]

Table I.   Détermination du statut de métaboliseur selon les génotypes du gène CYP2D6.

Statut de métaboliseur	Sans duplication du gène	Avec duplication du gène (*CYP2D6xN*)
UM		• ≥3 allèles à fonction normal ou accrue
EM	• 2 allèles à fonction normal • 1 allèle à fonction normale/1 allèle à fonction accrue ou réduite • 1 allèle à fonction accrue/1 allèle à fonction réduite	• 2 allèles à fonction normale/≥1 allèle à fonction réduite ou inactive • ≥3 allèles à fonction réduite
IM	• 1 allèle à fonction normale/1 allèle inactif • 2 allèles à fonction réduite • 1 allèle à fonction réduite/1 allèle inactif	• 1 allèle à fonction normale/≥2 allèles inactifs • 2 allèles à fonction réduite/1 allèle inactif • 1 allèle à fonction réduite/≥2 allèles inactifs
PM	• 2 allèles inactifs	• Seulement des allèles inactifs

UM, métaboliseur ultrarapide; EM, métaboliseur extensif; IM, métaboliseur intermédiaire; PM, métaboliseur pauvre.

1.2.2.2.4. CYP2E

Le CYP2E1 ne serait impliqué dans le métabolisme que de 3% des médicaments tels les anesthésiques halogénés ou la 6-hydroxylation du chlorzoxazone, un relaxant musculaire reconnu comme substrat-marqueur de l'activité de cette isoforme.[24,95,101] Le CYP2E1 représente tout de même 9% du contenu en CYP450 hépatique, en plus d'être exprimé dans divers tissus extrahépatiques comme le cœur, le cerveau ou les poumons.[26,80,102,103] Outre les médicaments, le CYP2E1 est impliqué dans la bioactivation de nombreux composés chimiques résultant en des métabolites à potentiel cancérigène, dans la formation de radicaux libres, ainsi que dans l'oxydation de l'éthanol et son potentiel tératogène.[104-109] Plusieurs études ont démontré des variations d'activité et d'expression du CYP2E1 jusqu'à 5 et 50 fois respectivement.[15] Contrairement à la majorité des CYP450s, la régulation du CYP2E1 se ferait davantage au niveau prétranslationnel, translationnel et post-translationnel que transcriptionnel par des mécanismes de stabilisation de la protéine et de son ARNm.[15,110] Le CYP2E1 peut notamment être induit par plusieurs substrats, par exemple l'éthanol, l'imidazole, l'acétone et le trichloréthylène, ainsi que dans des situations pathophysiologiques comme l'inflammation, le jeûne, le diabète et l'obésité.[111-113]   En plus de son potentiel à être induit, quelques polymorphismes du CYP2E1 ont été identifiés. Parmi ceux-ci, le variant *CYP2E1*1B* (9896C>G), qui résulte en l'absence d'un site de restriction TaqI, est le plus fréquent chez les caucasiens.[114] Très variable selon les populations, la fréquence du génotype hétérozygote pour l'allèle mineur (C/G) est de 24% et celle pour les homozygotes du variant (G/G) est de 1% chez les caucasiens.[115] Bien que ce variant ne semble pas modifier l'activité métabolique du CYP2E1 tel qu'évalué avec le substrat-marqueur chlorzoxazone, la présence de ce variant semble protéger contre la survenue de maladies alcooliques du foie.[116,117] Un autre variant souvent

étudié est la combinaison *CYP2E1*5B* (-1053C>T (RsaI) et -1293G>C (PstI)).[114] Chez les caucasiens, 7,5% exprimeraient le génotype hétérozygote, alors que seulement 0,1% environ serait homozygote muté.[115] Son effet sur l'activité métabolique du CYP2E1 n'est pas clairement déterminé. En effet, certains proposent une diminution du métabolisme de la théophylline en présence de l'allèle muté, alors qu'aucun effet du génotype du CYP2E1 sur la 6-hydroxylation du chlorzoxazone fut démontré dans une population contrôle caucasienne.[116,118] Outre son impact sur l'activité de l'isoforme du CYP450, le variant *CYP2E1*5B* semble associé avec une protection contre l'hépatotoxicité des médicaments anti-tuberculose et avec la survenue de certains cancers.[119,120]

1.2.2.2.5. CYP2J

Bien que le CYP2J2 soit présent au niveau du foie, son expression hépatique, de même qu'intestinale, ne représenterait que de 1-2% du contenu CYP450 total.[80] L'isoforme extrahépatique CYP2J2 a été identifiée en faible concentration (protéine et ARNm) au niveau du cerveau, du rein et du pancréas, mais serait le principal CYP450 au niveau cardiaque.[26,40,121] Bien que son principal rôle semble être le maintien de l'homéostasie cardiovasculaire dû notamment à son implication dans la biotransformation de l'acide arachidonique en acide époxyeicosatriénoïque, le CYP2J2 modulerait aussi la cardiotoxicité induite par les médicaments.[122-124] Le CYP2J2 partage un chevauchement important dans la spécificité des substrats avec le CYP3A. Les médicaments métabolisés par le CYP2J2 sont entre autres plusieurs antihistaminiques dont la terfénadine, l'astémizole et l'ébastine ou encore l'antiarythmique amiodarone et l'immunosuppresseur cyclosporine lesquels sont également des substrats du CYP3A4/5.[26,125] L'hydroxylation de l'antihistaminique ébastine est d'ailleurs un substrat-marqueur utilisé pour déterminer l'activité du CYP2J2.[126] Un des variants connu du

CYP2J2 est le *CYP2J2*6* (1210A>T). Bien que ce variant ait une fréquence allélique inférieur à 2% dans la population, son effet a été largement étudié.[125] La présence de l'allèle résulterait en une diminution de l'activité enzymatique, dénotée entre autres par une réduction du métabolisme de l'acide arachidonique.[40,127] Le polymorphisme *CYP2J2*7* (-50G>T) est pour sa part davantage répandu dans les différentes populations avec un fréquence allélique d'environ 17% chez les africains, 10% chez les caucasiens et un peu moindre dans différentes populations asiatiques.[125] L'allèle *CYP2J2*7* entrainerait une diminution d'environ 50% de la transcription résultant ainsi en une diminution de l'expression de la protéine et des concentrations de ses métabolites de l'acide arachidonique (acides *cis*-époxyéicosatriénoïques).[40,127,128] Bien que certains résultats semblent contradictoires ou dépendre du groupe ethnique étudié et que davantage d'études devraient être menées, la présence du polymorphisme *CYP2J2*7* serait un facteur de risque pour certaines maladies cardiovasculaires tels l'infarctus du myocarde, les maladies coronariennes et l'hypertension.[40,125,128-130] De plus, la présence du polymorphisme *CYP2J2*7* a été associée au développement précoce du diabète de type 2 (DT2), ainsi qu'à la progression de la maladie d'Alzheimer dans une population chinoise.[131,132]

1.2.2.3. CYP3

La famille CYP3 n'est constituée que d'une seule sous-famille, les CYP3As. Cette sous-famille compte quatre gènes fonctionnels, soit le CYP3A4, CYP3A5, CYP3A7 et CYP3A43.[15] L'isoforme CYP3A7 arbore une expression protéique hépatique surtout lors du développement fœtal, alors que de faibles niveaux de son ARNm sont détectés chez l'adulte.[133] Le CYP3A43 est quant à lui surtout exprimé au niveau de la prostate et serait responsable du métabolisme de stéroïdes.[134] Bien que son expression au cerveau soit associée au métabolisme

d'antipsychotiques, son rôle mineur dans le métabolisme des médicaments reste à déterminer.[135,136] Les deux isoformes CYP3A4 et CYP3A5 partagent 85% d'homologie en plus de présenter un important chevauchement dans la spécificité de leurs substrats.[137,138] Ces deux gènes de la sous-famille CYP3A seraient impliqués dans le métabolisme de plus du tier des médicaments, ce qui leur confère beaucoup d'attention et d'importance en clinique.[24]

1.2.2.3.1. CYP3A4 et CYP3A5

Les isoformes CYP3A4/5 constituent 82% du contenu hépatique total en CYP450.[80] L'isoforme CYP3A4 est majoritaire au niveau de l'intestin où les CYP3As représentent 40% des CYP450s.[80] Pour sa part, l'importance du CYP3A5 est surtout au niveau de son expression extrahépatique, notamment au niveau des poumons, du colon, des reins, de l'œsophage et de la glande pituitaire antérieure.[138] Bien que le CYP3A4 et le CYP3A5 présentent un important chevauchement dans la spécificité de leurs substrats, le CYP3A4 demeure l'isoforme la plus importante dans le métabolisme des médicaments au niveau du premier passage entéro-hépatique. Le CYP3A4 à lui seul métabolise environ 27% des médicaments prescrits en clinique.[24] On compte parmi les nombreux substrats du CYP3A4/5 des immunosuppresseurs comme la cyclosporine et le tacrolimus, des antirétroviraux tels les inhibiteurs de protéases, des inhibiteurs calciques, des statines, des benzodiazépines dont entre autres le midazolam, ainsi que des antidépresseurs.[15,138] Le midazolam et son métabolite 1'-hydroxymidazolam sont utilisés comme substrat-marqueur de l'activité CYP3A4/5.[95] On compte aussi plusieurs substrats endogènes du CYP3A4/5, notamment le cortisol et le cholestérol .[138] D'ailleurs, les ratios 6-hydroxycortisol sur cortisol et 4β-hydroxycholestérol (4β-OHC) sur cholestérol ont aussi été proposés comme marqueurs endogènes de l'activité CYP3A.[139,140] L'activité

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CYP3A4/5 présente une grande variabilité interindividuelle et leur grand nombre de substrats peut résulter ou accroître le nombre d'interactions médicamenteuses.

Pour le CYP3A4, cette variation dans l'activité de l'enzyme semble peu expliquée par la présence de polymorphismes génétiques alors qu'il est un gène hautement conservé. En effet, peu de données concluantes sur l'impact des polymorphismes sur l'activité du CYP3A4 sont disponibles. Plus récemment, l'allèle *CYP3A4*22* (6C>T) qui a une fréquence allélique de 5-7% chez les caucasiens a été identifié.[141] Il a été rapporté que ce polymorphisme réduirait l'expression et l'activité du CYP3A4, effet démontré cliniquement important dans le contexte de traitements avec le tacrolimus.[142-145] Par contre, en plus d'être relativement rare, l'impact du *CYP3A4*22* n'a pas été démontré dans d'autres études, notamment lorsque le marqueur d'activité utilisé était le métabolite CYP3A4 spécifique 4β-OHC.[146,147]

Pour leur part, plusieurs polymorphismes du CYP3A5 ont montré un effet significatif sur les niveaux d'activité de l'enzyme. Le polymorphisme *CYP3A5*3* (6986A>G) est le plus répandu dans la population générale et donne lieu à une protéine non fonctionnelle. Chez les caucasiens, la fréquence allélique du *CYP3A5*3* serait de 82 à 95%, alors qu'elle est de seulement 18% chez les populations d'Afrique qui présente un profil de polymorphismes génétiques distinctif des autres populations pour CYP3A5.[47,148] Un autre polymorphisme important est le *CYP3A5*6* (14690G>A) qui résulte en une absence d'expression et activité du CYP3A5.[148] Très rare chez les caucasiens et les asiatiques, sa fréquence allélique est d'environ 15% pour les populations africaines.[47,148]

Outre les polymorphismes, l'expression des CYP3A4 et CYP3A5 peut notamment être régulée au niveau transcriptionnel. Plusieurs récepteurs nucléaires tels PXR, CAR, HNF4α (*hepatocyte nuclear factor 4 alpha*), VDR (*vitamin D receptor*) et GR (*glucocorticoïd receptor*) sont impliqués dans la régulation transcriptionnelle des gènes *CYP3A*.[149,150] Bien que le CYP3A4 semblerait plus sensible à l'effet de nombreux inhibiteurs, l'activité des deux isoformes (CYP3A4 et 3A5) est diminuée par entre autres le kétoconazole, fluconazole, l'érythromycine et la clarithromycine.[149] Une inhibition irréversible du CYP3A4 au niveau de l'intestin par le jus de pamplemousse a été largement documentée et sa pertinence clinique est importante.[151] En effet, des interactions préjudiciables avec le jus de pamplemousse sont rapportées pour entre autres des statines (atorvastatine et simvastatine), des bloquants calciques (nifédipine), des immunosuppresseurs (cyclosporine) et des agents psychoactifs (buspirone) ou encore des antiarythmiques (amiodarone).[152] Finalement, une modulation à la baisse de l'expression et l'activité du CYP3A4 par des cytokines pro-inflammatoires a été observée *in vitro* et *in vivo*, complexifiant davantage les mécanismes sous-jacents à la grande variabilité interindividuelle observée pour les substrats des CYP3As.[62,64,153-156]

1.3. Les transporteurs

En plus des enzymes du métabolisme comme les CYP450**s**, les transporteurs membranaires régissent aussi la biodisponibilité, via l'absorption, des médicaments tel que mentionné à la section 1.1. En règle générale, les transporteurs d'influx favorisent l'absorption des médicaments, alors que les transporteurs d'efflux la limitent. Ainsi, la présence des transporteurs d'influx et d'efflux sera un déterminant majeur du devenir du médicament dans l'organisme. Les transporteurs régiront l'absorption, la distribution et l'élimination des médicaments et conséquemment, leurs concentrations plasmatiques ou tissulaires. Deux superfamilles de transporteurs sont largement impliquées dans le transport des médicaments, soit les ABC et les SLC.[157] Les transporteurs ABC sont reconnus comme des transporteurs d'efflux et effectuent un transport actif primaire des médicaments. C'est-à-dire qu'ils utilisent directement l'ATP (adénosine-tri-phosphate) comme source d'énergie pour transporter une molécule contre son gradient de concentration.[158] Les SLC, majoritairement transporteurs d'influx, transportent les médicaments par transport facilité ou actif secondaire. Le transport facilité ne nécessite pas de source d'énergie, car il se fait dans le sens du gradient de concentration et peut être bidirectionnel. Le transport actif secondaire utilise les gradients ioniques (H^+, Na^+ et Ca^{2+}) créés par transport actif primaire pour transporter des molécules contre leur gradient.[158]

1.3.1. ABC

Chez l'humain, 48 gènes des transporteurs ABC ont été identifiés et répartis dans 7 sous-familles.[159] Les transporteurs ABC les plus connus sont entre autres ABCB1 (glycoprotéine-P ou P-gp), les ABCC (MRP; *Multidrug resistance protein*), ABCG2 (BCRP; *breast-cancer resistance protein*), ABCG5/8 (transporteurs du cholestérol) et BSEP (transporteurs des sels biliaires).[159] Pour cette thèse, l'expression des transporteurs ABCB1 et ABCG2 a été évaluée au

niveau de l'intestin grêle. Ainsi, seuls les transporteurs étudiés au cours de mes travaux sont détaillés ci-bas.

ABCB1 ou P-gp, est un important transporteur d'efflux des médicaments qui est exprimé entre autres au niveau de l'intestin grêle, du foie, des reins et du cerveau.[159,160] Au niveau de l'intestin, ABCB1 est exprimé à la membrane apicale des entérocytes matures et limite l'absorption de nombreux substrats en les transportant de l'entérocyte vers la lumière intestinale.[161] Les transporteurs ABCB1 transportent une grande variété de médicaments comme des agents chimiothérapeutiques, des statines, des immunosuppresseurs, des bloqueurs calciques, des antirétroviraux et des antidépresseurs.[151] Plusieurs substrats du transporteur ABCB1 sont aussi des médicaments substrats du CYP3A4, par exemple, la cyclosporine, l'érythromycine, le tacrolimus et le vérapamil.[151,161] D'ailleurs, la cyclosporine et le vérapamil ont aussi un potentiel inhibiteur sur le ABCB1.[151]

Les transporteurs d'efflux ABCG2 ou BCRP présentent une distribution d'expression similaire au P-gp. Ils présentent une expression importante au niveau de la membrane apicale des entérocytes, des hépatocytes et des cellules épithéliales des tubules proximales des reins.[160,162] ABCG2 arbore un large spectre de substrats comme des agents anticancéreux, des inhibiteurs de la tyrosine kinase, des statines, des antibiotiques et des antiviraux.[151] Ainsi, de manière générale, les transporteurs ABCG2 limitent l'absorption des médicaments, limitent leur distribution tissulaire et favorisent leur élimination.

1.3.2. SLC

La famille des SLC est principalement constituée de transporteurs d'influx des médicaments. Plus de 362 gènes répartis en 55 sous-familles ont été identifiés chez l'humain.[163] Les *organic anion transporters* (OAT), *organic anion transporting polypeptides* (OATP), *organic cation transporters* (OCT) et *peptide transport proteins* (PEPT) font tous partie de la famille des transporteurs SLC.[163] Pour ma thèse, l'expression du gène OATP2B1 ou SLCO2B1 a été évaluée au niveau du duodénum et sont détaillés ci-bas.

L'OATP2B1 est surtout exprimé au niveau de la membrane basolatérale des hépatocytes des sinusoïdes du foie. Ils transportent donc les médicaments du sang vers l'intérieur des hépatocytes riches en enzymes du métabolisme tels les CYP450s.[158,164] Le transport OATP2B1 est largement exprimé dans l'organisme et sa présence au niveau de l'intestin grêle a aussi été rapportée.[160,164] Son expression intestinale serait surtout à la membrane apicale des cellules épithéliales.[164] Des polymorphismes du SLCO2B1 ont montré un effet significatif sur les paramètres pharmacocinétique et même pharmacodynamiques pour plusieurs drogues comme la rosuvastatine, le fexofénadine et céliprolol.[165-168]

1.4. Phénotypage des enzymes du métabolisme des médicaments; CYP450s

Chez les patients, la réponse aux différents médicaments est hautement variable. Dans certaines niches thérapeutiques, le taux de réponse favorable à un traitement peut être minoritaire dans la sous-population de patients traités. En général, le taux de réponse aux médicaments serait inférieur à 75% comme c'est le cas pour le diabète où celui-ci serait de 57%.[169] Un déterminant majeur de la réponse pharmacologique serait le métabolisme des médicaments via les CYP450s. La présence de polymorphismes génétiques, tel que discuté précédemment, est largement étudiée et même dans certains cas utilisés en clinique afin d'améliorer l'efficacité d'un traitement ou encore de minimiser la survenue d'effets indésirables. Cependant, le génotypage des CYP450s permet d'expliquer qu'une partie de la variabilité. En effet, dans un même groupe génotypique, les réponses aux médicaments restent variables. Outre les polymorphismes génétiques, plusieurs facteurs peuvent affecter l'activité des CYP450s; l'âge, le sexe, le statut pathologique, des facteurs environnementaux comme la cigarette, les substances chimiques environnementales ou encore un traitement médicamenteux concomitant.[170] Ces différents facteurs peuvent résulter en une phénoconversion du statut métabolique, soit en une divergence qui peut s'avérer cliniquement significative entre la capacité métabolique théorique basée sur le génotype et l'activité des CYP450s observée en pratique à un moment déterminé dans le temps. Afin de réduire les risques d'échec thérapeutique ou de réactions indésirables aux médicaments administrés aux doses usuelles, il apparait que la détermination du phénotype ou activité réelle des CYP450s serait préférable à la détermination du génotype seul. L'utilisation de substrats-marqueurs permet d'évaluer *in vitro* ou *in vivo* la

capacité métabolique (phénotype) réelle d'une enzyme par la quantification de leur biotransformation.

1.4.1. Substrats-marqueurs

Un substrat-marqueur est un médicament qui permet de déterminer le phénotype d'une enzyme par la quantification de son métabolisme, soit la mesure de son ou ses métabolites spécifiques à la voie métabolique étudiée. Un médicament marqueur doit donc être majoritairement ou exclusivement métabolisé par une isoforme des CYP450s pour laquelle nous voulons déterminer le phénotype. Le substrat-marqueur ne doit pas présenter d'autre source majeure de variabilité que la voie CYP450 étudiée. Finalement, le substrat-marqueur doit être sécuritaire et facilement disponible.[171,172] Le phénotypage peut se faire à l'aide d'un seul substrat-marqueur à la fois, mais aussi grâce à un *cocktail* qui permet de phénotyper plusieurs isoformes des CYP450s au même moment. Dans le cas d'un *cocktail* où plusieurs substrats-marqueurs sont administrés, ceux-ci doivent être spécifiques et exempts d'interaction. Certains *cocktail*s utilisés à ce jour permettraient de phénotyper jusqu'à 9 isoformes des CYP450s.[173,174] Suite à l'exposition ou administration du médicament marqueur, le ou les métabolite(s) spécifique(s) et substrat seront quantifiés dans la matrice et un ratio métabolique permettra de déterminer le phénotype d'activité de l'isoforme étudiée.[173]

1.4.1.1.   *In vitro*

Dans le cadre du développement de médicaments, des expériences *in vitro* sont surtout utilisées afin de phénotyper le profil de métabolisme d'une molécule et d'évaluer son potentiel d'interactions médicamenteuses. Les différents modèles utilisés *in vitro* lors d'étude du métabolisme des médicaments sont généralement les modèles d'enzymes recombinantes

humaines (rhCYP450), de fractions sous-cellulaires contenant le cytosol et les microsomes (fractions S9), cytosoliques ou microsomales, de tranches d'organes et de cultures cellulaires.[175] Pour cette thèse, les niveaux d'activité (phénotypes) des isoformes des CYP450s intestinaux ont été déterminés *in vitro* par incubation de substrats-marqueurs dans la fraction cellulaire S9 de biopsies duodénales, suivi d'une quantification par LC-MS/MS des métabolites d'intérêt. Les substrats-marqueurs pouvant être utilisés *in vitro* sont moins restrictifs qu'en *in vivo*, mais la qualité de ceux-ci a largement été documenté. La FDA a d'ailleurs publié des lignes directrices claires concernant les substrats-marqueurs valides et recommandés qu'utilisent l'industrie pharmaceutique.[176] Le tableau ci-dessous montre les réactions de substrats-marqueurs privilégiés par la FDA pour les études de métabolisme des principaux CYP450 *in vitro*, ainsi que les réactions de substrats-marqueurs les plus souvent utilisés selon le *Center for Drug Evaluation and Research (CEDR)*.

Table II. Réactions métaboliques spécifiques de différents substrats-marqueurs des CYP450s *in vitro*.

Enzyme	Réactions des substrats-marqueurs
CYP1A2	Phénacétine (O-dé-éthylation), 7-Éthoxyrésorufine (O-dé-éthylation), caféine (déméthylation)
CYP2B6	Éfavirenz (hydroxylation), Bupropion (hydroxylation), 7-éthoxy-4-trifluorométhyl coumarine (O-désalkylation)
CYP2C8	Paclitaxel (6α-hydroxylation), Amodiaquine (N-dé-éthylation)
CYP2C9	S-Warfarine (7-hydroxylation), Diclofénac (4'-hydroxylation), Tolbutamide (4′-hydroxylation)
CYP2C19	S-Méphénytoïne (4'-hydroxylation)
CYP2D6	Bufuralol (1'-hydroxylation), Dextrométhorphane (O-déméthylation), débrisoquine (4-hydroxylation), spartéine (oxydation)
CYP2E1	Chlorzoxazone (6-hydroxylation), débrisoquine, spartéine (oxydation), ρ-nitrophénol
CYP3A4/5	Midazolam (1'-hydroxylation), Testostérone (6β-hydroxylation) Nifédipine (oxydation), érythromycine (N-déméthylation), cyclosporine (oxydation), terfénadine (hydroxylation)

Adapté de *Drug Interactions & Labeling FDA Guidances* (2016)[177] et Yuan et al. (2002)[95].

Pour notre projet, le phénotypage de l'activité de 4 isoformes des CYP450s au niveau duodénal a été effectué séparément à l'aide de substrats-marqueurs reconnus. Le phénotype du CYP2B6 a été déterminé avec le bupropion (un anti-dépresseur et un antitabagique) qui est un substrat-marqueur validé.[178] Le CYP2B6 est le CYP450 responsable du métabolisme du

bupropion en hydroxybupropion, bien qu'une contribution négligeable du CYP2E1 est suggérée.[178-181] D'ailleurs, l'utilisation d'un anticorps du CYP2B6 inhibe 95% de la biotransformation du bupropion en hydroxybupropion.[179]

L'activité métabolique du CYP2C9 a été évaluée à l'aide du substrat-marqueur tolbutamide, un hypoglycémiant oral. Le CYP2C9 est responsable de 90% de la 4'-hydroxylation du tolbutamide. Le CYP2C8 ne contribuerait qu'à 10 % de la biotransformation du tolbutamide en hydroxytolbutamide.[182] La sélectivité de l'hydroxylation du tolbutamide envers CYP2C9 a été confirmée par des études d'inhibition par anticorps.[183] Bien que CYP2C19 aurait la capacité d'hydroxyler le tolbutamide, sa contribution serait mineure due à son bas niveau d'expression par rapport au CYP2C9.[184] Le tolbutamide reste le substrat-marqueur du CYP2C9 le plus utilisé en pratique (80%).[95]

Le phénotype du CYP2J2 a été déterminé *in vitro* avec le substrat-marqueur ébastine. L'intérêt envers le CYP2J2 dans un contexte pharmacologique est davantage récent et la FDA ne recommande pas de substrat-marqueur particulier. L'intérêt vient du fait que le CYP2J2 serait impliqué dans plusieurs physiopathologies et comorbidités.[185-189] Plusieurs médicaments ont récemment été identifiés comme substrat du CYP2J2, ce qui accroît l'importance de cette isoforme du CYP450, particulièrement au niveau extrahépatique tel au niveau cardiaque et intestinal.[40,121,190-192] Bien que l'hydroxylation de l'ébastine ne soit pas spécifique à la voie du CYP2J2, il a été montré que celle-ci était la plus importante puisque le rythme de formation du métabolite hydroxylé par CYP2J2 était environ 22 fois supérieur à CYP3A4.[126] De plus, une autre étude à montrer qu'au niveau intestinal, le CYP2J2 était l'hydroxylase dominante de l'ébastine par inhibition avec des anticorps, sans contribution significative du CYP3A4.[193]

Finalement, le phénotypage du CYP3A4 par substrat-marqueur a largement été documenté et plusieurs substrats ont été validés. Parmi ceux-ci, le midazolam est le standard clinique et est recommandé par la FDA et EMA. L'hydroxylation du midazolam en 1'-hydroxymidazolam est donc l'index phénotypique de l'activité du CYP3A4 privilégié.[194] Depuis longtemps, l'hydroxylation du midazolam par le CYP3A et sa spécificité ont été démontré *in vitro*.[195-198] Le substrat-marqueur midazolam s'est aussi prouvé utile pour refléter une modulation de l'activité du CYP3A4 par une multitude de facteurs comme l'âge, le sexe et des inducteurs ou inhibiteurs via la quantification de son métabolite majoritaire le 1'-hydroxymidazolam.[199,200] La validité de l'hydroxylation du midazolam comme marqueur phénotypique de l'activité du CYP3A a même été confirmée en validant les résultats observés *in vitro* avec les clairances du substrat *in vivo*.[201]

1.4.1.2. *In vivo*

La variabilité de réponse aux médicaments observée dans la population est multifactorielle. Entre autres, des facteurs non-pharmacologiques tels l'environnement, la génétique et la présence d'une pathologie peuvent influencer la pharmacocinétique et/ou pharmacodynamique d'un médicament. Tel que mentionné précédemment, le métabolisme des médicaments par les CYP450s est un déterminant majeur de la concentration plasmatique ou au site d'action d'un médicament, donc subséquemment de sa pharmacocinétique et pharmacodynamie. En clinique, le génotypage des CYP450s n'offre qu'une vision partielle de l'activité *in vivo* des isoformes puisque ceci ne considère pas l'impact des autres facteurs. Le phénotypage *in vivo* des isoformes des CYP450s pallie cette problématique en évaluant directement l'activité de l'enzyme résultant de l'influence de tous les déterminants. Pour déterminer le phénotype des CYP450s un substrat-marqueur peut être administré par différentes

voies d'administration seul ou en *cocktail*, soit plusieurs substrats-marqueurs afin de phénotyper plusieurs isoformes simultanément. Ensuite, la quantification du substrat et/ou de ses métabolites spécifiques dans la matrice biologique appropriée permettra de déterminer le phénotype. La FDA a émis des lignes directrices pour l'industrie concernant les substrats-marqueurs privilégiés pour le phénotypage des CYP450s *in vivo,* tel que répertorié dans la table ci-dessous.[177]

Table III. Substrats-marqueurs pour le phénotypage de l'activité métabolique des CYP450s *in vivo*.

Enzyme	Substrats sensibles[*]	Substrats sensibles modérés[*]
CYP1A2	Alosétron, Caféine[a], Duloxétine, Mélatonine, Rameltéon, Tasimeltéon, Théophylline[a], Tizanidine	Clozapine, Pirfénidone, Ramosetron
CYP2B6	Bupropion[a]	Éfavirenz
CYP2C8	Répaglinide	Montélukast, Pioglitazone, Rosiglitazone
CYP2C9	Célécoxib	Glimépiride, Phénytoïne, Tolbutamide[a], S-Warfarine[a]
CYP2C19	S-méphénytoïne[a], Oméprazole[a]	Diazépam, Lansoprazole, Rabéprazole, Voriconazole
CYP2D6	Atomoxétine, Désipramine[a], Dextrométhorphane[a], Éliglustat, Nébivolol, Nortriptyline, Perphénazine, Toltérodine, Venlafaxine	Amitriptyline, Encainide, Imipramine, Métoprolol, Propafénone, Propranolol, Tramadol, Trimipramine,
CYP2E1	Chlorzoxazone[a, b]	
CYP3A4/5	Alfentanil, Avanafil, Buspirone[a], Conivaptan, Darifénacine, Darunavir, Ébastine, Évérolimus, Ibrutinib, Lomitapide, Lovastatin[a], Midazolam[a], Naloxégol, Nisoldipine, Saquinavir, Simvastatine[a], Sirolimus, Tacrolimus, Tipranavir, Triazolam, Vardénafil	Alprazolam, Aprépitant, Atorvastatine[a], Colchicine, Éliglustat, Pimozide, Rilpivirine, Rivaroxaban, Tadalafil

Adapté de *Drug Interactions & Labeling FDA Guidances* (2016)[177] et Bjornsson et al. (2003)[171]

[a]Substrats recommandés par la FDA

[b]Classement non officialisé par la FDA

[*]Lors d'études d'interactions médicamenteuses en présence d'un inhibiteur puissant reconnu, un substrat sensible présente une augmentation de son AUC ≥ 5 fois et un substrat modéré démontre une augmentation de son AUC de ≥ 2 à <5 fois.

1.4.1.3. L'approche *cocktail*

L'approche utilisant un *cocktail*, soit une combinaison de substrats-marqueurs, est avantageuse car elle permet de déterminer le phénotype de plusieurs CYP450s simultanément optimisant donc : 1) le temps de participation des sujets, 2) la variabilité intra-individuelle, 3) la durée de l'étude et 4) les ressources nécessaires pour compléter l'étude. Cependant, certaines précautions doivent être considérées pour s'assurer de l'absence d'interaction métabolique et analytique lié aux substrats du *cocktail* afin d'éviter les effets indésirables aux médicaments, en plus de confirmer la validité des résultats. Par rapport à ces exigences, de nombreuses combinaisons ont été validées et décrites dans la littérature, par exemple les *cocktail*s « Inje », « Cooperstown 5+1 », « Karolinska », « Indianapolis » et « Pittsburg ».[202-210] Plus récemment, l'utilisation de *cocktail in vivo* pour le phénotypage des CYP450s tend à comprendre plus de 6 substrats-marqueurs, à utiliser de plus petites doses pour augmenter la sécurité et minimiser les interactions, ainsi qu'à développer des méthodes d'analyses sensibles et versatiles pour diminuer le nombre d'échantillons à traiter. Plusieurs options de substrats *in vivo*, combinaisons et ratios métaboliques pour substituer la fastidieuse utilisation de la clairance sont répertoriés dans différentes revues de littérature.[172,173,211] Tel que présenté dans la table ci-dessous, les combinaisons administrées par voie orale et comportant 6 ou plus substrats-marqueurs des CYP450s sont nombreuses.[173,211]

Table IV. Stratégies d'approches utilisant des *cocktails* pour le phénotypage *in vivo* des CYP450s chez l'humain.

Référence	Enzyme	Substrats marqueur (dose, mg)	Matrice biologique	Temps de prélèvement
Damkier et al.[212,213]	CYP1A2	Caféine (200)	Plasma	0-8 h
	CYP2C19	Méphénytoïne (100)	Urine	0-12 h
	CYP2C9	Tolbutamine (250)	Urine	0-6 h
	CYP2D6	Spartéine (100)	Urine	0-12 h
	CYP3A	Quinidine (200)	Plasma	0-48 h
	CYP3A	Cortisol (endogène)	Urine	0-48 h
Derungs et al.[214] *Donzelli et al.*[215] « Basel *Cocktail* »	CYP1A2	Caféine (100)	Plasma	4 h
	CYP2C19	Oméprazole (10)	Plasma	4 h
	CYP2C9	Losartan (12,5)	Plasma	6 h
	CYP2D6	Métoprolol (12,5)	Plasma	6 h
	CYP3A	Midazolam (2)	Plasma	2 h
	CYP2B6	Éfavirenz (50)	Plasma	6 h
Scott et al.[216] *Palmer et al.*[217] « GW *Cocktail* »	CYP1A2	Caféine (100)	Plasma	0-12 h
	CYP2C19	Méphénytoïne (100)	Urine	0-8 h
	CYP2C9	Diclofénac (25)	Urine	0-8 h
	CYP2D6	Débrisoquine (10)	Urine	0-8 h
	CYP2E1	Chlorzoxazone (250)	Plasma	0-12 h
	CYP3A	Midazolam (5)	Plasma	0-12 h
Bosilkovska et al.[218] « Geneva *Cocktail* »	CYP1A2	Caféine (25 ou100)	Plasma	2 h
	CYP2C19	Oméprazole (5)	Plasma	2-6 h
	CYP2C9	Flurbiprofène (25)	Plasma	3 h
	CYP2D6	Dextrométhorphane (10)	Plasma	3 h
	CYP3A	Midazolam (1)	Plasma	2 h
	CYP2B6	Bupropion (25)	Plasma	3 h
	P-gp	Fexofénadine (25)	Plasma	2-6 h
Petsalo et al.[219]	CYP1A2	Mélatonine (3)	Urine	0-8 h
	CYP2A6	Nicotine (1)	Urine	0-8 h
	CYP2B6	Bupropion (75)	Urine	0-8 h
	CYP2C8	Répaglinide (1)	Urine	0-8 h
	CYP2C9	Losartan (50)	Urine	0-8 h
	CYP2C19	Oméprazole (20)	Urine	0-8 h
	CYP2D6	Dextrométhorphane (12,5)	Urine	0-8 h
	CYP2E1	Chlorzoxazone (62,5)	Urine	0-8 h
	CYP3A4	Oméprazole (20)	Urine	0-8 h
	CYP3A4	Midazolam (3,75)	Urine	0-8 h
Lenuzza et al.[220] « CIME *Cocktail* »	CYP1A2	Caféine (73)	Plasma	0-168 h
	CYP2C8	Répaglinide (0,25)	Plasma	0-168 h
	CYP2C9	Tolbutamide (10)	Plasma	0-168 h
	CYP2C19	Oméprazole (10)	Plasma	0-168 h
	CYP2D6	Dextrométhorphane (18)	Plasma	0-168 h
	CYP3A4	Midazolam (4)	Plasma	0-168 h
	OATP	Rosuvastatine (5)	Plasma	0-168 h
	UGT	Acétaminophène (60)	Plasma	0-168 h
	(transport rénal)	Mémantine (5)	Plasma	0-168 h
	P-gp	Digoxine (0,25)	Plasma	0-168 h

Plus de 85% des médicaments prescrits en clinique seraient métabolisés par les isoformes CYP1A2, CYP2C9, CYP2C19, CYP2D6 et CYP3A4.[221] Il semble donc important d'inclure des substrats-marqueurs pour ces CYP450s dans les *cocktails* de phénotypage appliqués en recherche et développement. Le mode d'administration privilégié des substrats-marqueurs est orale (*per os*) puisque moins invasif qu'en intraveineux. Dans certains cas, l'administration du midazolam par ces deux voies d'administration est utilisée pour permettre d'évaluer en même temps la capacité métabolique totale du CYP3A (oral) par rapport à l'activité CYP3A au niveau systémique (intraveineux).[201] Bien que l'administration par intraveineuse permet d'évaluer l'activité au niveau hépatique en évitant les variations au niveau de la phase d'absorption ou au niveau du métabolisme intestinal, l'administration orale des composés est privilégiée, car elle démontre un meilleur profil d'effet indésirable en général, est moins invasive et informe sur la capacité métabolique totale. La quantification des substrats-marqueurs dans l'urine présente l'avantage d'être moins invasif et complexe que dans le plasma, mais nécessite souvent une collecte sur une plus longue durée et est parfois moins fiable pour certains substrats instables ou dépendants de la clairance rénale.[172,173,211]

Plusieurs substrats-marqueurs pour les différentes isoformes des CYP450s sont identifiés et leur utilisation dans les *cocktails* de phénotypage dépend de multiples facteurs. Pour le CYP1A2, le substrat-marqueur le plus utilisé est la caféine qui est le seul substrat pour lequel les paramètres de phénotypage ont été validés. Bien que très sécuritaire, la présence de caféine dans plusieurs breuvages et aliments complique son utilisation en nécessitant idéalement d'arrêter les produits contenant des xanthines pour l'étude. De plus le métabolisme de la caféine dépend de plusieurs enzymes, il est donc nécessaire de quantifier sa N-déméthylation en paraxanthine pour ne phénotyper que le CYP1A2.[222] Tel que présenté dans la table IV, le

phénotypage du CYP1A2 à l'aide de la caféine est davantage fiable dans le plasma, nécessitant donc d'effectuer des prélèvements sanguins. En effet, le ratio métabolique de la caféine dans l'urine dépend de la clairance rénale.[223] Pour éviter les prélèvements sanguins, *Petsalo et al.* a développé un *cocktail* quantifiable seulement dans l'urine en substituant la caféine par la mélatonine qui est un substrat-marqueur beaucoup moins validé.[219]

Bien que limité par sa disponibilité dans certains pays, le tolbutamide est un substrat-marqueur valide de l'activité du CYP2C9. Comme dans le « CIME *cocktail* », le ratio dans le plasma est préférable au ratio urinaire utilisé par *Damkier et al.* qui est moins bien démontré et davantage variable.[212,213,220,224] Tel qu'utilisé par *Petsalo et al.*, une bonne alternative au tolbutamide lorsqu'on veut doser dans l'urine est le ratio métabolique du losartan qui est davantage fiable que celui de tolbutamide.[219,225] L'utilisation du diclofénac comme dans le « GW *Cocktail* » n'est généralement pas recommandé pour sa grande variabilité au niveau de son absorption intestinale, mais surtout par rapport à l'instabilité de ses métabolites qui peuvent influencer le phénotypage du CYP2C9.[216,217,226,227]

Pour son meilleur profil d'effets indésirables et la caractérisation extensive de son index d'hydroxylation, l'oméprazole est le substrat recommandé du CYP2C19.[172,173,211] En revanche, il est instable à certains pH dans l'urine et l'utilisation de la méphénytoïne, bien que plus difficilement disponible et moins sécuritaire, est donc préférable dans cette matrice.[172,173,211,228,229]

Comme pour les autres isoformes des CYP450s, plusieurs substrats-marqueurs du CYP2D6 sont disponibles. Chacun présente des avantages et désavantages pour leur utilisation dans des *cocktails* de phénotypage *in vivo*. Le dextrométhorphane est reconnu comme le substrat-marqueur de référence pour sa disponibilité, son profil sécuritaire et sa validité dans

l'urine.[172,173,211] L'utilisation du métoprolol pour phénotyper le CYP2D6 comme dans le « Basel *Cocktail* » doit tenir compte que sa fiabilité semble réduite dans des populations non caucasiennes.[172,214,215,230] Un inconvénient majeur à l'utilisation de la débrisoquine et spartéine comme substrats-marqueurs du CYP2D6 est leur disponibilité qui est limitée à quelques pays seulement.[172,173,211]

Pour le projet de cette thèse, le phénotypage *in vivo* de sept isoformes des CYP450s a été réalisé à l'aide d'un *cocktail* de six médicaments : caféine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), oméprazole (CYP2C19), dextrométhorphane (CYP2D6) et midazolam (CYP3A4/5), et d'un substrat-marqueur seul pour le CYP2E1 (chlorzoxazone), tous administrés par voie orale. Plusieurs exemples et combinaisons de *cocktails* de substrats-marqueurs de l'activité des CYP450s sont validés et répertoriés.[173] Le protocole de recherche pour ce projet est disponible à la section 2.1.[231] Ensuite ces substrats-marqueurs et leurs métabolites spécifiques ont été quantifié dans des échantillons sanguins et/ou urinaires par LC-MS/MS par méthodes analytiques développées et validées au laboratoire.[228]

1.4.2. Biomarqueur endogène de l'activité des CYP450s

Bien que l'utilisation de substrats-marqueurs pour phénotyper les CYP450s ait évolué vers l'administration orale de petites doses mesurables dans un nombre réduit de prélèvements minimisant ainsi le côté intrusif et complexe du phénotypage *in vivo*, l'utilisation d'un biomarqueur endogène faciliterait davantage l'utilisation de cette stratégie en clinique. L'identification de biomarqueurs endogènes de l'activité des CYP450s est une avenue pertinente puisque les CYP450s sont impliqués dans environ 95% des réactions redox de plusieurs substances endogènes ayant un rôle dans divers processus homéostatiques.[15,232] Dans un contexte de substrat-marqueur endogène, le CYP3A4 a surtout été étudié puisqu'en plus de

son importance dans le métabolisme des médicaments, il serait impliqué dans 20% des réactions physiologiques.[232] D'ailleurs, plusieurs marqueurs endogènes de l'activité du CYP3A ont été proposés, tels les ratios du 6-hydroxycortisol sur cortisol dans l'urine et du 4β-hydroxycholestérol sur cholestérol dans le plasma.[139,140] Il a été déterminé *in vivo* suite à l'administration d'inhibiteurs et d'inducteurs, ainsi qu'*in vitro* dans des enzymes recombinantes que le 4β-hydroxycholestérol était un produit du métabolisme du cholestérol par la sous-famille du CYP3A, dont 90% par l'isoforme CYP3A4.[233,234] Par contre, très peu d'études ont validé le 4β-hydroxycholestérol comme marqueur phénotypique du CYP3A dans des populations pathologiques.[140] Un objectif secondaire de la recherche clinique présentée dans cette thèse palie à ce besoin. En effet, nous avons évalué la validité du marqueur endogène de l'activité du CYP3A dans notre population avec le DT2. (Section 2.2)

1.5. Statut pathologique et phénoconversion des CYP450s

Tel que mentionné à la section précédente, plusieurs facteurs autres que les variants génétiques peuvent influencer l'activité métabolique des isoformes des CYP450s. Un de ces déterminants est le statut pathologique. Bien que les mécanismes exacts restent à préciser, le statut inflammatoire des maladies serait impliqué dans la modulation de l'activité des CYP450s. En *in vitro* et *in vivo*, la modulation de l'activité métabolique des isoformes des CYP450s, généralement une diminution, a été associée au niveau de différentes cytokines pro-inflammatoires qui sont des médiateurs de l'inflammation.[235,236] Tel que schématisé à la figure 2, il a été démontré que le mécanisme, bien que gène spécifique, serait dû notamment à une suppression de la transcription des gènes engendrée par des cytokines pro-inflammatoires, IFN-γ, interleukines 1 bêta (IL-1β), IL-6 et *Tumor Necrosis factor alpha* (TNF-α) par exemple.[62,155,237] Bien que la suppression de la transcription des gènes résultant en une diminution de leur ARNm soit un des mécanismes majeurs de la diminution des CYP450s, d'autres voies de régulation post-transcriptionnelles ont été identifiées.[154,155] Un de ces mécanismes post-transcriptionnels est la déstabilisation de l'enzyme par l'oxyde nitrique (NO) suite à une induction de la synthase de l'oxyde nitrique (NOS) par l'inflammation.[154,155,238-241] L'implication de l'inflammation dans la modulation de l'activité des CYP450s est entre autres supportée par sa réversibilité suite à l'utilisation d'anticytokines. Par exemple, chez des patients atteints d'arthrite rhumatoïde et dans des modèles *in vitro*, la diminution d'activité du CYP3A en présence de marqueurs inflammatoires (IL-6 ou IL-1β) était restaurée par le tocilizumab, un anti-IL-6, ou par un antagoniste des récepteurs de l'IL-1β.[242-244]
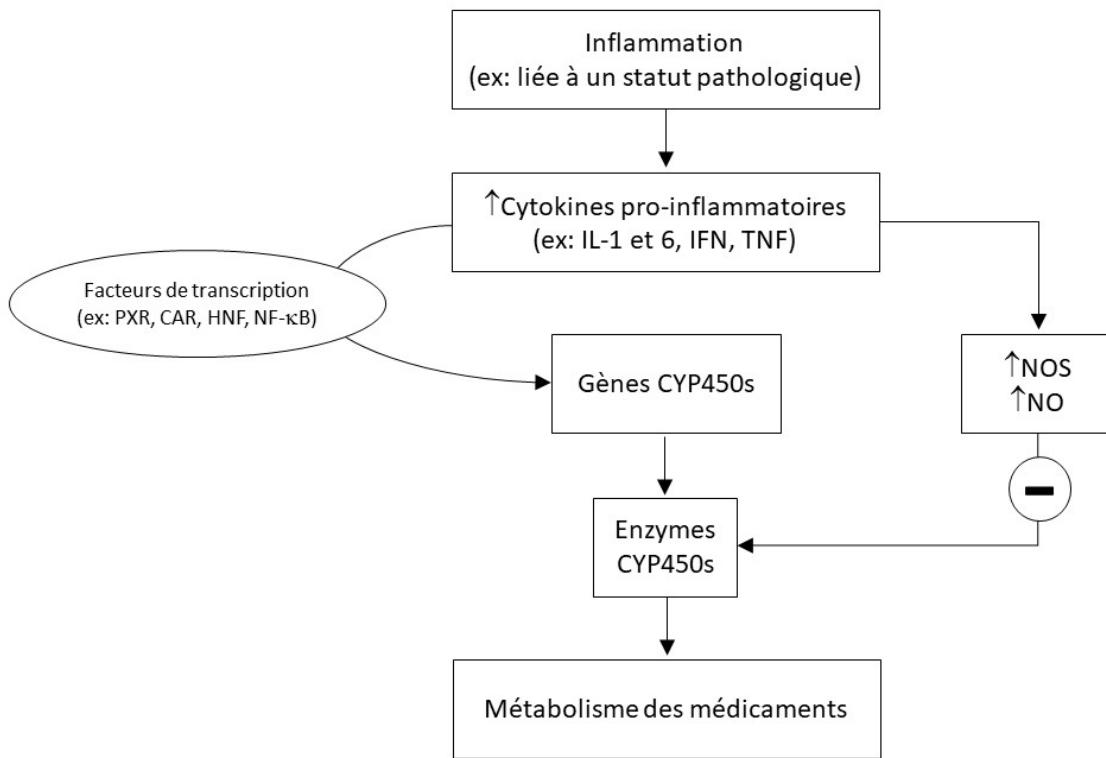
Figure 2.   Modulation de l'expression et de l'activité des CYP450s par des marqueurs inflammatoires.

Figure adaptée de Morgan ET, *et al*. (2008)[238]

Des évidences cliniques suggèrent que des maladies associées avec certaines de ces composantes inflammatoires influenceraient les niveaux d'activité *in vivo* des CYP450s entrainant parfois une phénoconversion, c'est-à-dire une divergence entre la capacité métabolique théorique et l'activité CYP450 observée en pratique. Cette phenoconversion peut s'avérer cliniquement significative par rapport à la clairance des médicaments, notamment dans des cas de médicaments à fenêtre thérapeutique étroite, lorsque l'effet thérapeutique nécessite une activation métabolique ou dans des situations où un patient nécessite la prise concomitante de plusieurs médicaments. Plusieurs pathologies à profil chronique telles le VIH, la maladie de

Crohn, l'hépatite B et C, le cancer, l'insuffisance cardiaque congestive, et l'arthrite rhumatoïde ont démontré un impact sur l'activité métabolique de différentes isoformes des CYP450s.[235] Dans cette optique, l'effet du DT2 sur l'activité métabolique des CYP450s a été évalué en clinique au cours de cette thèse.

1.5.1. Diabète de type 2 et inflammation

Le DT2 est une maladie multifactorielle qui survient en présence d'allèles à risque sur de multiples gènes et d'un environnement propice à la maladie. La forme monogénique de la maladie ne serait responsable que de 1 à 5 % de tous les cas de diabète.[245] La grande majorité des cas diagnostiqués sont hétérogènes de sorte qu'ils ne sont pas expliqués par la forme monogénique, par la forme gestationnelle ou ni par une maladie ou exposition spécifique. En effet, des déterminants comportementaux et environnementaux joueraient un rôle complémentaire dans la modulation de la susceptibilité génétique et le risque de développer le DT2. Bien qu'au moins 88 locus génétiques associés au DT2 aient été identifiés, leur effet cumulé n'expliqueraient qu'environ 10% de l'héritabilité du DT2.[246] De ce fait, plusieurs importantes composantes comportementales et environnementales comme la sédentarité, le surpoids, les suppléments alimentaires ou l'exposition à certains polluants ont été investigués et jugés importants dans la modulation des facteurs de risques génétiques et le développement du DT2.[245]

Bien que les causes de la maladie soient multiples, le DT2 résulte d'une dysfonction progressive des cellules β du pancréas pour compenser la résistance à l'insuline. Plusieurs mécanismes seraient impliqués dans le développement de la résistance à l'insuline et de la dysfonction des cellules β; le stress oxydatif, le stress au niveau du réticulum endoplasmique,

la présence de dépôt d'amyloïde au niveau du pancréas, les dépôts ectopiques de graisse dans les muscles, le foie et le pancréas, ainsi que la lipo- et gluco-toxicité.[247] Tous ces mécanismes faisant pression sur le système seraient associés à l'inflammation, soit en étant exacerbés par celle-ci ou en induisant la réponse inflammatoire.[247] Ces processus impliqués dans la pathophysiologie du DT2 lui procurent le statut de maladie auto-inflammatoire.[247,248] Chez les patients avec le DT2 plusieurs études ont montré des niveaux élevés de facteurs inflammatoires dans la circulation sanguine, telles des cytokines, des chimiokines et des protéines C-réactives (CRP).[249-252] Les niveaux d'IL-1β, IL-6 et CRP seraient d'ailleurs prédictifs du développement du DT2.[250,253]

Les niveaux sanguins des facteurs inflammatoires associés au développement du DT2 varient entre les individus et ne pourraient pas être représentatifs des niveaux d'inflammation spécifique dans des tissus sensibles à l'insuline ou des îlots pancréatiques.[247] La figure 3 ci-dessous montre un des phénomènes inflammatoires associés au diabète. Tel qu'illustré, des niveaux excessifs de certains nutriments, comme le glucose et les acides gras libres, vont impacter les îlots pancréatiques et les tissus sensibles à l'insuline comme le tissu adipeux, ou le foie et le muscle (non illustrés), conduisant à la production et libération locale de cytokines et chimiokines. Ces facteurs inflammatoires comprennent entres autres l'IL-1β et le TNF-α. En conséquence, des cellules immunitaires seront recrutées et la libération de cytokines et chimiokines dans la circulation favorisera ensuite l'inflammation à d'autres tissus.[247,254-256]
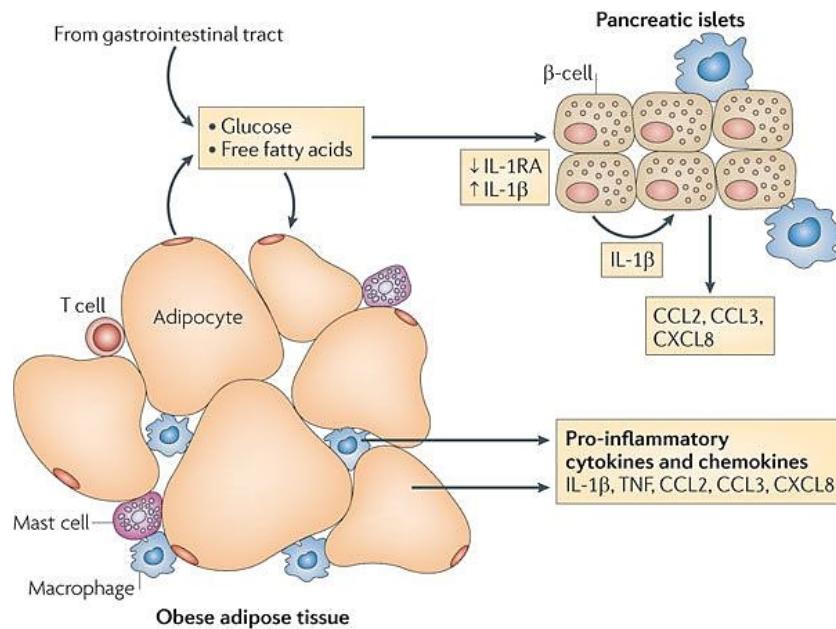
Figure 3. Diabète de type 2 et inflammation dans les tissus sensibles à l'insuline.

Tirée de Donath MY et Shoelson SE (2011)[247]

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[Springer Nature] [Nature Reviews Immunology] [Type 2 diabetes as an
inflammatory disease, Marc Y. Donath and Steven E. Shoelson] [Copyright ©
2011], 14 January 2011 (doi: https://doi.org/10.1038/nri2925)

En résumé, le DT2 est une pathologie multifactorielle auto-inflammatoire à composantes génétiques et environnementales qui affecte plusieurs tissus et fonctions normales du corps humain. Plusieurs mécanismes, cascades et facteurs inflammatoires comme l'hypoxie, la mort cellulaire, les voies de signalisations NF-κB et *JUN N-terminal Kinase* (JNK), les systèmes de l'IL-1 et 6, ainsi que les chimiokines et adipokines seraient impliqués dans la physiopathologie de la maladie et de ses complications.[247,248] Ces cytokines pro-inflammatoires qui sont augmentées da la pathologie du DT2 pourront ensuite avoir un effet sur l'expression et l'activité des CYP450s tel qu'illustré précédemment à la figure 2. Bien que l'implication de l'inflammation dans la physiopathologie du diabète soit établie, les mécanismes et répercussions exactes restent à préciser.

1.5.2. Impact du diabète sur l'activité métabolique des CYP450s

En clinique, il a été observé que les patients diabétiques présentent des réponses hautement variables à certains médicaments.[257-262] La physiopathologie du DT2 est liée à un statut inflammatoire, soit une augmentation de cytokines pro-inflammatoires chez les patients affectés.[249-252] Il a été démontré que l'inflammation modulait l'activité des CYP450s.[154,155,238-241] Ceci supporte que la variabilité dans la réponse aux médicaments observée chez les diabétiques en clinique puisse être expliquée en partie par une modulation de leur capacité à métaboliser les médicaments. En effet, la présence d'inflammation chez les diabétiques affecterait le métabolisme des médicaments via les CYP450s, lesquelles sont sensibles à certains des marqueurs inflammatoires impliqués dans la physiopathologie du DT2. Tel qu'observé dans des modèles précliniques et cliniques, la présence de la maladie pourrait donc affecter l'activité métabolique des isoformes des CYP450s de manière significative.

La prévalence du diabète étant en constante augmentation, les coûts sociétaux de la maladie explosent et un intérêt grandissant est attribué au DT2.[263-266] Plusieurs études sur l'impact du diabète sur les enzymes du métabolisme des médicaments ont été réalisées dans divers modèles de rats et souris. Or, les résultats de phénotypage des CYP450s obtenus chez la souris et le rat semblent inconsistants, en plus de dépendre du modèle de diabète utilisé (génétique ou obtenu par la diète).[267-279] De manière générale, les études d'expression et d'activité des CYP450s dans des modèles du diabète induits par la diète chez la souris s'accordent sur une diminution des niveaux d'expression et d'activité du cyp3a.[267,268,273,276-278] Une diminution du CYP3a est aussi observée chez un modèle du diabète induit par la diète chez le rat.[279] Les résultats sur les niveaux de cyp2b, cyp2c et cyp2d sont davantage variables. De plus, ces isoformes ont moins été étudiées dans le contexte du diabète, bien que leurs activités

apparaissent diminuées dans des modèles du diabète induits par la diète chez la souris.[273,276] Les effets du diabète sur les CYP450s observé dans des modèles génétiques chez le rat et la souris sont différents et hautement variables.[270-272,274,275] En contradiction avec le modèle induit par la diète, les modèles génétiques du diabète semblent plutôt suggérer une augmentation du cyp3a.[270,274,275] Certaines études rapportent aussi que l'effet de la maladie sur les CYP450s serait tissu spécifique en plus d'être isoforme-spécifique.[269,273,275]

Dans notre laboratoire, Maximos *et al.* ont caractérisé les effets du DT2 sur l'expression et l'activité métabolique des CYP450s dans un modèle de souris obèses-diabétiques induit par la diète.[273] Au total l'activité métabolique de 7 isoformes a été évaluée et l'expression relative d'ARNm de 15 isoformes des CYP450s a été mesurée au niveau hépatique, mais aussi dans les reins, les poumons et les cœurs. L'influence différentielle du DT2 sur les CYP450s des différents organes a donc pu être évaluée dans cette étude. L'article intégral se trouve en annexe 2 de cette thèse. En bref, les niveaux d'expression du cyp3a étaient réduits significativement chez les souris obèses-diabétiques d'un facteur de 2 à 14 fois au niveau du foie et de plus de 2 fois au niveau du rein. Cette réduction marquée de l'ARNm du cyp3a s'est reflétée dans les niveaux d'activité métabolique. En effet, des incubations du substrat-marqueur du cyp3a, le midazolam, dans les microsomes de foie et de rein des souris obèses-diabétiques a montré une réduction significative de l'activité métabolique d'environ 80% par rapport au groupe contrôle. Un autre point important de cette étude est que l'activité des isoformes des familles cyp2b et cyp2 était réduite dans tous les organes chez le modèle diabétique de souris, à l'exception du métabolisme de tolbutamide dans les microsomes de rein. Aucun impact ou seulement une influence mineure du DT2 a été observée pour les autres isoformes des CYP450s testées.[273] En

résumé, les rapports chez l'animal s'accordent pour suggérer une modulation tissue- et isoforme-spécifique des CYP450s par le diabète.[269,273,275]

Bien que l'intérêt se soit développé dans les dernières années, les études caractérisant l'impact du DT2 sur l'activité métabolique des différentes isoformes des CYP450s chez l'humain restent plutôt rares. Le CYP3A4/5 a été caractérisé dû à son importance dans le métabolisme de plusieurs médicaments en clinique et à son abondance relative dans le foie et l'intestin.[35,80,280] Les études semblent s'accorder sur une diminution de l'expression et/ou activité du CYP3A4/5 en présence du DT2.[277,281,282] En clinique, il a été rapporté que le DT2 n'influencerait pas l'activité métabolique du CYP1A2 tel que mesuré avec les substrats caféine et théophylline.[283-285] Par contre, une autre étude a mesuré une augmentation du métabolisme de la caféine chez des patients avec le DT2.[286] Dans une étude de métabolisme, le DT2 n'a montré aucun effet sur l'activité du CYP2D6 après l'administration d'une dose de dextrométhorphane.[283] L'effet du DT2 sur l'activité du CYP2C9 chez l'humain est très peu étudié. Une étude a montré que le métabolisme d'une dose de tolbutamide, substrat-marqueur du CYP2C9, était semblable chez des sujets normaux et diabétiques.[287] Le CYP2E1 est très étudié dans les contextes pathologiques pour son rôle dans le développement de certaines maladies.[288] Bien que l'effet semble dépendre du statut obèse, de l'avancement et du contrôle de la maladie, l'induction du CYP2E1 par le DT2 a été répertoriée dans plusieurs études.[282,289-291] Une étude a démontré que l'obésité était un facteur confondant dans l'augmentation de l'activité du CYP2E1, puisque les sujets non obèses avec DT2 présentaient des niveaux d'activité semblables aux sujets non diabétiques, alors que les sujets obèses, peu importe leur statut diabétique, présentaient une augmentation du CYP2E1.[290]

Seul un petit nombre d'études sont disponibles sur les effets du DT2 sur l'activité des CYP450s chez l'humain. Pourtant, cette population est sujette à une grande variabilité dans la réponse aux médicaments qui pourrait être due à leur capacité à les métaboliser. Le DT2 a d'ailleurs été associé à la réponse à divers médicaments entre autres le clopidogrel, la warfarine, la cyclosporine, le tacrolimus, le nisoldipine et les statines.[259,281,292-296] Davantage de recherche sur l'influence du DT2 sur le métabolisme des médicaments sont nécessaires afin de mieux comprendre la variabilité dans la réponse aux traitements et éventuellement améliorer les approches thérapeutiques et doses utilisées dans cette population de patients.

1.6. Objectifs généraux et spécifiques

L'objectif de ma thèse était de mieux comprendre les mécanismes sous-jacents à la variabilité dans la réponse aux médicaments observés chez les patients diabétiques en caractérisant l'impact chez l'humain du DT2 sur la capacité métabolique des CYP450s. Dans cette thèse nous rapportons les résultats obtenus lors d'une étude clinique *in vivo* caractérisant les impacts du DT2 sur sept isoformes des CYP450s. De plus, la modulation locale au niveau intestinal de l'activité de quatre isoformes des CYP450s, ainsi que l'expression de dix CYP450s, deux carboxylesterases et trois transporteurs par le DT2 a pu être investiguée *in vitro*.

L'objectif primaire de l'étude de pharmacocinétique *in vivo* chez des patients atteints du DT2 et des sujets non diabétiques était de déterminer l'effet du DT2 sur l'activité métabolique de 7 isoformes principales des CYP450s (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 et CYP3A4/5) suivant l'administration orale d'un *cocktail* de substrats-marqueurs. Cette étude nous permettait aussi de vérifier en objectif secondaire, l'influence sur l'activité des CYP450s de covariables comme des marqueurs de l'inflammation, des indices de résistances à

l'insuline, la présence de polymorphismes génétiques, ainsi que des variables démographiques et liées au diabète. De plus, les valeurs obtenues lors de cette étude pour le substrat-marqueur du CYP3A, le midazolam, ont servies à évaluer un biomarqueur endogène du CYP3A, le 4β-hydroxycholestérol, dans notre population non diabétique et diabétique.

Tel que discuté précédemment, le DT2 pourrait influencer l'expression et l'activité de diverses enzymes du métabolisme et transporteurs des médicaments au niveau intestinal chez l'humain. L'objectif primaire de notre étude *in vitro* était de déterminer si l'expression et l'activité des CYP450s intestinaux est modulée par le DT2 de manière tissu- et isoforme-dépendant à partir de biopsies duodénales obtenues chez des patients avec le DT2 versus des sujets non diabétiques. L'objectif secondaire de cette étude portait sur l'influence du DT2 sur l'expression relative d'ARNm des enzymes de métabolisme carboxylesterases et de transporteurs dans le duodénum chez l'humain. L'influence de marqueurs inflammatoires, de polymorphismes génétiques des CYP450s et de variables démographiques a aussi été examinée.

Section 2 : Manuscrits

2.1. Manuscrits 1 et 2

Manuscrit 1 (article de protocole): « Evaluating the impact of type 2 diabetes mellitus on

CYP450 metabolic activities: protocol for a case–control pharmacokinetic study. »

- **Gravel S,** Chiasson J-L, Dallaire S, Turgeon J, Michaud V. *BMJ Open.* 2018;8(2). PMID: 29439084 / PMCID: PMC5829905 / DOI: 10.1136/bmjopen-2017-020922

&

Manuscrit 2: « Modulation of CYP450 activities in patients with type 2 diabetes. »

- **Gravel S**, Chiasson JL, Turgeon J, Grangeon A, Michaud V. *Clin Pharmacol Ther.* 2019. PMID: 31099895 / DOI: 10.1002/cpt.1496

2.1.1. Introduction:

Plusieurs patients avec le DT2 présenteraient en clinique une réponse hautement variable à certains médicaments dont le clopidogrel, la warfarine, la cyclosporine, le tacrolimus, ainsi que certains antihypertensifs et hypocholestérolémiants.[257-262] Cette variation dans l'effet des médicaments peut s'expliquer par une différence interindividuelle dans la pharmacocinétique (PK) et/ou pharmacodynamique (PD) des médicaments. Dans plusieurs cas, le niveau d'activité des enzymes de métabolisme est un facteur déterminant de la relation PK-PD pour un médicament donné. La superfamille d'enzymes majoritairement impliquées dans le métabolisme des médicaments est les CYP450s. Tel que mentionné précédemment, l'activité des CYP450s peut être régulée par de nombreux facteurs comme la génétique, l'environnement, la prise concomitante de médicaments et le statut inflammatoire. Plusieurs composantes inflammatoires ont été rapportées dans la physiopathologie du diabète de type 2. Des études sur l'activité des CYP450s dans d'autres conditions pathologiques à statut inflammatoire ont démontré une influence significative sur l'activité des CYP450s.[235] L'impact du DT2 sur les CYP450s pourrait donc être un facteur important dans la variabilité de réponse aux médicaments observée chez les patients avec DT2.

Hypothèse : La grande variabilité interindividuelle observée dans la réponse aux médicaments chez les patients avec le DT2 serait due à une modulation de l'activité des isoformes des CYP450s.

Objectifs:

- L'objectif primaire de cette étude était de déterminer l'effet du DT2 sur l'activité métabolique de 7 isoformes principales des CYP450s (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 et CYP3A4/5) suivant l'administration orale d'un *cocktail* de substrats-marqueurs chez des patients avec le DT2 versus des sujets non diabétiques.

- En objectif secondaire, l'influence sur l'activité des CYP450s de covariables comme des marqueurs de l'inflammation, des indices de résistances à l'insuline, la présence de polymorphismes génétiques, ainsi que des variables démographiques et liées au diabète a été investiguée.

2.1.1.1. **Manuscrit 1**: « Evaluating the impact of type 2 diabetes mellitus on CYP450 metabolic activities: protocol for a case–control pharmacokinetic study. »

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ABSTRACT

Introduction: Diabetes affects more than 9% of the adult population worldwide. Patients with type 2 diabetes mellitus (T2DM) show variable responses to some drugs which may be due, in part, to variability in the functional activity of drug metabolizing enzymes including cytochromes P450 (CYP450s). CYP450 is a superfamily of enzymes responsible for xenobiotic metabolism. Knowledge must be gained on the impact of T2D and related inflammatory processes on drug metabolism and its consequences on drug response. The aim of this study is to characterize the activity of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3As in T2DM *vs* non-T2DM subjects following the administration of a *cocktail* of probe drug substrates.

Methods and analysis: This single-center clinical study proposes the first detailed characterization of T2DM impacts on major CYP450 drug metabolizing enzyme activities. We intend to recruit 42 controlled T2DM patients (A1C<7%), 42 uncontrolled T2DM patients (A1C≥7%) and 42 non-T2DM control subjects. The primary objective is to determine and compare major CYP450 activities in T2DM *vs* non-T2DM patients by dosing in plasma and urine probe drug substrates and metabolites following the oral administration of a drug *cocktail*: caffeine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6), chlorzoxazone (CYP2E1) and midazolam (CYP3As). Secondary objectives will evaluate the influence of variables such as glycaemia, insulinemia, genetic polymorphisms and inflammation. The value of an endogenous biomarker of CYP3A activities is also evaluated. The first patient was recruited in May 2015 and patients will be enrolled up to completion of study groups.

Ethics and dissemination: Approval was obtained from the ethic review board of the CHUM research center (Montreal, Canada).

Trial registration number: NCT02291666

Strengths and limitations of this study:

- Study population including a control group of non-T2DM subjects and T2DM patients (T2DM patients will be stratified according to their glycemic control) will provide valuable information to better understand T2DM impacts on drug metabolism and to dissect the role of glycaemia *vs* inflammatory factors.

- The use of a validated *cocktail* of 7 probe-substrates along with highly precise, fast and robust quantification methods will permit the simultaneous determination of phenotypes for 7 major CYP450 isozymes involved in drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5.

- An exhaustive data collection and review of medical and pharmacological records for all participants will permit evaluation of covariables and investigation of mechanisms underlying expected modulation of CYP450 isozyme activities.

- The use of endogenous 4β-hydroxycholesterol plasma concentrations will be assessed as a putative biomarker of CYP3A activity.

- Study population is limited to individuals with a BMI≤35 which, although reducing confounding factors, prevents the extrapolation of our results to morbid obese individuals.

INTRODUCTION

The increasing prevalence of diabetes mellitus, a pathology which is associated with numerous comorbidities, is of major concern due to its important burden on society and health care systems.[1,2] In 2014, diabetes was estimated to affect more than 9% of the worldwide adult population.[3] Type 2 diabetes mellitus (T2DM), the most common form of diabetes, is characterized by insulin resistance, an incapacity of the body to respond adequately to insulin action and by beta cell dysfunction, an inability to respond to a glucose challenge by an appropriate insulin release. The resulting elevated blood glucose levels can lead to the development of costly and serious complications and comorbidities such as neuropathy, nephropathy, retinopathy, heart disease and an elevated incidence of stroke.[4-6] In 2013, diabetes mellitus was the 7th leading cause of death in the United States.[7]

Prevention and treatment of T2DM and its complications and comorbidities often require the use of multiple drugs to address these issues. Polypharmacy, *per se*, increases the risk of multi-drug interactions leading to adverse drug reactions (ADRs) which account for about 6.5% of all hospital admissions.[8] Furthermore, the risk of ADRs and treatment failure appears to be increased in patients with T2DM. Indeed, it has been previously reported that patients with T2DM tend to show highly variable responses to different drugs; while some patients are resistant to some drugs, some are more sensitive to other drugs (*e.g.* warfarin, clopidogrel, cyclosporine, tacrolimus, and antihypertensive agents).[9-14]

Cytochrome P450 (CYP450) is a superfamily of enzymes responsible for drug metabolism that represents a major source of variability in drug pharmacokinetics and response. CYP450 metabolizing activities can be regulated by many intrinsic and extrinsic factors such as

genetics and environment. For instance, pro-inflammatory cytokines have been shown to decrease CYP3A and CYP2Cs activities.[15-17] Thereby, chronic inflammatory diseases such as T2DM can impact CYP450 isoformes and modulate the patients' response to drugs.[18] The impact of T2DM on drug metabolizing enzymes could be an important factor to consider clinically to improve treatment of T2DM patients by managing efficiently the use of multiple concomitant drugs to reduce ADRs or to optimize efficacy. To reach an optimized drug utilization, knowledge has to be gained on the influence of T2DM per se on drug metabolism.

Our hypothesis is that T2DM and related inflammatory processes alter activities of CYP450s involved in drug disposition which may explain variability in drug response. The specific aims are 1) to determine CYP450 phenotypes in patients with T2DM vs non-diabetic subjects following a single oral administration of a *cocktail* of CYP450 probe drugs, and 2) to compare CYP450 phenotypes according to the glycemic control.

METHODS AND ANALYSIS

This clinical study is an open-label explorative pharmacokinetic research investigating the effects of T2DM on major CYP450 activities, namely CYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1 and 3As. Study protocol (Trial #14.066) was approved by the ethic review board of the CRCHUM (Centre de recherche du centre hospitalier de l'Université de Montréal, Montreal, Canada) and registered at the US National Institutes of Health website (http://www.clinicaltrial.gov; NCT02291666).

Study population

A total of 126 participants, gender-matched between groups, are to be recruited from the Diabetes Registry at the CRCHUM outpatient clinic to constitute three study groups:

A) 42 T2DM patients with good glycemic control defined here as an A1C ≤ 7%;

B) 42 T2DM patients with inadequate glycemic control defined here as an A1C > 7%; and,

C) 42 non-T2DM participants serving as control.

Eligibility criteria include participants ≥18 years old and with a body mass index (BMI) ≤35 kg/m^2. They have to be non-smoker for at least two months prior to study and to abstain from grapefruit juice within two weeks before drug administration. Exclusion criteria also include a review of all subjects' pharmacologic record to control for the use of CYP450 inhibitor or inducer drugs. Detailed eligibility criteria are presented in **Table 1**. Written informed consent is to be obtained from all participants prior to any initiation of study procedure.

Table 1. *Eligibility criteria*

INCLUSION CRITERIA	• Male and female (non-pregnant) aged 18 years and over • Glomerular filtration rate > 50 ml/min/1,73 m2 • Acceptable hepatic function (alt and ast levels below three times the normal range) • Bmi ≤ 35 kg/m2 • Non-smoker for at least 2 months

EXCLUSION CRITERIA	• Use of:
	  o antibiotics, antivirals, antiretrovirals, monoamine oxidase inhibitors, immunosuppressants, interferons and antibodies
	  o CYP450 inductors (*e.g.*; carbamazepine, phenobarbital, phenytoin, rifampicin, St-John's worth)
	  o CYP450 inhibitors (e.g.; amiodarone, fluvoxamine, fluoxetine, verapamil, terbinafine).
	• Uncontrolled thyroid function
	• Presence of an important inflammatory condition
	• A diagnosed gastrointestinal pathology
	• Patients with an active cancer
	• Patients who underwent a transplant or a bariatric surgery

Primary endpoints

Our primary endpoint is to compare phenotypes for 7 major CYP450 drug metabolizing enzymes by calculating metabolic ratios in our study groups, *i.e.* T2DM (A1C≤7% and A1C >7%) and non-T2DM (control), by using well-characterized isoform selective orally administered probe-substrates:

a) a CYP450 probe drugs *cocktail* (CRCHUM-MT *cocktail*) comprising:

- Caffeine (Wake Ups® 100 mg, Adem); CYP1A2

- Bupropion (Bupropion SR® 100 mg, Sandoz); CYP2B6

- Tolbutamide (Orinase® 250 mg, AA pharma inc.); CYP2C9

- Omeprazole (APO-Omeprazole® 20 mg, Apotex); CYP2C19

- Dextromethorphan (Bronchophan Forte DM® 30 mg, Atlas Laboratories); CYP2D6

- Midazolam (Midazolam injection®, USP, 2 mg, Fresenius Kabi); CYP3As

b) Chlorzoxazone alone (Acetazone Forte® 250 mg, Rougier); CYP2E1

All participants will be studied at the Phase 1 Unit at the CRCHUM starting at 7h00 AM. Subsequent to *cocktail* administration, blood and urine samples will be collected at specified times (for 24 and 8 hours, respectively). All drug concentration-time data will be analyzed by standard non-compartmental methods using Kinetica® 5.1 (Thermo Kinetica, Thermo Fisher Scientific, USA). The area under the curve 0-8h (AUC_{0-8}) will be obtained by use of the mixed log-linear trapezoidal rule enabling for interpolation and using the limit of quantification (LOQ) to compute last AUC triangle when the 8h time point is below the limit of quantification (BLQ). Phenotypic indices of major CYP450 activities will be determined as previously validated using metabolic ratios (MR) of urinary or plasmatic concentrations.[19-24] Each of our probe drugs are selective for a specific isoform of the CYP450s and do not affect other isoform activities. All probe drugs will be administered at the same time as a *cocktail* (7h00 AM), except for chlorzoxazone which is administered separately in the evening of the experimental day with a urine sample collected overnight and blood sample collected the next morning.

Secondary endpoints

As a secondary endpoint, we will investigate the influence of different T2DM characteristics and covariates on the enzymatic activity of measured CYP450s activities. Principal variables investigated are:

- Insulin resistance markers: Insulin, blood glucose and glycated haemoglobin levels.

- Genetic polymorphisms associated with CYP450s under investigation which are known to modulate enzymatic activity.

- Pro-inflammatory cytokines: interferon-gamma (IFN-γ), interleukin 1-beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels.

- Covariates such as body mass index (BMI), age, co-medication and time since diagnosis.

Another exploratory end point will be to investigate the potential use of the plasmatic ratio cholesterol/4β-hydroxycholesterol as an endogenous biomarker of CYP3A activity. Indeed, recent data suggest that this plasmatic ratio could be used as an endogenous marker of CYP3A activity *in vivo*.[25,26]

Experimental protocol.

On experimental day, participants will be admitted to the clinical research unit of the CRCHUM at 7h00 AM after an overnight fast and will remain fasted until the 4h time point is collected. Serial blood samples will be drawn in 6 mL K2-EDTA vacutainers via indwelling venous catheters immediately before (time 0) and at 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8 and 24h post administration of the CYP450 probe drugs *cocktail* for measurement of probe drugs and their metabolites. Blood samples will be kept on ice and centrifuged for 10 minutes at 4°C and 1,500g less than 15 minutes after collection. Plasma samples will then be stored in aliquots at -80°C for later analysis. Urine will be collected prior to probe drug administration (baseline) and over an 8 hour period after the *cocktail* administration. Urine will constantly be kept on ice and after measurement of total urine volume, aliquots will be stored at -20°C until analysis. Patients will be discharged after the 8h urine and plasma sample collection. As an outpatient, on the same

night, subjects will be asked to take a chlorzoxazone oral dose prior to bedtime and collect all their urine for a minimal period of 8 h after drug administration. This urine collection will be returned on the next morning to the research center. After measurement of total urine volume, aliquots will be stored at -20°C for later analysis.

Two blood samples collected in SST II vials prior to dosing will be sent to the CHUM's biochemistry lab for insulin and glucose measurements. Furthermore, a blood sample will be collected in 6 mL K2-EDTA vacutainers prior to dosing for the measurement of inflammatory markers and plasmatic ratio cholesterol/4β-hydroxycholesterol.

Sample size calculations and statistical analyses

Sample size was calculated for a significant difference of 30% between groups, with a power of 80% and an alpha value of 0.05. The required sample size (n=38 subjects per group) was calculated to meet the primary objective considering the reported variances of CYP3A activities found in the literature (greatest changes have been observed with CYP3A4). Considering an anticipated drop-out rate of 10%, 42 subjects per group will be recruited for a total of 126 participants.

Statistical analyses will be used to answer primary objective which consists of determining if T2DM disease modulates CYP450 activities. A stepwise statistical analysis will be performed: 1) each phenotypic probe will be compared between T2DM (including controlled and uncontrolled glycemia) *vs* non-T2DM individuals, and 2) T2DM group will be sub-categorized based on A1C ≤7% *vs* A1C >7% *vs* non-T2DM group. This aim will be tested for significance by performing an analysis of variance (ANOVA) with a post-hoc.

In a secondary analysis, the influence of covariables will be tested using multiple regression analyses. Different variables will be considered in the models: CYP450 genetic polymorphisms, BMI, insulin resistance markers, pro-inflammatory cytokines, age and time since diagnosis. Potential confounding variables will be defined according to: 1) variables associated with a specific CYP450 that have been reported in literature and 2) variables with a correlation coefficient above 0.2 or below -0.2 in T2D groups *vs* non-diabetic group.

Finally, plasmatic concentration ratios of the endogenous markers (cholesterol/4□-hydroxycholesterol) will be compared between the 3 study groups and correlation will be established with activity observed for the probe-substrate, midazolam, administered in the CRCHUM-MT *cocktail*.

Data management

In this project, confidentiality of collected data will be preserved by using an attributed identification number to all participants. Subjects' identities and numbers will only be accessible to designated investigators and all data will be treated in a non-nominal manner. All documentation related to study will be kept in locked drawers. DNA samples will solely be used for genetic analyses associated with CYP450s or other enzymes/transporters of drugs relevant for T2D, associated co-morbidities and drug response variability. It will not be used to serve any other purpose than this present study. DNA will be kept for a maximum period of 10 years before complete destruction (hydrochloric acid).

Ethics and dissemination

This study will be carried out in compliance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines. All participants will be

informed of the objectives, risks and inconveniences of the trial and be provided with written information on the study, contact details of the principal investigators as well as copies of their signed consent/assent forms. Subjects will be clearly informed of their right to withdraw from the study at any time during the process, and this without consequences for their future care. Immediate intervention or treatment is available in case of an acute adverse event, *e.g.* anaphylactic reaction.

A manuscript presenting and discussing all results of the primary objective as well as the covariables analyses will be submitted and published in a peer-reviewed journal. A second distinct manuscript addressing the use of an endogenous marker of CYP3A activity will be submitted in a peer-reviewed journal as well.

Upon completion of the trial and publication of the results, a request for experimental data can be submitted to principal investigator at the CRCHUM in Montreal, Canada.

DISCUSSION

Many drugs in addition to antidiabetic medications are prescribed to T2D patients to treat their numerous co-morbidities. However, few information is available on the efficacy and dosing of these drugs in this sub-population of patients. Indeed, during drug development processes, pharmacokinetic and pharmacodynamic studies of new medications for various indications are rarely tested in a population of patients with T2D. Available data for this group of patients are often derived from secondary analyses of clinical trials. This research protocol will generate valuable results enabling a better understanding of factors affecting drug metabolism *in vivo*. New knowledge gained on metabolic capacity in patients with T2D will improve our understanding of the underlying mechanisms responsible for the high interindividual variation in drug response observed in this group of patients. Thereby, it will provide new information to help clinicians improving use of drugs to reduce therapeutic failure and toxicity entailed by sub-optimal or inappropriate use of pharmaceutical treatments.

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2.1.1.2. Discussion

Cet article présente notre protocole qui vise à évaluer l'influence du DT2 sur la capacité métabolique de sept isoformes des CYP450s. Il se base sur l'administration orale d'un *cocktail* de substrats-marqueurs composé de caféine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), oméprazole (CYP2C19), dextrométhorphane (CYP2D6) et midazolam (CYP3A), suivi d'une administration de chlorzoxazone (CYP2E1) seul. Notre *cocktail* de substrats-marqueurs est du même type et adapté d'autres combinaisons largement utilisées en recherche comme les *cocktail*s « Inje », « Cooperstown 5+1 », « Karolinska », « Pittsburg » et « Indianapolis ».[202-210]

Le ratio métabolique utilisé pour déterminer le phénotype du CYP1A2 est le ratio de la concentration plasmatique de paraxanthine, métabolite spécifique de la voie CYP1A2, sur caféine 4 heures post-dose. Bien que son métabolisme soit complexe, la caféine est le substrat-marqueur du CYP1A2 le plus utilisé en clinique.[297] Sa grande disponibilité et son profil sécuritaire favorise son utilisation chez l'humain et plusieurs études ont testés différents ratios métaboliques dans le plasma et l'urine.[172,173,211] Bien que la clairance systémique soit le meilleur index de l'activité du CYP1A2, le ratio plasmatique paraxanthine/caféine entre 3 et 6h post-dose est un bon indice phénotypique qui nécessite un seul prélèvement et un temps de collecte plus court que dans l'urine.[172,173,211,298,299]

L'intérêt de phénotyper l'activité du CYP2B6 *in vivo* est plus récent. Basé sur les résultats des études proposant des ratios métaboliques de bupropion et de son métabolite hydroxylé dans l'urine ou le plasma, ce marqueur fut utilisé dans le cadre de cette étude.[173,177,218] Notre étude a utilisé le ratio métabolique de la quantité d'hydroxybupropion/bupropion excrétée dans l'urine

sur une collecte de 8 heures. La quantification de bupropion et d'hydroxybupropion dans l'urine dans l'optique du phénotypage a été préalablement décrite par *Petsalo et al.*.[219]

Pour le phénotypage de CYP2C9, nous avons utilisé le substrat recommandé, soit le tolbutamide, un marqueur largement utilisé.[177] En effet, plus de 10 *cocktails* utilisant le tolbutamide comme substrat-marqueur du CYP2C9 ont été répertoriés, ce qui fait de lui le plus utilisé.[173] Pour notre étude, la clairance 24h du tolbutamide a été préférée au ratio métabolique plasmatique et urinaire. Seulement une petite quantité de tolbutamide inchangée est excrétée dans l'urine limitant donc l'utilisation du ratio urinaire qui nécessiterait une collecte sur une longue durée pour réduire la grande variabilité qui est rapportée.[85,224,300-302]

Pour CYP2C19, le méphénytoïne serait le substrat-marqueur le plus souvent utilisé, mais nous avons privilégié l'oméprazole pour son accessibilité au Canada et son meilleur profil d'effet indésirable.[173] L'oméprazole n'étant pas stable dans l'urine, nous avons utilisé le ratio métabolique plasmatique du 5'-hydroxyoméprazole/oméprazole sur 8 heures.[228,229] Dans le contexte de notre étude, ce ratio est préférable à la mesure ponctuelle entre 2-4 heures aussi validée qui présente un risque plus élevé d'observer des niveaux non détectables chez certains individus, en plus d'être associée à une plus grande variabilité.[172,173,211]

Tel que recommandé par la FDA nous avons utilisé le substrat-marqueur dextrométhorphane pour phénotyper le CYP2D6.[177] Il est suggéré qu'une collecte urinaire de longue durée est recommandée pour pouvoir bien distinguer les différents phénotypes, alors que le ratio plasmatique dextrorphane/dextrométhorphane serait fiable à partir d'aussi peu que 3 heures post-dose.[172,173,211] Par rapport à notre protocole de recherche, nous avons utilisé le ratio métabolique plasmatique sur 8 heures, lequel est fréquemment utilisé en clinique.

Dans l'approche *cocktail in vivo*, le midazolam est le substrat-marqueur de l'activité du CYP3A de référence. Administré par voie orale, le midazolam reflète l'activité des CYP3As au niveau intestinal et hépatique. L'utilisation du midazolam présente l'avantage qu'il n'est pas substrat des P-gp contrairement au test respiratoire à l'érythromycine qui est le deuxième standard utilisé pour le phénotypage du CYP3A4 seulement. Bien que des mesures ponctuelles réduites aient été proposées, le ratio métabolique plasmatique des AUC de 1'-hydroxymidazolam/midazolam reste le plus utilisé.[172,173,211] Par rapport à notre devis et en accord avec d'autres études, nous utilisons le ratio plasmatique de 1'-hydroxymidazolam/midazolam sur 8 heures qui semble adéquat compte tenu de la demi-vie et du temps pour atteindre la concentration plasmatique maximale du midazolam qui sont d'environ 3 h et 21 min respectivement.[303]

Finalement, le chlorzoxazone, substrat-marqueur recommandé du phénotype de CYP2E1 a été administré séparément du *cocktail* pour notre étude.[177] Puisque le midazolam et le chlorzoxazone peuvent interagir, ce substrat-marqueur a été administré seul au moins 12h heures après l'administration du *cocktail*.[172,173,211] En effet la prise du chlorzoxazone se faisait le soir au coucher, ce qui est aussi pratique puisque le chlorzoxazone est un relaxant musculaire qui peut causer de la somnolence. Le ratio métabolique urinaire sur une collecte de 8 heures, tel que rapporté précédemment, a été préféré au ratio métabolique plasmatique puisque la prise du médicament se faisait chez les participants.[172,173,211]

Ce protocole de recherche, qui vise à évaluer l'influence du DT2 sur la capacité à métaboliser les médicaments via les CYP450s, présente un devis d'étude prospective incluant un groupe témoin non diabétique. Ce protocole permet de contrôler pour l'effet de la maladie et

du contrôle glycémique, en plus de considérer des facteurs génétiques et démographiques. La stratégie de phénotypage de sept isoformes des CYP450s à l'aide d'un *cocktail* de substrats-marqueurs a été préalablement utilisée en clinique. Chaque substrat-marqueur de notre *cocktail* avait préalablement été décrit et validé, seul ou en combinaison.[172,173,211]

2.1.1.3. **Manuscrit 2:** « Modulation of CYP450 activities in patients with type 2 diabetes. »

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CONFLICT OF INTEREST

FUNDING

ABSTRACT

We conducted a comprehensive *in vivo* study evaluating the influence of type 2 diabetes (T2D) on major CYP450 activities. These activities were assessed in 38 T2D and 35 non-T2D subjects after a single oral administration of a cocktail of probe drugs: 100mg caffeine (CYP1A2), 100mg bupropion (CYP2B6), 250mg tolbutamide (CYP2C9), 20mg omeprazole (CYP2C19), 30mg dextromethorphan (CYP2D6), 2mg midazolam (CYP3As), and 250mg chlorzoxazone (alone) (CYP2E1). Mean metabolic activity for CYP2C19, CYP2B6 and CYP3A were decreased in T2D subjects by about 46%, 45% and 38% ($p<0.01$), respectively. CYP1A2 and CYP2C9 activities appeared slightly increased in diabetic subjects and no difference was observed for CYP2D6 or CYP2E1 activities. Several covariables such as inflammatory markers (IL-1ß, IL-6, IFN-γ and TNF-α), genotypes, diabetes- and demographic-related factors were considered in our analyses. Our results indicate that low chronic inflammatory status associated with T2D modulates CYP450 activities in an isoform specific manner.

INTRODUCTION

The increasing prevalence of diabetes mellitus is a major socio-economical concern due to its important burden on healthcare systems and society. In 2014, diabetes was estimated to affect more than 9% of the worldwide adult population and was the 7th leading cause of death in the United States.[1,2] Type 2 diabetes mellitus (T2D) is associated with the apparition of numerous complications and comorbidities such as neuropathy, nephropathy, retinopathy, heart diseases and strokes.[1] Multiple medications from various pharmacological classes are often required in patients with T2D to prevent complications and treat comorbidities. While polypharmacy is a valuable therapeutic strategy, it increases the risk of drug-drug interactions which can lead to adverse drug reactions (ADRs).

Patients with T2D tend to show highly variable responses to different drugs: some patients are resistant to some drugs while others are more sensitive.[3,4,5] This unpredictability in drug response can largely be explained by inter-subject variability in drug pharmacokinetics and pharmacodynamics (PK-PD). For several medications, the activity level of drug metabolizing enzymes is a key determinant of their PK-PD relationship with the Cytochrome P450 (CYP450) superfamily comprising the main group of enzymes. CYP450 activities can be regulated by several intrinsic and extrinsic factors such as genetics, environment, concomitant medications and inflammatory markers.[6]

The involvement of a chronic inflammatory status in the pathophysiology of T2D is well established as well as for the more complex metabolic syndrome condition. Furthermore, some data suggests that pathological conditions with an inflammatory component, such as T2D, influence the functional activity of CYP450s.[7,8] Indeed, numerous studies have investigated the

influence of inflammatory mediators, such as cytokines, on CYP450-dependent drug metabolism.[7-9] Therefore, the impact of T2D on drug metabolizing enzymes could represent a major determinant of the variability observed in drug response in T2D patients with polypharmacy.

The primary objective of our study was to determine the impact of T2D on the activity of 7 major CYP450 isoforms (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5) following the oral administration of a cocktail of probe drugs in patients with T2D and in non-diabetic subjects. As secondary objectives, influence of covariables such as inflammatory markers, insulin resistance markers, genetic polymorphisms, as well as demographic and disease-related covariates on CYP450 activities were investigated.

RESULTS

In total, 73 subjects successfully completed all study procedures without encountering any clinically significant adverse events. Table 1 describes the demographics and medications used in the study population. Of these participants, 38 were classified as T2D patients and 35 were registered as non-T2D subjects according to medical records and physical examination. Patients included in the T2D group were slightly older than the non-diabetic group and were mostly males. Although T2D patients presented a higher mean BMI than the non-diabetic subjects, both groups were overweight pre-obese according to WHO guidelines. Most of the participants enrolled in our study were Caucasians (n=69) and only 4 subjects were Asians (3) and Blacks (1), respectively.

Table 2 reports on the effects of T2D on CYP450 activities using metabolic ratios or oral clearance values, as used previously. In addition, analyses of covariances (including the potential confounding variables of age and gender) were performed and adjusted p-values are also provided in Table 2. Metabolic activity for CYP2B6, as measured by its metabolic ratio in urine, was decreased by about 45% in patients with T2D compared to the non-diabetic controls (2.24±3.09 *vs.* 4.05±4.24, p=0.01). Activity levels for CYP2C19 and CYP3A were also reduced by about 46% and 38%, respectively, in patients with T2D compared to controls (p=0.001 and <0.0001, respectively). T2D also modulated CYP1A2 activity: the metabolic ratio for CYP1A2 was increased in T2D (0.59±0.17) compared to non-T2D subjects (0.48±0.19, p=0.008 and p=0.07 when adjusted for age and gender). Moreover, there was a significant negative relationship with caffeine metabolic ratios and the use of oral contraceptive (containing ethinylestradiol) (p<0.001). CYP2C9 activity tended to increase in patients with T2D

(1.23±0.35 *vs.* 0.98±0.23, p=0.0008) although it barely fails to maintain statistical significance when adjusted for age and gender (p=0.05). CYP2D6 and CYP2E1 activity were not affected by diabetic status (p=0.75 and 0.78, respectively).

All 73 participants (n=35 non-diabetic and n=38 T2D subjects) were genotyped for major variants of studied CYP450 isoforms. Allelic frequencies in both groups are presented in Table S1. No major differences were observed in the variant allele frequencies between the two study groups. Phenotype was assigned to each individual based on genetic information — all concomitant medications were stopped the night before (except for insulin) to avoid drug-drug interactions — according to CPIC guidelines and literature. As presented in Table S2, although limited by sample size and high variability, results of two-way ANOVA analyses showed no interaction between genotype/phenotype classification and diabetes status on CYP450 activities. However, non-T2D individuals having variant alleles of CYP450 genes showed different metabolic activities. Due to the small number of subjects (n≤1) for PM groups, some statistical comparisons could not be performed.

There is no substantial difference in the measured CYP2C19 metabolic ratios among various CYP2C19 phenotypes (IM, EM and UM) for T2D patients while an expected incremental activity is observed in non-T2D subjects (Table S2). Our results showed a relationship between the bupropion metabolic ratios and CYP2B6 genotype subgroups: metabolic ratios of bupropion were higher in group of subjects classified as extensive metabolizers of CYP2B6 compared to poor metabolizers among the entire cohort and when adjusted for diabetes (p=0.03) Also, the ratio of bupropion metabolic ratios (CYP2B6) between EMs and PMs was smaller in T2D (5) *vs.* non-T2D subjects (7). Finally, CYP3A4 EMs with T2D exhibit significantly lower activity than EMs in the non-T2D group (p<0.0001), reaching

the level of activity measured in IM subjects (Table S2). All these results point towards phenoconversion observed in T2D subjects. The decreased activities observed in T2D patients are not due to drug-drug interactions as all medications (except for insulin) were stopped for at least 24 hours before study, and patients taking strong inhibitors, mechanism-based inhibitors or inducers of CYP450s were excluded (see Method section). Drug-drug interactions with probe drugs contained in the cocktail with prescribed medication due to competitive inhibition are therefore unlikely. Among T2D patients taking a proton pump inhibitor (PPI) as comedication (Table 1), 6 subjects received pantoprazole, 2 esomeprazole, one omeprazole and one lansoprazole. No difference in the metabolic ratio of omeprazole was observed between patients receiving a PPI or no-PPI (0.21 *vs.* 0.31, p=0.3).

For continuous variables, univariate linear regression analyses were performed as a first step to determine the influence of demographics, T2D-related and inflammation-related factors on measured metabolic ratios (CYP450s activities). Variables with p-value <0.25 (Table 4) were considered to build the subsequent multivariate models. As presented in Supplementary Table S3, results from multivariate analyses could explain 60%, 33%, 52%, 42%, 31%, 9% and 55% of variability observed in CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A activities, respectively. Independent variables included in the multiple linear regression analyses differed between the various CYP450 isoforms. For instance, intersubject variability in CYP1A2 activity was associated with diabetic status, HOMA-β, use of oral contraceptive pill (containing ethinylestradiol) as well as with pro-inflammatory cytokines IFN-γ and TNF-α (Supplementary Table S3). Intersubject variability in CYP2C9 activity was associated with HbA1C, BMI and gender (Supplementary Table S3) while for CYP2C19, diabetic status, insulin

levels, IFN-γ and TNF-α contribute to 42% of intersubject variability in measured activity level (Figure 1 and Supplementary Table S3).

Gender, genotype of CYP3A5 and HOMA-IR explained up to 55% of the intersubject variability in CYP3A activity (Supplementary Table S3). Female gender was associated with an increase in CYP3A activity while an increase in HOMA-IR, which corresponds to increased insulin resistance, resulted in lower CYP3A activity. As expected, subjects carrying an active allele of the CYP3A5 isoform presented an increased MR for CYP3A. In this study, CYP3A phenotypic activity could be described with the following equation: Y = -0.80 + (0.336*gender) + (0.359*CYP3A5 genotype_IM) + (-0.222*(log(HOMA-IR)). Predicted versus observed data plot for CYP3A activity is shown in Figure 1.

Multivariate models for CYP2B6 (Figure 1 and Supplementary Table S3), CYP2D6 (Supplementary Table S3) and CYP2E1 (Supplementary Table S3) could explain less than 33% of intersubject variability in CYP450 activities. Intersubject variability for the activity of CYP2B6 was best explained (r^2=0.33) by 5 independent variables: diabetic status, genotype, HOMA-IR, IFN-γ and TNF-α. Both diabetes and inflammation have a negative impact on CYP2B6 activity. Intersubject variability in CYP2D6 activity was predominantly explained (r^2=0.31) by genotype (β = 1.37, p < 0.0001). Only 9% of intersubject variability in CYP2E1 activity was explained by three independent variables, namely IL-1β levels and diabetic status with HbA1C >7%.

DISCUSSION

Our analyses demonstrate that CYP2C19, CYP2B6 and CYP3A4 activities are reduced in patients with T2D by approximately 1.5-2-folds. For the other isoforms, metabolic activities appeared slightly increased or not affected in patients with T2D.These findings support the hypothesis that various levels of inflammatory states, such as T2D, can modulate CYP450 activities in an isoform dependent manner. The interindividual variability observed for all CYP450 activities measured in our study (using non-T2D group as a reference) was in the same range as observed previously.

CYP2C19 is involved in the metabolism of various drugs such as anti-depressants and PPIs. Using the probe substrate omeprazole, we demonstrated a 2-fold reduction in CYP2C19 activity in patients with T2D. These results are in agreement with previous studies showing the modulatory role of inflammation on CYP2C19 activity both *in vitro* and *in vivo*.[8,10-12] In human hepatocytes, CYP2C19 expression was downregulated by treatment with pro-inflammatory cytokines.[10] Provoked inflammation in healthy male volunteers resulted in a decrease in CYP2C19 activity.[11] Reduced CYP2C19 activity was correlated with levels of the pro-inflammatory cytokine TNF-α in patients with congestive heart failure.[12] In our multivariate analysis, model for CYP2C19 activity did include levels of inflammatory markers IFN-γ and TNF-α, both interacting with diabetic status. Furthermore, there are numerous reports of genotype-phenotype mismatch for CYP2C19 in patients with inflammation-related diseases such as patients with cancer, multiple myeloma and liver disease.[8] These patients exhibit a general shift from their genotype status towards lower metabolic activity phenotypes.[8] A similar observation was observed in our T2D population.

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Drug-disease interactions for the CYP2C19 activated pro-drug clopidogrel have been thoroughly studied in recent years. In various studies, T2D was linked to clopidogrel high on treatment platelet reactivity (HTPR) which may be associated with thromboembolic events.[13,14] In a clopidogrel treated population, a higher incidence of major adverse cardiovascular events was observed among the diabetic sub-population.[13-15] This association between diabetes and HTPR seems to be absent in patients treated with the antiplatelet agents ticagrelor and prasugrel, two P2Y12 inhibitors that do not need activation by CYP2C19.[16] Since the FDA black box warning on clopidogrel for patients with a CYP2C19 PM genotype, some meta-analyses have confirmed the implication of CYP2C19 loss of function allele on poor response to clopidogrel.[17-19] Consequently, decreased CYP2C19 activity by T2D leading to a potential phenoconversion to a CYP2C19 PM phenotype may be of clinical interest for prodrugs such a clopidogrel.

Severe inflammation has been associated with variability in voriconazole's pharmacokinetics.[20,21] Voriconazole, a first line antifungal agent for the treatment of invasive aspergillosis, is mainly metabolized by CYP2C19 and to a lesser extent by CYP3A and CYP2C9. Due to the large variability in voriconazole exposure and its narrow therapeutic index, a therapeutic drug monitoring is recommended. In adults and children aged >12 years, studies reported that severe inflammation as reflected by C-reactive protein values was associated with higher voriconazole through concentrations. It was proposed that inflammatory stimuli lead to downregulation of CYP isoforms, particularly CYP2C19, resulting in reduced voriconazole metabolism.[21,22] Further investigations are required to evaluate if there is a difference between severe inflammation *e.g.* infection vs low chronic inflammatory conditions *e.g.* diabetes, heart failure on CYP450 activities.

Inflammation has been shown to downregulate CYP2B6 mRNA expression and protein levels in human hepatocytes.[10] However, studies on the effect of diabetes on CYP2B6 are limited. We and others have previously demonstrated a downregulation of Cyp2b activity and mRNA expression in diabetic mouse models of diet-induced obesity.[23,24] To our knowledge, our study is the first to report the modulatory role of T2D on CYP2B6 in patients.

The importance of investigating the effects of T2D on CYP3A4 activity is well-justified by the fact that this isoform is responsible for the elimination of approximately 40% of all therapeutic agents, several of which are used in T2D patients with comorbidities.[25] As for CYP2C19, inflammation reduces CYP3A expression and activity in human hepatocytes.[10,26] In patients with some inflammatory conditions such as HIV, active Crohn's disease, hepatitis C, cancer and rheumatoid arthritis, CYP3A activity is decreased.[7] Moreover, different models of non-alcoholic fatty liver disease have demonstrated a reduction in the expression and activity of CYP3A.[27] In this study, we found that CYP3A activity was reduced by about 1.6-fold in our T2D population. This is in agreement with previous studies indicating lower *in vivo* metabolism of CYP3A substrates in human with T2D.[4,28] For instance, a study compared CYP3A activity and expression in human liver microsomes obtained from T2D patients and demographically matched nondiabetic individuals.[29] They demonstrated a reduction of about 1.6-fold in CYP3A4 content in human liver microsomes and a marked decrease in CYP3A metabolic activity (using two different probes, including midazolam and testosterone) in patients with T2D.[29] Along with our findings, these results suggest that care should be taken when polypharmacy is used in a diabetic population to prevent ADRs as cases of CYP3A mediated disease-drug-drug interactions have been observed in patients with inflammation before.[30]

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The isozyme CYP1A2 is predominantly expressed in the liver and only a few studies have investigated its activity in the context of inflammation or other diseases.[7] Results differ between studies in patients with T2D. In one study with 15 type 1 diabetes and 16 T2D patients, CYP1A2 activity was increased in the type 1 diabetes group, but no significant change was observed in the T2D group.[31] In contrast, no change in caffeine pharmacokinetics was reported for both type 1 and T2D when assessing liver function.[32] Another study reported unaltered theophylline clearance in a cohort of T2D patients.[33] Our study, like the one by Urry *et al.*, demonstrated an increase in paraxanthine to caffeine ratio, indicative of an increase in CYP1A2 activity in patients with T2D.[34] Current evidence suggests that part of the variability observed in the CYP1A2 activity could be explained by gender: CYP1A2 activity being higher in males than in females.[35,36] In agreement with previous studies, we observed a lower caffeine metabolic ratio (MR_{4h}) in females compared to males (0.44 *vs.* 0.59, p=0.001) (univariate analysis Table 3). It has been suggested that use of oral contraceptive pill and hormonotherapy could also contribute to decreased CYP1A2 activity.[36,37] Our results did not show any difference in caffeine metabolic ratio among females using estrogen derivatives (combining oral contraceptive and hormonotherapy) *vs.* non-users (p=0.3). However, our results showed that the use of oral contraceptive pill was negatively associated with CYP1A2 activity when analyses were conducted with the entire cohort (Table S3). The overall contribution of oral contraceptive use on the modulation of CYP1A2 activity remains to be confirmed in a large group of individuals. Hong *et al*. reported a positive relationship between endogenous insulin levels and CYP1A2 activity.[38] Our univariate analysis did not reveal any significant effects of baseline insulin levels and CYP1A2 activity (p=0.33) (Table 3). In our study, 17 patients with T2D received insulin injections as part of their treatment regimen. There was no significant difference in the caffeine

metabolic ratio (MR_{4h}) among T2D patients receiving insulin *vs.* no-insulin (0.54 *vs.* 0.61, p=0.25). However, our results demonstrated a positive relationship between CYP1A2 activity with glycemia, and with HbA1C levels (p=0.01 and 0.02, respectively). The exact mechanism on CYP1A2 modulation by inflammatory factors is not known but studies may suggest the role of aryl hydrocarbon receptor activation in immune responses or under oxidative stress.

In line with our results, Villeneuve and Pichette reported that CYP2D6, CYP2C9 and CYP2E1 activities were less or not affected by liver disease.[39] In human hepatocytes CYP2C9 mRNA expression is decreased by cytokines, but in cancer patients with increased levels of IL-6 and TNF-α, CYP2C9 activity was not altered.[10,40] Furthermore, no difference in tolbutamide clearance was previously reported in a cohort of patients with T2D.[41] No change in CYP2D6 activity was observed in patients with T2D using the probe substrate dextromethorphan and other studies report no change in CYP2D6 activity in patients with different pathologies related to inflammation.[7,31] Finally, CYP2E1 activity has been shown less sensitive to modulation by disease state, with some studies showing a modulatory role of inflammation and others not.[6,7,39] In agreement with our findings, two studies reported no modulation of CYP2E1 activity and expression in T2D patients.[42,43] However, other studies reported an increase in CYP2E1 activity in subjects with T2D.[29,44] Contradictory results may be due to demographic characteristics, co-morbidities, co-medication or disease progression.[29] It was noted that obesity seems to be responsible for increased CYP2E1 activity among patients with T2D as well as in obese individuals.[44,45] Increase in CYP2E1 activity was also linked to poor control of diabetes and increase in HbA1C.[46,47] The lack of effect of T2D observed in our study might be due to the fact that our average diabetic population was non-obese (mean BMI<30 kg/m^2) patients with well controlled diabetes (mean HbA1C=7.1%).

Participants did not receive on a regularly basis any drugs contained in the cocktail except for omeprazole and caffeine. In this current study, two subjects received esomeprazole and one took omeprazole on a regular basis. There is no evidence indicating that omeprazole and esomeprazole are subjected to auto-inhibition for their own metabolism by the CYP2C19 isoform or to mechanism-based inhibition. Moreover, plasma concentrations for all probes were measured at baseline. For omeprazole, its plasma concentration was undetectable for all patients taking either omeprazole or esomeprazole, except for one patient taking esomeprazole who had barely detectable levels (1.7 ng/mL, LLOQ 1 ng/mL). Furthermore, hydroxy-omeprazole was undetectable at baseline for all subjects. The potential of bias introduced by this condition and the risk for competitive inhibition are therefore very unlikely.

Concomitant medications taken on a regular basis by participants and known as CYP450 inhibitors, mechanism-based inhibitors or inducers could produce a CYP450 phenoconversion and introduce a bias in our analysis. Subjects receiving any of these drugs were excluded. Furthermore, all medications, except for insulin, were stopped the night before study day. We are therefore confident that, under these conditions, competitive inhibition between their regular medications and drugs contained in the cocktail is unlikely. Conceptually, 12-24 hours after the administration of a drug (long after its Tmax), very little of this drug remains in the proximal parts of the intestinal space (where most of our probe drugs are absorbed) or is present at high concentration in the portal vein. Some of the drug coming back to the liver does so mostly through the hepatic artery at very low concentrations (compared to concentrations during the absorption phase in the intestine or in the portal vein). Thus, the possibility of competitive inhibition 12-24 hours post-administration a drug with our probe drug cocktail is very low.

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In conclusion, our results indicate that low chronic inflammatory status associated with T2D modulates CYP450 activities in an isoform specific manner. We propose that T2D effects on drug metabolizing enzymes could represent a major determinant of the inter-subject variability observed in drug response in T2D patients receiving prodrugs, drugs associated with toxicity and/or narrow therapeutic index drugs.

METHODS

Study protocol. Study protocol (Trial #14.066) was approved by the ethic review board of the CHUM research center (Montreal, Canada) and registered at the US National Institutes of Health website (http://clinicaltrials.gov; NCT02291666). This clinical trial was carried out in compliance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines. Written informed consent was obtained from all participants prior to any initiation of study procedure. Detailed protocol for this case-control pharmacokinetic study was published previously.[48] In brief, this study evaluates the impact of T2D on major CYP450 isoform activities. Phenotype indices of CYP450 activities were measured in a group of patients with T2D *vs.* a group of non-diabetic subjects after oral administration of (a) a CYP450 probe drug cocktail consisting of caffeine (CYP1A2), bupropion (CYP2B6), tolbutamide (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4) and (b) chlorzoxazone alone (CYP2E1). A total of 73 participants were recruited to constitute 2 study groups; a group of 35 subjects classified as non-diabetic volunteers according to their medical histories, physical examinations and pertinent laboratory tests and a second group of 38 patients diagnosed with T2D. Detailed list of all exclusion and

inclusion criterion and procedures have been published elsewhere.[48] In order to minimize risk of drug-induced CYP450 phenoconversion, exclusion criteria comprised a broad range of drugs known to appreciably influence CYP450 activities (CYP450 inhibitors, CYP450 with strong affinity for the selected isoform and with a long half-life, mechanism-based inhibitors and inducers). In addition to co-administered drugs, the potential of disease states associated with inflammation inducing CYP450 phenoconversion was also considered and exclusion criteria comprised diseases such as arthritis, HIV, hepatitis, bowel disease, *H. Pylori*, any infections (active or in the last 4 weeks), as well as heart failure. In overall, subjects were in a good and stable health conditions.

The primary study aim was to evaluate the effects of T2D on seven major CYP450 isoforms. The planned study design also aimed to investigate the influence of glycemic control by enrolling patients with adequate (A1C≤7) and poor glycemic control (A1C>7). To overcome difficulties with recruitment of patients with poor glycemic control, the 2 groups of T2D patients have been combined in this current analysis. Then, our primary analyses were performed on two groups rather than three to increase our statistical power.

Phenotype assessment.   Utilization of our probe drug cocktail CRCHUM-MT enables to characterize the effect of T2D on metabolism and elimination of drugs by targeting specific activity of different CYP450 isoforms. Each of our probe drugs is selective for a particular isoform of the CYP450s and is free of interaction with one another. Phenotypic indices were determined as previously validated using metabolic ratios (MR) of urinary or plasmatic concentrations, as well as oral clearance (see Table 2 and references in Supplementary file). For all subjects, plasma and urine concentrations of all probe substrates and metabolites were

determined by liquid chromatography tandem mass spectrometry as described previously.[49] The LC-MS/MS analytical method was validated in our laboratory and implemented in the pharmacokinetics core facilities at the CRCHUM. All metabolic ratios were calculated using the concentrations for the metabolite(s) of interest divided by the corresponding parent drug concentration (molar units). Oral clearance, defined as the ratio of the dose administered to the plasma area under the curve (AUC) of the parent drug tolbutamide, was used as a phenotypic marker for CYP2C9. All drug-concentration-time data were analyzed by standard non-compartmental methods using Kinetica® 5.1 (Thermo Kinetica, Thermo Fisher Scientific, USA). The $AUC_{0\text{-}8\,hr}$ were obtained by use of the mixed log-linear trapezoidal rule enabling for interpolation and using LOQ to compute last AUC triangle when the 8h time point was BLQ.

CYP450s' genotype analysis. A blood sample for genotyping was obtained after signature of written informed consent during the screening visit prior to study day. Samples were kept at room temperature and DNA was extracted from leukocytes according to standard procedures within 7 days using the GenElute™ Blood Genomic DNA kit (Sigma Aldrich, Oakville, Can). Resulting purified genomic DNAs were stored at -20°C until genotyping. Overall, variants for the isoforms CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 and CYP3A5 were detected using the TaqMan® qRT-PCR SNP Genotyping Assay (Life Technologies, Burlington, Can). The PCR assay was performed and analyzed using the QuantStudio™ 6 Flex System (Life Technologies, Burlington, Can). The SNP Genotyping assay was completed using specific probes for all SNPs (Life Technologies, Burlington, Can). Distinctively, variant *CYP2B6*4* (rs2279343) was determined by PCR-RFLP. For CYP2D6, copy number variation (CNV), gene deletion (*CYP2D6*5*) and duplication (*CYP2D6*2xN*), were determined by TaqMan® copy number assay on the QuantStudio™ 6 Flex System (Life

Technologies, Burlington, Can). Genotype-predicted phenotypes were assigned for CYP2C19, CYP2C19 and CYP2D6 based on CPIC guidelines, PharmGKB and PharmVar websites. CYP2B6 genotypes were categorized as poor metabolizer (*CYP2B6*6/*6*) intermediate (*CYP2B6*1/*6, *4/*9, *4/*6, *5/*6*), and normal metabolizer (*CYP2B6*1/*1, *1/*4, *1/*5, *4/*5*).

Determination of Proinflammatory cytokine levels. Blood samples for the quantification of proinflammatory cytokines were kept on ice and rapidly sent to the research laboratory and centrifuged for 10 min at 4°C and 1,100 g within 1 hour of sampling. Plasma was then aliquoted and stored at -80°C until use. Levels of inflammatory markers INF-γ, IL-1β, IL-6 and TNF-α were quantified by electrochemiluminescence immunoassays using the V-PLEX Proinflammatory Panel 1 Human Kit, QuickPlex SQ120 Imager and WORKBENCH software (MSD®, Rockville, MD).

Statistical analyses. Continuous and nominal variables were expressed using mean ± SD or percentage (%) respectively. The comparison between subjects with T2D and non-diabetic subjects for characteristic variables expressed in percentage were analyzed using Fisher's exact test. Primary end points (urine or plasma MR and oral clearance) were analyzed using a one-way linear model to compare between subjects with T2D and non-diabetic subjects with heterogeneous variances. An attempt to reduce the model to a one-way analysis while keeping the same variance between groups was also performed. In case of heteroscedasticity, the statistical analyses were performed using separate residual variances for each group. The Satterthwaite's degree of freedom statement was added for unequal variance structures. Analyses were adjusted for confounding age, gender and genotype effects. Furthermore, all

characteristic variables and CYP450 genotyping were investigated to predict the urine or plasma MR as well as oral clearance. Relationship between continuous variables and primary end points were checked for the assumption of linearity using a regression linear model. All primary end points were log transformed to fulfill this assumption. Variables whose univariate regression analyses with the primary end points, had a p-value < 0.25 were candidates for the multivariate model building. The selection of variables was performed using two statistical approaches. First, the stepwise and backward selections of variables were used in the multivariate regression model with interaction effects between independent candidate variables. An alternative procedure to select variables was to use the best subset selection containing one to seven variables. Akaike's and Sawa's Bayesian information criteria were computed to validate the model selected. To assess the goodness-of-fit of the model, visual representation of studentized residuals as well as Shapiro-Wilk test for normality were performed. A robust statistical regression approach (M-estimation) was performed for each response variable to investigate the stability of estimates. Both regression approaches gave similar estimates. Data were analysed using the statistical package SAS version 9.4 (SAS Institute Inc, Cary, NC) and R software, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

STUDY HIGHLIGHTS

What is the current knowledge on the topic?

Inflammation modulates CYP450 activities. Some diseases such as HIV infection, rheumatoid arthritis, hepatitis C and T2D are associated with chronic inflammation. In clinic, patients with T2D show highly variable responses to different drugs that could be explained by a modulation of CYP450 activities.

What question did this study address?

What is the impact of T2D on drug metabolizing activity for 7 major CYP450 isoforms in human.

What does this study add to our knowledge?

A modulation of CYP450 activities was observed in patients with T2D in an isoform specific manner. Our results show that patients with T2D had a lower metabolic activity for CYP3A4/5, CYP2C19 and CYP2B6. In opposite, CYP1A2 and CYP2C9 activities tended to be enhanced while CYP2D6 and CYP2E1 were not affected by T2D in this study.

How might this change clinical pharmacology or translational science?

A better understanding of T2D influence on CYP450 metabolizing capacity, especially in patients with polypharmacy, could contribute to improve drug response and prevent adverse drug reactions.

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AUTHOR CONTRIBUTIONS

V.M. designed the research; S.G. and JL.C. performed the research; S.G., V.M. and J.T. analysed the data; S.G., V.M. and J.T. wrote the manuscript; A.G. developed LC-MS/MS analytical methods.

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Table 1. Demographic and clinical characteristics

Parameters	Non-T2D participants	Patients with T2D
No. (%) of subjects	35 (48)	38 (52)
Sex: No. (%) M:F	15:20 (43:57)	30:8 (79:21)*
Age (years)	46 ± 16	66 ± 10*
Body Weight (Kg)	73.8 ± 15.5	84.5 ± 15.5*
BMI (kg/m^2)	25.7 ± 3.9	29.1 ± 6.5*
Insulin (pmol/L)	63.7 ± 40.7[a]	327.6 ± 1,121.7*,[b]
Glycaemia (mmol/L)	4.6 ± 0.5	8.7 ± 2.4*
HbA1C (%)	5.2 ± 0.5	7.1 ± 1.0*
HOMA-IR	1.9 ± 1.3[a]	18.3 ± 61.8*,[b]
HOMA-β	180.6 ± 117.8[a]	200.3 ± 633.6*,[b]
Creatinine clearance (ml/min)	108.8 ± 32.4	92.4 ± 28.3*
Time since diagnostic (years)	*na*	15.2 ± 8.8
Medication use, No. (%) of subjects		
Metformin	0	33 (87)*
Sulfonylurea	0	18 (47)*
DPP4-I	0	21 (55)*
Insulin	0	17 (45)*
Statins	5 (14)	28 (74)*
ACEI	0	14 (37)*
ARB	2 (6)	18 (47)*
CCB	0	15 (39)*
β-Blockers	3 (9)	11 (29)*
Aspirin	2 (6)	18 (47)*
other NSAID	2 (6)	4 (11)
Antidepressants	2 (6)	4 (11)
PPI	1 (3)	10 (26)*
OC	3 (9)	0
Hormonotherapy containing estrogen	5 (14)	2 (5)

Continuous variables are presented as mean ± SD

* Demographic parameters are significantly different between study groups (p<0.05).

[a] data available for n = 33.

[b] data available for n = 37.

na, not applicable; Non-T2D, non-diabetic patient group; T2D, patients with a diagnostic of Type 2 diabetes group. BMI, body mass index; HbA1C, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β, homeostatic model assessment of beta cells function; DPP4-I, dipeptidyl peptidase-4 inhibitors; ACEI, angiotensin-converting-enzyme inhibitors; ARB, angiotensin II receptor blockers; CCB, calcium channel blockers; NSAID, non-steroidal anti-inflammatory drugs; PPI, proton pump inhibitors; OC, oral contraceptive (containing ethinylestradiol).

Table 2. Mean Metabolic Ratios for the 7 CYP450 isoforms among the two study groups.

Isoform	Matrix	Phenotypic parameter	Non-T2D participants	Patients with T2D	p-value	Adjusted p-value[a]
CYP1A2	plasma	$C_{4\,hr}$ paraxanthine/$C_{4\,hr}$ caffeine	0.48 ± 0.19	0.59 ± 0.17	0.008	0.07
CYP2B6	urine	$Ae_{0-8\,hr}$ OH-bupropion/$Ae_{0-8\,hr}$ bupropion	4.05 ± 4.21	2.24 ± 3.09	0.01	0.01
CYP2C9	plasma	Tolbutamide dose /$AUC_{0-24\,hr}$ tolbutamide	0.98 ± 0.23	1.23 ± 0.35	0.0008	0.05
CYP2C19	plasma	$AUC_{0-8\,hr}$ OH-omeprazole/$AUC_{0-8\,hr}$ omeprazole	0.52 ± 0.32	0.28 ± 0.23	0.0009	0.003
CYP2D6	plasma	$AUC_{0-8\,hr}$ dextrorphan/$AUC_{0-8\,hr}$ dextromethorphan	1.27 ± 0.99	1.27 ± 1.59	0.75	0.78
CYP2E1	urine	Ae OH-chlorzoxazone/Ae chlorzoxazone	9329 ± 6142	8512 ± 5315	0.78	0.54
CYP3A	plasma	$AUC_{0-8\,hr}$ OH-midazolam/$AUC_{0-8\,hr}$ midazolam	0.57 ± 0.23	0.36 ± 0.13	<0.0001	0.003

Metabolic ratios are presented as mean ± SD

C, concentration; Ae, amount excreted; AUC, area under the concentration-time curve.

[a] ANCOVA analysis, p-value adjusted for sex and age.

Phenotypic indices were determined using metabolic ratios of urinary or plasmatic concentrations and oral clearance as previously reported (references are presented in the Supplementary Reading list)

Table 3. Univariate regression analysis of covariates for studied CYP450 activities (from all subjects n=73).

Variable	Caffeine (CYP1A2)	Bupropion (CYP2B6)	Tolbutamide (CYP2C9)	Omeprazole (CYP2C19)	Dextromethorphan (CYP2D6)	Chlorzoxazone (CYP2E1)	Midazolam (CYP3A)
HbA1C	8.9 ± 3.9 (0.02)	-17.7 ± 10.0 (0.08)	10.4 ± 2.4 (<0.0001)	-27.0 ± 8.1 (0.001)	-1.31 ± 13.03 (0.9)	5.5 ± 6.9 (0.4)	-19.2 ± 3.9 (<0.0001)
Log (Glucose)	0.34 ± 0.13 (0.01)	-0.73 ± 0.34 (0.03)	0.29 ± 0.08 (0.0009)	-0.86 ± 0.28 (0.003)	-0.35 ± 0.44 (0.4)	0.01 ± 0.24 (1.0)	-0.74 ± 0.13 (<0.0001)
Log (Insulin)	-0.05 ± 0.05 (0.3)	-0.26 ± 0.13 (0.04)	0.09 ± 0.03 (0.006)	-0.50 ± 0.10 (<0.0001)	0.11 ± 0.16 (0.5)	0.11 ± 0.09 (0.2)	-0.26 ± 0.05 (<0.0001)
Log (HoMA-β)	-0.17 ± 0.05 (0.0004)	-0.07 ± 0.13 (0.6)	-0.01 ± 0.03 (0.7)	-0.17 ± 0.11 (0.1)	0.26 ± 0.16 (0.1)	0.10 ± 0.09 (0.3)	0.02 ± 0.06 (0.7)
Log (HoMA-IR)	0.0002 ± 0.044 (1.0)	-0.24 ± 0.10 (0.02)	0.09 ± 0.03 (0.001)	-0.42 ± 0.08 (<0.0001)	0.03 ± 0.13 (0.8)	0.076 ± 0.076 (0.3)	-0.25 ± 0.04 (<0.0001)
Age	0.011 ± 0.003 (0.0002)	0.095 ± 0.05 (0.04)	0.003 ± 0.002 (0.08)	-0.04 ± 0.04 (0.3)	-0.002 ± 0.010 (0.8)	-0.004 ± 0.005 (0.5)	-0.010 ± 0.003 (0.003)
Weight	0.004 ± 0.003 (0.2)	0.0005 ± 0.008 (1.0)	0.012 ± 0.001 (<0.0001)	-0.009 ± 0.006 (0.2)	0.005 ± 0.010 (0.6)	0.004 ± 0.005 (0.4)	-0.013 ± 0.003 (<0.0001)
Height	1.05 ± 0.49 (0.03)	2.25 ± 1.27 (0.08)	1.19 ± 0.30 (0.0002)	0.76 ± 1.09 (0.5)	-2.03 ± 1.63 (0.2)	-0.83 ± 0.87 (0.3)	-1.18 ± 0.56 (0.04)
BMI	0.003 ± 0.011 (0.8)	-0.03 ± 0.03 (0.2)	0.039 ± 0.006 (<0.0001)	-0.05 ± 0.02 (0.02)	0.05 ± 0.04 (0.2)	0.027 ± 0.019 (0.2)	-0.04 ± 0.01 (0.0005)
Log (IFN-γ)	0.18 ± 0.09 (0.04)	-0.76 ± 0.21 (0.0008)	0.03 ± 0.06 (0.7)	-0.06 ± 0.20 (0.7)	0.06 ± 0.30 (0.8)	-0.13 ± 0.16 (0.4)	-0.10 ± 0.10 (0.3)
Log (IL-1β)	0.10 ± 0.09 (0.3)	-0.28 ± 0.23 (0.2)	0.03 ± 0.06 (0.6)	-0.22 ± 0.19 (0.2)	0.13 ± 0.29 (0.7)	-0.24 ± 0.15 (0.1)	-0.08 ± 0.10 (0.4)
Log (IL-6)	0.09 ± 0.07 (0.2)	-0.44 ± 0.16 (0.007)	0.11 ± 0.04 (0.01)	-0.30 ± 0.14 (0.04)	0.28 ± 0.21 (0.2)	0.02 ± 0.12 (0.9)	-0.21 ± 0.07 (0.004)
TNF-α	0.19 ± 0.06 (0.002)	-0.47 ± 0.15 (0.003)	0.13 ± 0.04 (0.001)	-0.14 ± 0.14 (0.3)	-0.09 ± 0.21 (0.7)	-0.14 ± 0.11 (0.2)	-0.19 ± 0.07 (0.007)

Values presented as Coefficient β ± SE (p-value).

HbA1C, glycated hemoglobin; HOMA-β, homeostatic model assessment of beta cells function; HOMA-IR, homeostatic model assessment of insulin resistance; BMI, body mass index; IFN-γ, interferon-gamma; IL-1β, interleukin-1beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

CYP2C19
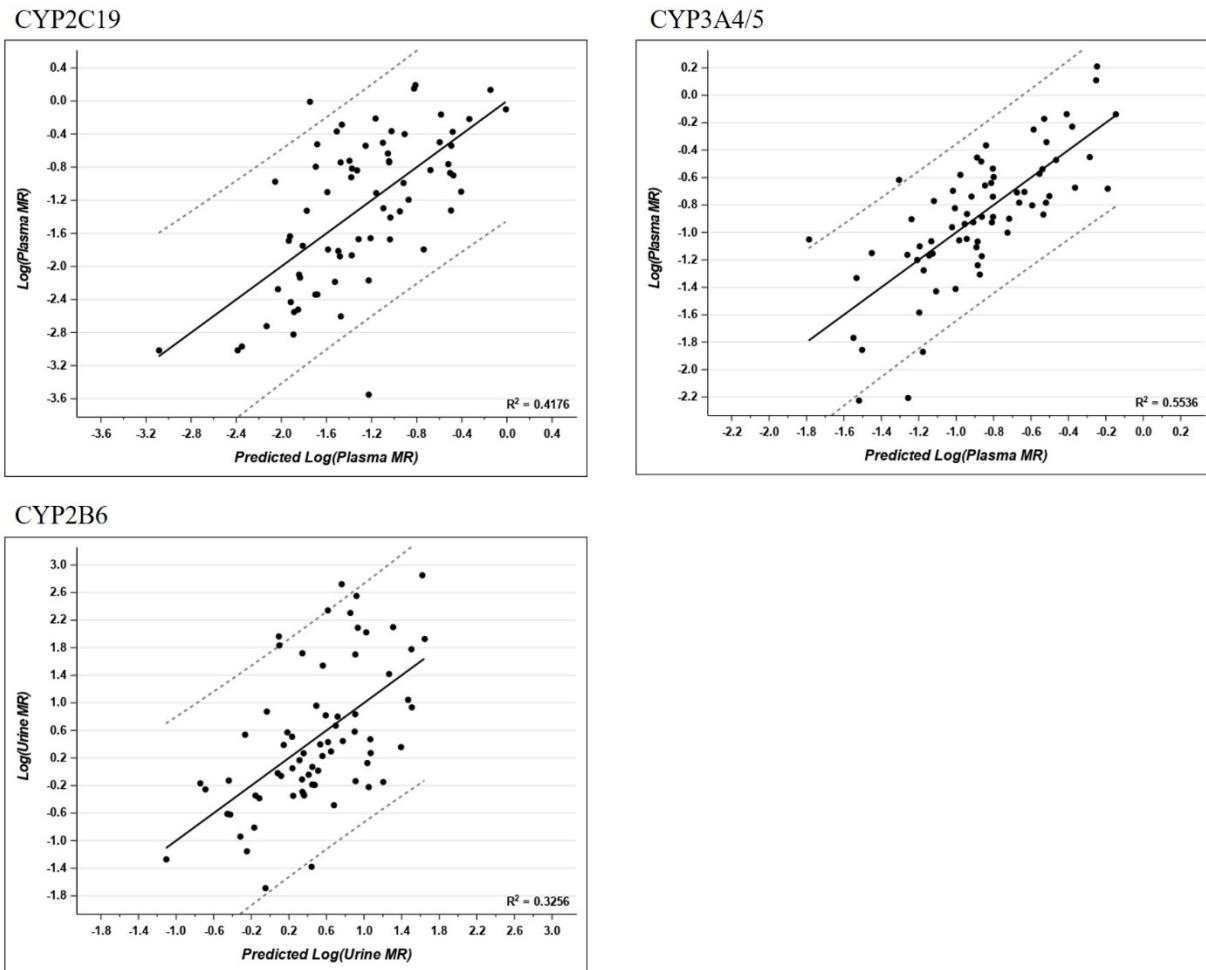
CYP3A4/5

CYP2B6

Figure 1 Plots of observed vs predicted metabolic ratios estimated from multi-regression models for selected CYP450 activities are illustrated: CYP2C19, CYP3A4/5 and CYP2B6 (dotted lines show the 95% confidence intervals). Plots are presented for CYP450 isoforms for which a statistically significant correlation between T2D and CYP activities was obtained from ANCOVA (*i.e.* CYP2B6, CYP2C19 and CYP3A4/5).

SUPPLEMENTARY MATERIAL

Table S1. Variant allele frequencies (%) across study population.

	Non-T2D participants (n)	Patients with T2D (n)
CYP2B6		
*1	61 (43)	64 (49)
*4	4 (3)	5 (4)
*5	17 (12)	8 (6)
*6	17 (12)	22 (17)
CYP2C9		
*1	84 (59)	79 (60)
*2	13 (9)	20 (15)
*3	3 (2)	1 (1)
CYP2C19		
*1	61 (43)	68 (52)
*2	21 (15)	13 (10)
*3	0 (0)	0 (0)
*17	17 (12)	18 (14)
CYP2D6		
*1	36 (25)	53 (40)[b]
*2	20 (14)	17 (13)
*3	1 (1)	1 (1)
*4	17 (12)	10 (8)
*5	6 (4)	3 (2)
*6	1 (1)	3 (2)
*10	10 (7)	4 (3)
*41	9 (6)	9 (7)
CNV (xN)[a]	9 (3)	5 (2)
CYP3A4		
*1	99 (69)	96 (73)
*22	1 (1)	4 (3)
CYP3A5		
*1	9 (6)	7 (5)
*3	90 (63)	93 (71)
*6	1 (1)	0 (0)

Variant allele frequencies are provided in percent with number of alleles (n).
CNV, Copy Number Variation.
[a] CNV; frequency of subjects in percent carrying >2 copy number with number of individuals (n).
[b] Allele frequency is significantly different between study groups (p=0.02).
Genotype-predicted phenotypes were assigned for CYP2C19, CYP2C19 and CYP2D6 based on CPIC guidelines, PharmGKB and PharmVar websites.[1,2] CYP2B6 genotypes were categorized as poor metabolizer (*CYP2B6*6/*6*) intermediate (*CYP2B6*1/*6, *4/*9, *4/*6, *5/*6*), and normal metabolizer (*CYP2B6*1/*1, *1/*4, *1/*5, *4/*5*).[3-6]

Table S2. Genotype-predicted phenotype status and CYP450 isoform activities (mean ± SD (n)) across study groups.

Isoform	Study group	Metabolizer Status			
		PM	IM	EM	UM
CYP2B6	Non-T2D	0.64 ± 0.35‡ (2)	3.88 ± 3.65 (8)	4.38 ± 4.53 (25)	
	T2D	0.53 ± 0.35‡ (2)	1.75 ± 2.78* (13)	2.66 ± 3.36 (23)	
CYP2C9	Non-T2D	0.58 (1)	0.96 ± 0.25 (9)	1.01 ± 0.22 (25)	
	T2D	0.71 (1)	1.27 ± 0.41* (14)	1.22 ± 0.30* (23)	
CYP2C19	Non-T2D		0.37 ± 0.23 (15)	0.63 ± 0.35 (13)	0.64 ± 0.32 (7)
	T2D		0.28 ± 0.15* (10)	0.30 ± 0.30* (18)	0.24 ± 0.17* (10)
CYP2D6	Non-T2D	0.02 (1)	0.67 ± 0.51† (17)	1.73 ± 0.76 (15)	3.58 ± 0.42 (2)
	T2D		0.53 ± 0.50† (13)	1.70 ± 1.86 (24)	0.61 (1)
CYP3A4	Non-T2D		0.27 (1)	0.58 ± 0.22 (34)	
	T2D		0.38 ± 0.18 (3)	0.35 ± 0.13** (35)	
CYP3A5	Non-T2D	0.55 ± 0.22‡ (29)		0.68 ± 0.27 (6)	
	T2D	0.34 ± 0.13*,‡ (33)		0.46 ± 0.07* (5)	

Two-way ANOVA analyses showed no significant interactions for all isozymes when applicable.
p-value for interaction reported for two-way ANOVA analyses using Tukey-Kramer's multiple comparison tests (comparison between phenotypes among one study group: †p<0.05 IM vs EM and ‡p<0.05 PM vs EM).
p-value for interaction reported for two-way ANOVA analyses using Mann Whitney non-parametric t-test (comparison between T2D vs non-T2D : *p<0.05 vs non-T2D participants and **p<0.0001 vs non-T2D participants)
PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; UM, ultra-rapid metabolizer.

Table S3. Multivariate analysis of CYP450 activities.

CYP450 isoform	Independent variables	parameter estimate (β)	r^2
CYP1A2	Intercept	-3.05	0.60
	Diabetic status	0.01	
	Log (HOMA-β)	-0.13	
	Log (IFN-γ)	0.12	
	TNF-α	2.21	
	(TNF-α)^2	-0.73	
	(TNF-α)^3	0.08	
	None oral contraceptive user	0.78	
CYP2C9	Intercept	-0.97	0.52
	HbA1C	4.45	
	BMI	0.03	
	Gender-female	-0.15	
CYP2C19	Intercept	-0.81	0.42
	Diabetic status	0.33	
	Log (Insulin)	-0.35	
	Log (IFN-γ)	-0.38	
	TNF-α	0.91	
	Diabetic status_Log (IFN-γ)	0.84	
	Diabetic status_TNF-α	-0.92	
CYP3A	Intercept	-0.80	0.55
	Log (HOMA-IR)	-0.22	
	CYP3A5 genotype_IM	0.36	
	Gender_female	0.34	
CYP2B6	Intercept	0.75	0.33
	Diabetic status	-0.17	
	CYP2B6 genotype_IM	0.78	
	CYP2B6 genotype_EM	1.23	
	Log HOMA-IR	-0.14	
	Log (IFN-γ)	-0.56	
	TNF-α	-0.17	
CYP2D6	Intercept	-2.53	0.31
	CYP2D6 genotype_EM/UM	1.37	
	Log (HOMA-β)	0.30	
CYP2E1	Intercept	7.94	0.09
	Diabetes status_HbA1C>7%	0.43	
	Log (IL-1β)	-0.27	

Diabetic status: T2D =1 and non-T2D=0

T2D, patients with type 2 diabetes; BMI, body mass index; HbA1C, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β, homeostatic model assessment of beta cells function; Insulin (pmol/L); IFN-γ, interferon-gamma; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; EM, extensive metabolizers; IM, intermediate metabolizers; PM, poor metabolizers; UM, ultra-rapid metabolizers; oral contraceptive containing ethinylestradiol.

None oral contraceptive users = 1 and Oral contraceptive users = 0

Gender: female=1 and male=0

CYP3A5 genotype metabolizer: IM=1 (carrier of one CYP3A5 active allele) and PM=0

CYP2D6 genotype: due to the low number of PM and UM subjects, they were combined with IM and EM groups, respectively.

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Supplemental reading list. Additional references

In this study, phenotypic indices were determined using metabolic ratios of urinary or plasmatic concentrations and oral clearance as previously reported.[1-13]

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2.1.1.4. Discussion

Nos résultats démontrent que l'effet du DT2 sur la capacité métabolique, déterminé à la suite de l'administration par voie orale d'un *cocktail* de substrats-marqueurs des CYP450s, est isoforme-dépendant. L'activité métabolique du CYP2B6, CYP2C19 et CYP3A4/5 est réduite chez des patients avec le DT2 comparativement à des sujets non diabétiques. Inversement, les activités métaboliques du CYP1A2 et du CYP2C9 apparaissent élevées chez les patients avec le DT2, mais l'effet ne demeure pas statistiquement significatif lorsqu'ajusté pour l'âge et le sexe. Aucun effet significatif de la pathologie n'a été observé pour les CYP2D6 et CYP2E1. Nos résultats indiquent que le DT2, une pathologie à composante inflammatoire, modulerait l'activité de certaines isoformes des CYP450s pouvant ainsi jouer un rôle dans la variabilité de réponse aux médicaments observée dans cette sous-population de patients qui nécessitent souvent une polymédication.

L'isoforme CYP1A2 est majoritairement exprimée au niveau hépatique et serait responsable d'environ 9% des réactions de métabolisme des médicaments utilisés en clinique.[24] Malgré son importance, peu de rapports sur l'impact de l'inflammation ou de diverses maladies sur la capacité métabolique du CYP1A2 ont été publiés.[235] En lien avec le statut inflammatoire associé à diverses conditions pathologiques, une diminution faible à modérée de l'activité du CYP1A2 a été corrélée avec les niveaux de différents marqueurs inflammatoires chez des patients atteints d'hépatite B ou C ou d'influenza, d'insuffisance cardiaque congestive.[235,304-306] Dans une étude chez des patients atteints du VIH, les niveaux d'activité du CYP1A2 étaient similaires aux sujets sains tel que mesuré à l'aide de la caféine.[235] Ces différences d'effets pourraient être dû aux différences dans les niveaux des cytokines inflammatoires chez les divers groupes de patients et le type d'inflammation pour les diverses pathologies. Aussi, bien que la

corrélation entre les niveaux des marqueurs inflammatoires et les concentrations rapportées des substrats pour CYP1A2 soit inverse, plusieurs facteurs peuvent moduler l'activité des CYP450s et une comparaison avec un groupe contrôle en prenant compte des caractéristiques démographiques semblent adéquat pour évaluer l'effet d'un statut pathologique.

Les résultats des quelques études mesurant l'effet du DT2 sur l'activité du CYP1A2 sont variables.[283-286] Dans trois populations diabétiques de tailles limitées (n ≤ 16) aucun impact de la maladie ne fut observé sur la clairance ou les ratios métaboliques de la caféine ou théophylline, 2 substrats-marqueurs connus du CYP1A2.[283-285] Plus récemment, Urry *et al.* a rapporté que le ratio métabolique paraxanthine/caféine, index phénotypique du CYP1A2, était augmenté dans une population de 57 patients atteints du DT2.[286] En accord avec cette étude, nous avons mesuré chez 38 sujets diabétiques une augmentation du ratio métabolique de la caféine, indiquant que le DT2 augmenterait l'activité du CYP1A2. Il est intéressant de noter que pour les trois études n'ayant rapporté aucun effet du DT2 sur l'activité du CYP1A2, la consommation de produits contenants de la caféine avait été arrêtée au moins 24h avant l'administration du substrat-marqueur.[283-285] Or, dans l'étude rapportée par Urry *et al.* et la nôtre, la consommation de produits caféinés n'a pas été restreinte pour une période d'élimination du produit suffisante avant l'étude.[231,286] Toutefois, une étude clinique à devis croisé aurait démontré que la restriction de consommer des produits contenant de la caféine avant l'étude n'affecterait pas les résultats de phénotypage obtenus dans la salive et le plasma.[307] Cependant, l'effet du DT2 sur le CYP1A2 que nous avons observé semble être expliqué par l'âge et le sexe de nos participants. En accord avec ce qui a été rapporté auparavant, nos participants de sexe féminin présentaient un ratio métabolique pour la caféine significativement moindre que chez

nos participants males présents en plus grande proportion dans notre groupe de sujets atteints du DT2.[308,309]

Un autre facteur à considérer est la prise concomitante de médicaments. La prise de contraceptifs oraux ou d'hormonothérapie pourrait contribuer à une diminution des niveaux d'activité du CYP1A2.[309,310] Aucune différence entre les ratios métaboliques des femmes utilisant un dérivé de l'œstrogène et celles sans ne fut observé dans notre étude. Toutefois, l'utilisation de contraceptifs oraux contenant de l'éthynylestradiol était négativement associée aux ratios métaboliques de caféine. La modulation de l'activité du CYP1A2 par la prise de contraceptifs oraux devrait être confirmée dans une population plus importante. L'insuline a été rapporté comme un facteur influençant l'activité du CYP1A2.[311] Dans notre étude, les 17 individus recevant de l'insuline pour le traitement de leur DT2 ne présentaient pas un ratio métabolique du CYP1A2 différent des diabétiques non-utilisateurs d'insuline. Toutefois, une relation positive entre l'activité du CYP1A2 et la glycémie ainsi que les niveaux de HbA1c a été mesurée dans notre population.

La régulation du CYP2B6 par l'inflammation et en condition pathologique a été peu étudiée. En présence de cytokines inflammatoires, l'expression en ARNm et protéines du CYP2B6 était diminuée dans des hépatocytes humains en culture.[62] De plus, une diminution de l'expression d'ARNm et de l'activité du Cyp2b est rapportée dans des modèles de souris du diabète induit par la diète.[267,272,273] À notre connaissance, nous sommes les premiers à rapporter une diminution de l'activité du CYP2B6 chez des patients atteints du DT2.

Comme pour le CYP2B6, l'exposition d'hépatocytes humains en culture à différentes cytokines inflammatoires a résulté en une diminution de l'expression d'ARNm de l'isoforme

CYP2C19.[62] La modulation *in vivo* du CYP2C19 par l'inflammation et en présence de maladies a été étudiée précédemment.[236,304,312] Les études s'accordent sur une diminution de l'activité du CYP2C19 en présence d'inflammation.[236,304,312] Par exemple, l'exposition à l'oméprazole, un substrat de CYP2C19, était environ deux fois plus élevée chez des patients atteints d'arthrite rhumatoïde.[236] Aussi, les niveaux d'activité du CYP2C19 tel que mesuré à l'aide du méphénytoïne étaient fortement et inversement corrélés aux niveaux de TNF-α et IL-6 chez des patients atteints d'insuffisance cardiaque congestive.[304] En accord avec la littérature, nous avons observé une diminution de l'indice d'hydroxylation de l'oméprazole, soit de l'activité du CYP2C19. Dans une étude chez des volontaires sains, le lien entre l'inflammation induite par une injection de lipopolysaccharide et une diminution de l'activité du CYP2C19 a été mis en évidence.[312] Aussi, chez des patients souffrant d'insuffisance cardiaque congestive, des niveaux élevés des cytokines pro-inflammatoires IL-6 et TNF-α corrélaient avec une activité réduite du CYP2C19.[304] Plusieurs études effectuées chez des patients atteints d'un cancer, de myélomes multiples ou d'une maladie du foie ont rapporté des discordances entre les niveaux d'activité attendus en fonction des génotypes et les phénotypes d'activité mesurés pour CYP2C19.[236] En effet, dans ces populations, plusieurs patients avec un génotype correspondant à un métaboliseur extensif tendaient à présenter un phénotype d'activité du CYP2C19 réduit qui serait semblable à un métaboliseur lent.[236]

Puisque nous avons observé dans notre étude une diminution significative de l'activité du CYP2C19 chez nos patients atteints du DT2, il est intéressant de s'attarder à l'interaction médicament-pathologie rapportée entre le clopidogrel et le diabète. Le clopidogrel est un pro-médicament qui doit être métabolisé, notamment par le CYP2C19, afin d'exercer son effet antiplaquettaire. Chez les patients traités avec le clopidogrel, une incidence plus élevée d'effets

indésirables cardiaques majeurs fut rapportée dans une sous-population de patients diabétiques.[313-315] Depuis que la FDA a émis un *black box warning* concernant le clopidogrel pour les patients présentant un génotype CYP2C19 de métaboliseur lent, des méta-analyses ont confirmé l'implication de l'allèle inactif dans la réponse sous-optimale au traitement.[316-318] Conséquemment, une réduction de l'activité métabolique du CYP2C19 chez les patients atteints du DT2 pouvant mener à une phénoconversion vers un phénotype de métaboliseur lent pourrait avoir une importance clinique pour des pro-médicaments comme le clopidogrel.

Un autre exemple de médicament à index thérapeutique étroit pour lequel la réponse pourrait être potentiellement affectée par une modulation du CYP2C19 et la prise concomitante de médicaments chez les diabétiques est le Voriconazole.[319] Cet agent antifongique est principalement métabolisé par le CYP2C19 et moindrement par les CYP3A et CYP2C9. Une variabilité dans sa pharmacocinétique, soit une augmentation de ses concentrations, a été rapportée en présence d'inflammation et il est proposé que cette différence soit dû à une diminution de son métabolisme par les CYP450s.[320,321] Toutefois, de plus amples études sur les différences d'effets entre l'inflammation sévère, comme dans le cas d'infections, et l'inflammation chronique modérée, comme pour le diabète, sur les niveaux d'activités des CYP450s sont encore requises afin de déterminer les mécanismes exacts et la pertinence clinique de ces effets.

Les niveaux d'activité mesurés lors d'expériences *in vitro*, l'expression en ARNm et les niveaux de protéines du CYP3A étaient diminués en présence accrue de marqueurs inflammatoires.[62,243] Dans notre étude, nous avons mesuré des niveaux d'activité métabolique environ 40% moindre chez nos sujets atteints du DT2 comparativement aux sujets non diabétiques. Dû à l'important rôle du CYP3A dans le métabolisme des médicaments, plusieurs

études ont mesuré ses niveaux d'activité dans diverses populations de patients.[235,277,322] Notamment, une activité moindre du CYP3A a été aussi rapportée chez des patients atteints du VIH, de la maladie de Crohn en phase active, de l'hépatite C, d'un cancer, d'arthrite rhumatoïde ou d'une stéatose hépatique non alcoolique.[235,277] Par exemple, une étude chez les patients atteints d'arthrite rhumatoïde présentant des niveaux élevés d'IL-6 rapporte que ces patients présentent une exposition à la simvastatine, un substrat du CYP3A, plus élevée que des sujets contrôles. L'inflammation chez ces patients atteints d'arthrite rhumatoïde réduirait donc l'activité du CYP3A. Dans cette étude-ci, l'administration de tocilizumab, un anti-IL-6, restaurait l'activité du CYP3A tel que mesuré par une exposition à la simvastatine diminuée à des niveaux similaires à des sujets contrôles, soit d'environ 66%.[242] Dans le cas du diabète, la diminution de l'activité du CYP3A que nous avons mesurée chez nos sujets avec le DT2 est en accord avec d'autres études. [281,282,295] En effet, chez des patients diabétiques le métabolisme de la cyclosporine et du nisoldipine était diminué, indiquant une diminution de l'activité métabolique de l'isoforme CYP3A.[281,295] Une autre étude a démontré que l'expression et l'activité du CYP3A dans des microsomes de foies humains étaient diminuées chez les individus atteints du DT2 comparativement à des sujets non diabétiques appariés pour leurs caractéristiques démographiques.[282] Compte-tenu des rapports déjà publiés, les résultats de notre étude suggèrent que des précautions devraient être prises lorsque plusieurs médicaments substrats des CYP450s sont utilisés conjointement et ce particulièrement pour les substrats des CYP3As chez des patients diabétiques afin de prévenir et minimiser la survenue d'effets indésirables. D'ailleurs, plusieurs cas d'interactions pathologie-médicament médiées par les CYP3As ont été observées chez des sous-populations de patients présentant une condition inflammatoire.[242]

Dans notre étude, aucun impact significatif du DT2 n'a été mesuré sur le CYP2D6 et le CYP2E1. Bien que l'activité du CYP2C9 semble augmentée chez nos patients atteints du DT2, la différence entre nos deux groupes ne restait pas significative lorsqu'on ajustait pour le sexe et l'âge. Intéressement, un rapport par Villeneuve et Pichette décrit que ces trois isoformes seraient moins affectées par une maladie du foie.[323] Bien que dans des hépatocytes humains en culture l'exposition à l'IL-6 et le TNF-α diminuait l'expression en ARNm du CYP2C9, l'élévation de ces cytokines chez des patients cancéreux n'altérait pas l'activité métabolique *in vivo* du CYP2C9.[62,324] De plus, aucune modulation de la clairance du tolbutamide n'a été rapportée dans une cohorte de patients atteints du DT2.[287] Dans notre étude, l'activité du CYP2D6 n'était pas affectée par le DT2. D'autres études ne rapportent aussi aucun changement des niveaux d'activité du CYP2D6 chez des cohortes de patients atteints d'une pathologie à composante inflammatoire.[235,283] Les résultats concernant la modulation du CYP2E1 chez des sujets diabétiques sont variables. Comme dans notre étude, deux autres projets cliniques n'ont rapporté aucun effet du DT2 sur l'activité du CYP2E1.[290,325] Par contre, d'autres études ont mesuré une augmentation de l'activité du CYP2E1 chez des sujets atteints du DT2.[282,291] Plusieurs facteurs comme les caractéristiques démographiques, les comorbidités, la comédication ou la progression de la maladie peuvent être responsables de ces résultats contracdictoires.[282] Il a d'ailleurs été suggéré que l'obésité pourrait être responsable de l'augmentation de l'activité du CYP2E1 parmi les sujets diabétiques, de même que chez les obèses non diabétiques.[290,291,326] En plus du statut diabétique, l'augmentation de l'activité du CYP2E1 est liée au contrôle de la maladie et au niveau HbA1C.[327,328] Ainsi, l'absence d'effet du DT2 sur l'activité du CYP2E1 dans notre étude pourrait être dû au fait que notre population

diabétique est non-obèses (indice de masse corporelle <30 kg/m^2) et présente un bon contrôle glycémique (HbA1C moyen de 7.1%).

Cette étude nous a permis d'évaluer l'influence du DT2 sur l'activité métabolique de sept isoformes des CYP450s. Suivant l'analyse de plusieurs covariables, l'effet du diabète s'avère isoforme-dépendant. Les patients atteints du DT2 présentent une réduction significative de l'activité des isoformes CYP2B6, CYP2C19 ainsi que CYP3A, mais aucun impact significatif de la maladie n'est observé sur l'activité des isoformes CYP1A2, CYP2C9, CYP2D6 et CYP2E1.

2.2 Manuscrit 3

Manuscrit 3: « Use of 4β-hydroxycholesterol plasma concentrations as an endogenous biomarker of CYP3A activity: Clinical validation in individuals with Type 2 diabetes. »

2.2.1. Introduction

Chez l'humain, le CYP3A4/5 est hautement exprimé au niveau hépatique et intestinal.[35,80] Le CYP3A joue un rôle important dans le métabolisme de plus de la moitié des médicaments utilisés en clinique.[280] Par ses nombreux substrats, le CYP3A est impliqué dans de nombreuses interactions médicamenteuses pouvant même mener à la survenue de toxicités menant au retrait de certaines molécules du marché.[329-331] De plus, une importante variabilité interindividuelle, qui est multifactorielle, est observée pour le métabolisme des médicaments par le CYP3A chez l'humain.[332,333] Présentement, plusieurs médicaments substrats-marqueurs sont utilisés pour évaluer l'implication des variables génétiques, ethniques et environnementales dans la variabilité du métabolisme des médicaments. Pour le phénotypage du CYP3A *in vivo*, un des substrats-marqueurs recommandés et validés pour les études de métabolisme, d'induction, d'inhibition et d'interactions médicamenteuses est le midazolam.[172,173,211] Tout comme pour le métabolisme des médicaments, le CYP3A est aussi grandement impliqué dans les réactions redox de substances endogènes.[232] De ce fait, la mesure plasmatique du métabolite CYP3A dépendant du cholestérol, le 4β-hydroxycholestérol, a été identifiée comme biomarqueur endogène potentiel de l'activité métabolique du CYP3A.[140] Contrairement aux

médicaments substrats-marqueurs, l'utilisation d'un biomarqueur endogène de l'activité du CYP3A ne nécessiterait pas d'administrer un composé exogène et ne nécessiterait qu'un seul prélèvement, ce qui faciliterait son application dans un contexte de pratique clinique. *In vivo*, la valeur du 4β-hydroxycholestérol plasmatique comme biomarqueur endogène de l'activité du CYP3A a été rapporté pour des études de métabolismes en présence d'inducteurs et d'inhibiteurs.[140] En revanche, les études évaluant son potentiel d'utilisation dans différentes sous-populations pour mesurer les niveaux basaux d'activité du CYP3A4 sont plutôt rares.

Hypothèse : La concentration plasmatique du 4β-hydroxycholestérol, suggérée dans la littérature comme biomarqueur de l'activité du CYP3A, pourrait être utilisée en clinique à titre d'indice phénotypique endogène du CYP3A.

Objectifs :

- L'objectif de cette étude était de valider l'utilisation des concentrations plasmatiques de 4β-hydroxycholestérol, ainsi que son ratio molaire sur le cholestérol total, comme biomarqueur endogène de l'activité métabolique du CYP3A dans 1) une population non diabétique, puis 2) chez des patients avec le DT2. Le midazolam a été utilisé à titre de référence pour valider le 4β-hydroxycholestérol comme biomarqueur endogène de l'activité basale du CYP3A dans notre population non diabétique et diabétique.

2.2.2. **Manuscrit 3:** « Use of 4β-hydroxycholesterol plasma concentrations as an endogenous biomarker of CYP3A activity: Clinical validation in individuals with Type 2 diabetes. »

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CONFLICT OF INTEREST

None.

ABSTRACT

The relevance of endogenous 4β-hydroxycholesterol (4β-OHC) plasma concentrations or of the 4β-OHC/total cholesterol concentration ratio (4β-OHC ratio) as surrogate markers of CYP3A activity was evaluated in individuals with (n=38) or without (n=35) type 2 diabetes (T2D)). Midazolam was used as a comparator to validate exploratory measures of phenotypic CYP3A activity. Metabolic ratios (MR) of orally administered midazolam in the non-diabetic and diabetic populations correlated significantly with 4β-OHC (r_s=0.64 and 0.48: p≤0.003) and 4β-OHC ratio (r_s=0.69 and 0.46; p≤0.003), respectively. Activity of CYP3A was lower in the T2D population compared to non-diabetic subjects: this decrease was reflected in 4β-OHC concentrations (24.33 vs 12.58 ng/ml; p<0.0001) and 4β-OHC ratio (0.13 vs 0.09 (x10^4); p<0.0002). These results suggest that 4β-OHC should be considered as a valid, convenient and easy to use endogenous biomarker of CYP3A activity in patients.

INTRODUCTION

Cytochrome P450 3As (CYP3As) represent ~25% and 80% of all CYP450s expressed in human liver and intestines, respectively.[1-5] CYP3As play a critical role in the metabolism of about half of all marketed drugs.[6] Consequently, CYP3As are implicated in numerous drug-drug interactions (DDI) for a variety of therapeutic agents including several HMG-CoA reductase inhibitors, calcium channel blockers, serotonin reuptake inhibitors, macrolide antibiotics, antifungals and protease inhibitors.[7] In recent years, drugs such as terfenadine, mibefradil, astemizole, cisapride, alosetron, cerivastatin or nefazodone have been removed from the market due to toxicities observed in the context of CYP3A-mediated DDI.[8,9] Moreover, a large inter-individual variability, up to 30- to 40-fold, in CYP3A-mediated drug metabolism rates has been observed in human. This variation in CYP3A activities appears to be due mostly to various nongenetic factors and in part to genetic factors.[10,11]

Several probe drug substrates are used to assess the implication of genetic, ethnic and environmental factors on drug metabolism variability for various CYP450 isoforms.[12] Marker substrates are selected to provide information on each CYP450 isoform activity *in vivo* (phenotype) under conditions of enzyme induction, inhibition or drug-drug interactions. The probe substrate midazolam has been widely used and validated as the gold standard for phenotyping CYP3A activity *in vivo*. Its selectivity allows for its use alone as well as in a combination with other probe drugs (cocktail) to determine simultaneously several CYP450 phenotypes.[13-18]

In addition to their role in the metabolism of drugs, CYP450s are also involved in redox reactions of endogenous substances as they participate in 94% of important homeostatic

processes.[19] As for drug metabolism, CYP3As could be involved in 18% of those reactions.[19] This observation supports the concept that endogenous biomarkers of CYP3A activity could be identified. If so, such endogenous biomarkers would represent some advantages as they may not require the administration of a xenobiotic and could be measured, under various conditions, with a single rather than multiple blood draws. This could make such endogenous marker more efficient and more amenable to clinical practice for the determination of CYP450 activities or phenotypes. Furthermore, administration of exogenous xenobiotics may be more difficult in infants and patients taking multiple drugs as well as be associated with anxiety and adverse drug events (allergic reactions, side-effects).

Urinary 6-hydroxycortisol to cortisol ratio and plasma 4β-hydroxycholesterol to cholesterol ratio (4β-OHC ratio) have been proposed as markers of CYP3A activities.[20,21] *In vivo* determination of 4β-OHC concentrations in the presence of CYP3A4 inducers, inhibitors as well as *in vitro* characterization of cholesterol metabolism using recombinant CYP450 isozymes confirmed that 4β-OHC was the product of the CYP3A subfamily. This metabolic pathway could be mediated to an extent of more than 90% by the isoform CYP3A4.[22,23] However, only few studies have measured 4β-OHC in different populations (such as HIV, patients with epilepsy or with gallstone) as an indicator of basal CYP3A4 activity before the administration of an inducer or an inhibitor.[21] Therefore, the objectives of this study were 1) to validate the use of both plasma 4β-OHC concentrations and 4β-OHC ratio (the molar ratio of 4β-OHC to total cholesterol plasma concentrations) as an endogenous biomarker of CYP3A activity in healthy subjects; 2) to evaluate its reliability as a marker of CYP3A activity in a cohort of patients with type 2 diabetes (T2D). Midazolam was administered and used as a reference to validate 4β-

OHC concentrations and ratio as biomarkers of basal CYP3A activity in our control and T2D populations.

RESULTS

Data obtained from 35 non-diabetic participants and 38 patients with T2D were considered in the validation process of 4β-OHC as an endogenous biomarker of CYP3A activity (characteristics of individuals are presented in Table 1). Midazolam-based metrics were also derived in this population. For all subjects, plasma concentrations of 4β-OHC and of total cholesterol were determined using a validated LC-MS/MS assay. Two analyses were required due to the large range of concentrations between 4β-OHC and cholesterol. Values for 4β-OHC and its normalized ratio to total cholesterol concentrations (4β-OHC ratio) varied from 7.5 to 49.0 (24 ± 11 ng/ml; mean ±SD) and from 0.05 to 0.32 (0.13 ± 0.06 (x10^4); mean ±SD), respectively (Table 2). AUC$_{0-8h}$ for 1-OH-midazolam and midazolam were calculated from the measured plasma concentrations using a specific LC-MS/MS assay: midazolam metabolic ratio (MR) are reported and used as a reference of CYP3A activity. The resulting MRs varied between 0.24 and 1.24 with a mean value of 0.57 (Table 2). The high inter-individual variability observed for the 4β-OHC-based metrics were also observed for the midazolam-based metric.

The frequency distribution of midazolam MR, 4β-OHC concentrations and 4β-OHC ratios for the non-diabetic population showed similar patterns as illustrated in Figure 1a. Most subjects (10/16) found below the 25th percentile or above the 75th percentile were the same for all three markers.

As depicted in Figure 1b, frequency distributions of 4β-OHC, 4β-OHC ratios and midazolam MR, follow the same trend and are shifted to the left compared to the non-diabetic

population. Indeed, mean values for the three markers of CYP3A activity are similarly reduced by 1.4 to 2-fold when compared to the non-diabetic population (Table 2).

When calculations were performed for the entire population, *i.e.* the 35 non-diabetic subjects along with the results from the 38 diabetic patients, the correlation between the midazolam MR and the cholesterol-based markers of CYP3A activity were significant with spearman's rank correlation coefficients of 0.69 and 0.67 ($p < 0.0001$) for 4β-OHC and 4β-OHC ratio, respectively (Figure 2). These values were $r_s = 0.48$ and 0.46 ($p = 0.003$) for T2D individuals and $r_s = 0.64$ and 0.69 ($p < 0.0001$) in non-T2D individuals (Figure 2).

Numerous factors may affect the activity of CYP3A and valid markers should show sensitivity to these factors. For instance, genetic variants have been shown to modulate CYP3A activities. As presented in Table 3, relationships of tested CYP3A phenotypic parameters and common genetic variants known to impact CYP3A activity were considered. The tested endogenous biomarkers 4β-OHC and 4β-OHC ratio were not affected by the CYP3A5 genotype ($p = 0.1$ and $p = 0.09$) although a trend was observed. In contrast, a statistically significant relation was observed for midazolam MR ($p = 0.03$). No difference between carriers and non-carriers of the *CYP3A4*22* variant was observed for all phenotypic markers. This is mostly due to the limited number of subjects studied. In addition to the midazolam MR, multiple midazolam-based parameters were derived and tested, and analyses based on oral clearance of midazolam are presented in Table 4.

DISCUSSION

This study, using a sensitive LC-MS/MS method for the determination of 4β-OHC, demonstrated that this endogenous compound could be used as a potential biomarker of basal CYP3A activity. Concentrations of 4β-OHC alone or normalized for total cholesterol concentrations (4β-OHC ratio) demonstrated a high correlation with midazolam MR, a gold standard for the determination of phenotypic CYP3A activities. Finally, we demonstrated that 4β-OHC concentrations were a valid marker of CYP3A activities in individuals without T2D and in patients with T2D, although CYP3A activity was decreased and showed a tighter distribution in this population.

The phenotypic potential of an endogenous cholesterol-based metric was evaluated for plasma 4β-OHC concentrations with or without normalizing by cholesterol levels (4β-OHC ratio). In principle, normalized 4β-OHC concentrations could be more precise in a population where levels of cholesterol are highly variable or in a population with individuals taking cholesterol-lowering drugs.[24] Indeed, in individuals without T2D, normalized 4β-OHC concentrations were more closely correlated to midazolam clearance.[25] But in contrast, numerous studies have shown that the variation in cholesterol levels affected only slightly (<10%) the concentrations of 4β-OHC or showed that the 4β-OHC concentrations performed as efficiently as the 4β-OHC/total cholesterol ratio to reflect CYP3A activity.[26-29] Spearman's rank correlation coefficient between 4β-OHC and 4β-OHC ratio in our global population was r_s = 0.93. Our results demonstrate that the measured correlation using only 4β-OHC concentrations rather than the 4β-OHC ratio was a powerful biomarker of CYP3A activity ($p \leq$ 0.003) for both study groups or in our entire cohort. This represents a practical advantage as

only 4β-OHC would have to be quantified from plasma samples reducing cost and analytical challenges due to a large gap in relative abundance of cholesterol (mg/ml) compared to 4β-OHC (ng/ml).

In our population, cholesterol levels varied from 0.85 to 2.68 mg/ml and 49% of our study population was on statins and/or ezetimibe. All drugs were stopped the day before the administration of midazolam to avoid direct competitive inhibition. This strategy, however, had a limited impact on 4β-OHC and cholesterol levels in patients taking cholesterol lowering drugs. Nevertheless, there was no difference observed in the relationship between 4β-OHC metrics and midazolam MR, either in non-diabetic or T2D patients, whether they were or not taking statins.

Several articles reporting the value of 4β-OHC as an endogenous biomarker only present the extent of changes in its concentrations after exposure to known CYP3A inducers or inhibitors. Although relevant in the context of DDI studies, these reports do not demonstrate the ability of 4β-OHC to reflect intrinsic CYP3A activity. To validate 4β-OHC as an endogenous biomarker of CYP3A basal activity, its concentrations should correlate with known and validated phenotypic substrate markers such as midazolam. Several studies do not include data with another phenotypic marker as reference and only few reports are available in untreated non-diseased populations.[25,30,31] Our study includes both a reference phenotypic marker, midazolam, and a control population. Using the MR of orally administered midazolam and both cholesterol-based metrics, 4β-OHC and 4β-OHC ratio, we showed that they correlated significantly and strongly with spearman rank correlation coefficient of 0.64 and 0.69, respectively.

Our non-diabetic study group included demographically divergent and sometimes poly-medicamented participants. Notwithstanding these conditions, our analyses were associated with correlations much better than those observed previously.[25,30,31] Shin *et al.* (2013) administered midazolam intravenously and determined midazolam clearance and 4β-OHC concentrations in 24 healthy males ($r^2 = 0.381$, $p < 0.001$).[31] Bjorkhem-Bergman *et al.* (2013) studied the correlation between orally administered midazolam and 4β-OHC concentrations in a population of 24 healthy Caucasian males and females.[30] Although their sampling strategy and sensitivity of their analytical method did not allow for 1-OH-midazolam quantification and required determination of extrapolated AUC for midazolam, they demonstrated a significant correlation between the oral clearance of midazolam (over 10 hours) and 4β-OHC ratio ($r^2 = 0.29$, $p<0.01$).[30] Using a limited sampling strategy, Tomalik *et al.* (2009) conducted four separate studies and determined the correlation between 4β-OHC and both, orally and intravenously administered midazolam to healthy Caucasian males.[25] Correlation was significant only in one of their studies (D) with 16 subjects and when pooling all volunteers from the 4 studies (n= 50).[25] These divergent results between their separate sub-studies may be due to their respective limited sample size (n≤16) which may not be appropriate to evaluate activity of an enzyme known to be highly variable among different populations. In another study, Woolsey *et al.* (2016) found no correlation between oral midazolam and 4β-OHC in healthy subjects.[32] However, they used a single time-point sampling strategy for determination of midazolam as their reference metrics which can obviously be associated with some limitations.

Multiple studies have evaluated cholesterol-based endogenous biomarkers of CYP3A activity in the presence of inducers or inhibitors in different patient cohorts.[21] However, very few evaluated the ability of 4β-OHC to characterize intrinsic CYP3A activities in patients with different diseases using a validated comparator. It has been shown that CYP450 activities are modulated by certain pathological conditions, especially when inflammation status is increased.[33-35] To our knowledge, we are the first to report on the evaluation of 4β-OHC as an endogenous biomarker of CYP3A activity in a group of patients with T2D. The need for a fast and non-invasive technique to evaluate CYP3A activity in patients is of great interest, particularly diabetic patients who are often prone to polypharmacy and tend to show highly variable responses to different drugs clinically.[36-39] CYP3A activity was decreased in patients with T2D (midazolam MR lower in T2D patients: 0.36 vs 0.57; $p<0.0001$). This decrease in CYP3A activity was also unmasked by the 4β-OHC and 4β-OHC ratio metrics ($p \leq 0.0002$). This suggests that the endogenous biomarker 4β-OHC could be used in a T2D population to evaluate CYP3A activity and phenotype.

We have given great thoughts and considered *i.v.* administration of midazolam while designing this study. One could consider that an *i.v.* administration would mimic better conditions associated with the production of an endogenous substrate, especially in the case of CYP3A probe substrates as this isoform is expressed in the intestine and the liver. However, we have concluded that an oral administration was a preferred choice for several reasons:

1. First and foremost, the overall objective of this study was to demonstrate the value of 4β-OH-cholesterol as a marker of CYP3A activities in order to predict the dose of CYP3A substrates to be administered in patients. These substrates (calcium channel

blockers. HMG COA reductase inhibitors, benzodiazepines, etc …) are administered orally in ambulatory patients. Thus, the endogenous marker selected should be compared to a probe marker (midazolam) administered orally, not *i.v.*

2. A significant amount of circulating cholesterol is coming from our diet (30-40%). Hence, the amount of circulating 4β-OH-cholesterol could be influenced not only by liver CYP3A activities but also by intestinal CYP3As.

3. We have indicated that the aim of the study was to identify a non-invasive biomarker of CYP3A activities. While a single blood draw could be considered an invasive procedure, it could be argued that *i.v.* administration of a probe drug would appear even more invasive for most people.

4. Finally, as clearly mentioned, this study is a sub-study of a clinical trial assessing Cytochrome P450 activities in Type 2 Diabetes patients following the administration of a cocktail of probe drugs. Midazolam was included in this validated cocktail and administered by the oral route.

The influence of CYP3A5 genetic variants on midazolam and 4β-OHC have been widely considered in previous studies and data on the impact of *CYP3A5*1* allele on midazolam pharmacokinetic and 4β-OHC are variable.[25,27,32,40,41] We detected a significant impact of the CYP3A5 genotype on midazolam MR with CYP3A5 expressers exhibiting increased midazolam MR. However, this increase was not significant for both cholesterol-based metrics (although the same tendency was observed). This lack of significance for the cholesterol-based metrics could also reflects that 4β-OHC is mostly a product of CYP3A4, making it less dependent on CYP3A5 genotype than midazolam.[22,23] Woolsey *et al.* and Tomalik *et al.* also did not observed a significant difference between the non-expressers and expressers of

CYP3A5.[25,32] A possible cause of the lack of significance observed in our study, as for Woolsey *et al.* (2016) and Tomalik *et al.* (2009), is the limited sample size of carriers of the *CYP3A5*1* allele and ethnicity of participants. Indeed, allele frequency of *CYP3A5*1* is more important among populations other than Caucasians, like in African decent individuals. In our study population, although 11 individuals were expressers of CYP3A5, no participant carried two active alleles of CYP3A5. A more extensive study across different ethnic groups and larger number of participants carried out by Diczfalusy *et al.*, concluded that CYP3A5 functional alleles had a significant impact on 4β-OHC.[27] Other studies also agreed and reported a significant impact of CYP3A5 expressers on studied parameters.[40,41]

In this study, we also evaluated the influence of the newly identified *CYP3A4*22* reduced function allele. Few studies are available on the impact of this SNP on cholesterol-based metrics. Although limited by a small number of individual carrying the *22* allele (as for Woolsey *et al.*), we did not observe an impact of *CYP3A4*22* on cholesterol and midazolam metrics.[32] A significant impact of *CYP3A4*22* on weight-corrected cholesterol-based metric was reported in a multivariate analysis carried in 147 renal transplant recipients receiving tacrolimus.[42] Our results are in agreement with a more extensive study (n=655) recently published by Hole *et al.* who reported no significant difference between levels of 4β-OHC in individuals expressing the *CYP3A4*22* SNP and those wild-type for CYP3A4.[59] Due to its relatively low allele frequency (5-7% in Caucasians), it would be important and interesting to carry larger sample size studies in order to evaluate the impact of *CYP3A4*22* on cholesterol-based metrics.[43]

Urinary 6-hydroxycortisol to cortisol is another endogenous marker that has been proposed to assess CYP3A activity. Although urinary 6-hydroxycortisol to cortisol would be non-invasive, a urine collection of at least 24h seems optimal.[44] While 6-hydroxycortisol to cortisol presented a rapid response to change in CYP3A activity, its high intra-individual variability due in part to diurnal variation constitutes a considerable limitation.[30]

In brief, our results support the hypothesis that 4β-OHC is a suitable endogenous biomarker to evaluate *in vivo* CYP3A intrinsic activity. Our study demonstrates its usability in a population of patients affected by T2D and addresses important gaps on 4β-OHC knowledge. Further investigations are required to establish the appropriate threshold for phenotypic designation. Such determination will allow the use of biomarker levels to adjust dose for CYP3A drugs.

METHODS

Subjects.   Study protocol (Trial #14.066) was approved by the ethic review board of the CHUM research centre (Montreal, Canada) and registered at the US National Institutes of Health website (http://www.clinicaltrial.gov; NCT02291666). This study was carried out in compliance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines. Written informed consent was obtained from all participants prior to any initiation of study procedure. Evaluation of 4β-OHC as an endogenous biomarker of CYP3A activity was an exploratory endpoint of a case-control pharmacokinetic study characterizing the effects of T2D on major CYP450 activities.[13] A total of 73 participants were recruited to constitute 2 study groups; a group of 35 subjects classified as non-diabetic volunteers according to their medical histories, physical examinations and pertinent laboratory tests and a second

group of 38 patients diagnosed with T2D. Detailed eligibility criteria and protocol have been described and published previously.[13]

Determination of midazolam metabolism. To determine systemic CYP3A metabolic activity, relevant pharmacokinetic parameters of the validated probe drug midazolam were derived. On dosing day, injectable midazolam (Midazolam injection®, USP, 2 mg/2 mL, Fresenius Kabi) was administered orally as part of a CYP450 probe drug cocktail as described in Gravel *et al.*.[13] Briefly, injectable midazolam formulation was added in 10 mL of syrup containing dextromethorphan. Participants were admitted to the clinical research unit of the CRCHUM, serial blood samples were drawn before and at 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8 and 24 h following the administration of 2mg midazolam. Subjects were allowed to leave the clinical research unit between the interval of 8-24 hours. For all subjects, plasma concentrations of midazolam and its metabolite 1-hydroxymidazolam were determined by liquid chromatography tandem mass spectrometry as described previously.[45]

Midazolam metabolic ratio was calculated using the plasma concentrations over 8h of 1-hydroxymidazolam and midazolam (AUC_{0-8h} 1-OH-midazolam /AUC_{0-8h} midazolam). Oral clearance was calculated using the $AUC_{0-infinity}$ of midazolam plasma concentrations (Dose/AUC_{0-inf}). Drug concentration-time data were analysed by standard non-compartmental methods using Kinetica® 5.1 (Thermo Kinetica, Thermo Fisher Scientific, MA, USA) and the AUCs were obtained by use of the mixed log-linear trapezoidal rule.

Determination of cholesterol and 4β-OHC. To measure concentrations of cholesterol and its metabolite 4β-OHC, a plasma sample was obtained, on dosing morning, prior to dosing with the cocktail of probe drugs containing midazolam. Blood samples were drawn in 6 mL K_2-

EDTA vacutainers via an indwelling venous catheter and plasma was retrieved immediately by centrifuging samples for 10 minutes at 4°C and 1,500g. Plasma samples were then aliquoted and stored at -80°C until use.

The LC-MS/MS quantification of cholesterol and 4β-OHC in plasma samples was carried out using calibration standards purchased from Avanti Polar Lipids (Alabastar, AL) and Toronto Research Chemical (Ontario, Canada), respectively. Standards were prepared in ethanol-PBS as surrogate matrix. Due to the difference in expected plasma concentrations of cholesterol (mg/ml) and 4β-OHC (ng/ml), two different sample preparations were implemented. For the analysis of cholesterol, the plasma samples were diluted with PBS prior extraction. For 4β-OHC, plasma was undiluted. Internal standard working solution (ISWS) was added to aliquoted samples; $^{2}H_7$-cholesterol (Toronto Research Chemical, Toronto, Canada) for the analysis of cholesterol and $^{2}H_7$-4β-OHC (Toronto Research Chemical, Toronto, Canada) for the analysis of 4β-OHC. Then, 1M potassium hydroxide in 95:5 methanol:water solution was used for alkaline hydrolysis to obtain the free hydroxycholesterol from the long fatty acid esters. Afterwards, n-hexane was added to the sample and gently mixed by rotation for 20 minutes, then centrifuged and the organic layer was transferred into a clean borosilicate tube and evaporated to dryness at 30°C under a gentle stream of nitrogen. The dried extract was resuspended with mobile phase consisting of 75:25 acetonitrile and 10 mM ammonium formate pH 3. Chromatographic separation was achieved using isocratic elution with a Phenomenex Luna C8 analytical column (150 x 2.0 mm I.D., 5 μm) operating at 50°C. The mobile phase consisted of acetonitrile and 10 mM ammonium formate pH3 at ratio 75:25, respectively. The flow rate was fixed at 500 μl/min. 4β-hydroxycholesterol and cholesterol eluted at 7.0 and 12.3 minutes, respectively. Two microliters for the analysis of cholesterol and five microliters for the

analysis of 4β-hydroxycholesterol of the extracted sample was injected the total run time was set at 14 min. To obtain reliable results, it was necessary to ensure chromatographic separation of 4β-OHC with other isomeric oxysterols, including 4α-OHC (Figure S1). The mass spectrometer, Thermo Scientific TSQ Quantiva triple quadrupole was interfaced with the Thermo Scientific Dionex 3000 UHPLC system using an atmospheric chemical ionization ion source (APCI). MS detection was performed in positive ion mode, using selected reaction monitoring (MRM). The precursor-ion reactions selected for cholesterol, ^{2}H$_7$-cholesterol, 4β-OHC and ^{2}H$_7$-4β-OHC were set to 369.4 → [147.2+161.2], 376.4 → 147.2, 385.4 → [97.1+109.1] and 392.4 → 109.1, respectively. A linear regression (weighted 1/concentration) produced the best fit for the concentration-detector relationship for cholesterol and 4β-OHC. The concentrations were determined based on peak area ratio of the analyte to its internal standard. The calculated coefficients of determination (r^2) were ≥ 0.9974 for an analytical range of 0.025 to 2.0 mg/ml for cholesterol and ≥ 0.9992 for an analytical range of 2.00 to 300 ng/ml for 4β-OHC. Intra- and inter-day relative standard deviations for cholesterol were less than 3.6% and 3.5%, respectively and the accuracy ranged 94.0 to 107.7%. For 4β-OHC, the intra- and inter-day were less than 6.6% and 9.8%, respectively and the accuracy ranged from 88.6 to 102.4%. These precisions and accuracies are within generally accepted criteria.[46]

Since calibration standards and quality control samples are prepared in a surrogate matrix (ethanol-PBS), the matrix effect was evaluated by plotting the concentration-dependent responses recorded from the analytes (cholesterol and 4β-OHC) in a biological matrix to those recorded from the analytes in the surrogate matrix solution. A regression line was constructed.

Peak area ratios were plotted; those obtained from the extraction of biological matrix on the y-axis, and those obtained from the analysis of pure solution on the x-axis. Deviation of the slope from unity is indicative of absolute matrix effect. Moreover, the relative matrix effect, which is the comparison of matrix effect values between different lots of biofluids, was evaluated in six different lots. The variability of standard line slopes in different lots of biofluids expressed as coefficient of variation, CV (%) serves as a good indicator of relative matrix effect. The precision value should not exceed 4% for the method to be considered reliable and free from relative matrix effect.[47] Results are presented in Table 5. Although relatively stable, 4β-OHC and its isomer 4α-OHC can be formed by auto-oxidation. The presence of 4α-OHC and its relative abundance to the 4β-OHC peak was monitored throughout the study to account for sampling, handling and storing quality.[48,49]

CYP450 genotype analyses. Blood samples for genotyping were obtained during the screening visit and DNA was extracted from leukocytes according to standard procedures within 7 days using the GenElute™ Blood Genomic DNA kit (Sigma Aldrich, Oakville, Canada). Purified genomic DNAs were stored at -20°C until genotype analyses were performed. Variants were detected using the Taqman® qRT-PCR SNP Genotyping Assay (Life Technologies, Burlington, Canada). The PCR assay was performed using the QuantStudio™ 6 Flex System (Life Technologies, Burlington, Canada). The SNP Genotyping assay was completed using specific probes for all SNPs (Life Technologies, Burlington, Canada). The tested SNP for the isoform CYP3A4 was *CYP3A4*22* (rs35599367) with specific probe C_59013445_10. For CYP3A5, presence of two SNPs was tested; *CYP3A5*3* (rs776746) and *CYP3A5*6*

(rs10264272). The specific probes used were C_26201809_30 and C_30203950_10, respectively.

Data analysis. Validity of 4β-OHC as an endogenous biomarker of CYP3A activity was determined by calculating correlations between its concentrations, as well as its normalised to total cholesterol concentrations, and midazolam metabolic ratio (MR). To this end, Spearman's rank correlation coefficients were considered using a two-sided p-value ($\alpha = 0.05$). Influence of CYP3A4 and CYP3A5 genotypes on midazolam and cholesterol metrics was evaluated using Mann Whitney non-parametric t-test. All correlation, linear regression, non-parametric and descriptive statistics analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

STUDY HIGHLIGHTS

The responses to all four questions should be limited to 150 words or less in total.

1. What is the current knowledge on the topic?

    The 4β-OHC endogenous biomarker of CYP3A activity has been evaluated in different settings for its use in drug-drug interaction studies. Only few studies reported results using a reference exogenous probe or from populations with various diseases. Extensive pharmacokinetic data for a validated probe substrate should be evaluated to gain knowledge on 4β-OHC usability to determine basal CYP3A phenotype in the presence of pathological conditions.

2. What question did this study address?

    Are endogenous cholesterol-based phenotypic metrics suitable markers of CYP3A activity in patients with chronic inflammatory diseases such as type 2 diabetes.

3. What does this study add to our knowledge?

    Scope of application for this endogenous biomarker using results from a robust pharmacokinetic study of midazolam in both, non-diabetic and diabetic patients.

4. How might this change clinical pharmacology or translational science?

    A better evaluation of the CYP3A phenotype using a single blood sample could contribute to improve drug utilization and reduce adverse drug reactions, especially in

polymedicated sub-population prone to high variability in drug response such as diabetic patients.

AUTHOR CONTRIBUTIONS

S.G., J.T. and V.M. wrote the manuscript; V.M. designed the research; S.G., JL.C., S.D. and F.B. performed the research; S.G., V.M. and J.T. analysed the data; F.B. developed genotyping methods; F.G. developed and performed LC-MS/MS analytical methods.

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Table 1. Demographic and clinical characteristics

Parameters	Non-T2D	T2D
No. (%) of subjects	35 (48)	38 (52)
Sex: No. (%) M:F	15:20 (43:57)	30:8 (79:21)*
Age (years)	46 ± 16	66 ± 10*
BMI (kg/m^2)	25.7 ± 3.9	‡29.1 ± 6.5*
HbA1C (%)	5.2 ± 0.5	7.1 ± 1.0*
Creatinine clearance (ml/min)	108.8 ± 32.4	92.4 ± 28.3*
Cholesterol (mg/ml)	1.78 ± 0.36	1.40 ± 0.31*
Medication use, No. (%) of subjects		
Metformin	0	33 (87)*
Sulfonylurea	0	18 (47)*
DPP4-I	0	21 (55)*
Insulin	0	17 (45)*
Statins/Ezetimibe	6 (17)	30 (79)*
ACEI	0	14 (37)*
ARB	2 (6)	18 (47)*
CCB	0	15 (39)*
β-Blockers	3 (9)	11 (29)*
Aspirin	2 (6)	18 (47)*
other NSAID	2 (6)	4 (11)
Antidepressants	2 (6)	4 (11)
PPI	1 (3)	10 (26)*

Continuous variables are presented as mean ± SD

* Demographic parameters are significantly different between study groups (p<0.05).

na, not applicable; Non-T2D, non-diabetic patient group; T2D, patients with a diagnostic of Type 2 diabetes group. BMI, body mass index; HbA1C, glycated hemoglobin; DPP4-I, dipeptidyl peptidase-4 inhibitors; ACEI, angiotensin-converting-enzyme inhibitors; ARB, angiotensin II receptor blockers; CCB, calcium channel blockers; NSAID, non-steroidal anti-inflammatory drugs; PPI, proton pump inhibitors.

Table 2. Mean values (±SD) of CYP3A phenotype markers in non-diabetic and diabetic patients.

| Study group | Midazolam-based metrics | Cholesterol-based metrics | |
	MR	4β-OHC (ng/ml)	4β-OHC R (x 10^4)
Non-T2D (n = 35)	0.57 ± 0.23	24.33 ± 11.07	0.13 ± 0.06
T2D (n = 38)	0.36* ± 0.13	12.58* ± 6.13	0.09** ± 0.03
All (n = 73)	0.46 ± 0.21	18.21 ± 10.59	0.11 ± 0.05

MR: plasma metabolic ratio of midazolam calculated as $AUC_{metabolite}/AUC_{substrate}$ over 8 hours; 4β-OHC, 4β-hydroxycholesterol concentration in plasma expressed in ng/ml; 4β-OHC ratio, molar ratio of 4β-hydroxycholesterol to cholesterol in plasma; Non-T2D: study group of healthy participants without type 2 diabetes; T2D: study group of patients with T2D.
*$p<0.0001$ vs non-T2D participants; ** $p=0.0002$ vs non-T2D participants

Table 3. Association of *CYP3A* gene variants with corresponding phenotypic markers in all study population.

Status[a]	CYP3A4*22			CYP3A5		
	Carrier	Noncarrier		Non-Expresser	Expresser	
	n = 4	n = 69	p-value	n = 62	n = 11	p-value
MR	0.35 ± 0.08	0.47 ± 0.03	0.3	0.44 ± 0.03	0.58 ± 0.07	0.03
4β-OHC (ng/ml)	13.40 ± 4.61	18.49 ± 1.28	0.3	17.56 ± 1.35	21.88 ± 3.00	0.1
4β-OHC R (x 10⁴)	0.10 ± 0.03	0.11 ± 0.01	0.6	0.10 ± 0.01	0.13 ± 0.02	0.09

Values presented as mean ±SEM
[a]Non-expressers are *CYP3A5*3* homozygous; Expressers carry at least one *CYP3A5*1* allele
MR, Plasma Metabolic Ratio of midazolam calculated as $AUC_{metabolite}/AUC_{substrate}$ over 8 hours; 4β-OHC, 4β-hydroxycholesterol concentration in plasma expressed in ng/ml; 4β-OHC ratio, molar ratio (x 10^4) of 4β-hydroxycholesterol to cholesterol in plasma. Statistical comparisons were performed using Mann Whitney non-parametric t-test.

Table 4. CYP3A phenotyping using midazolam apparent oral clearance as a probe: A) correlation between midazolam oral clearance and 4β-OHC phenotype biomarkers and B) association with CYP3A4 and CYP3A5 genotypes.

A. Descriptive Statistics and Correlation Analyses

	Midazolam-based metrics		Correlation analysis with Cholesterol-based metrics	
	CLo (L/h)		4β-OHC	4β-OHC R
Study group	Mean	± SD	r_s (p-value)	r_s (p-value)
Non-T2D (n = 35)	94.5	38.6	0.36 (p = 0.03)	0.53 (p = 0.001)
T2D (n = 38)	52.7	26.9	0.42 (p = 0.008)	0.37 (p = 0.02)
All (n = 73)	72.7	38.9	0.59 (p < 0.0001)	0.58 (p < 0.0001)

B. Association of CYP3A4 and CYP3A5 genetic variants with oral midazolam clearance

Status[a]		CLo (L/h)		Mann Whitney non-parametric t-test
		Mean	± SEM	p-value
CYP3A4*22	Carrier (n = 4)	62.6	16.8	0.6
	Noncarrier (n = 69)	73.3	4.7	
CYP3A5	Non-Expresser (n = 62)	71.9	5.1	0.4
	Expresser (n = 11)	77.8	9.8	

CLo, Midazolam oral clearance calculated as Dose/AUC$_{extrapolated}$ where AUC$_{extrapolated}$ = AUC$_{0-24h}$ + AUC$_{24h-\infty}$; 4β-OHC, 4β-hydroxycholesterol concentration in plasma expressed in ng/ml; 4β-OHC ratio, molar ratio (x 10^4) of 4β-hydroxycholesterol to cholesterol in plasma; r_s, spearman's rank correlation coefficient; Non-T2D, study group of healthy participants, study group of patients with T2D.

[a] Non-expressers are *CYP3A5*3* homozygous; Expressers carry at least one *CYP3A5*1* allele

Table 5. Biological matrix effects in quantitative LC-MS/MS analyses of cholesterol and 4β-OHC.

Compound	Cholesterol		4ß-hydroxycholesterol (4ß-OHC)	
Lot	mean slope /donor ±SD (n=6)	RSD (%)	mean slope /donor ±SD (n=6)	RSD (%)
1	0.980 ± 0.006	0.6	1.089 ± 0.021	2.0
2	1.001 ± 0.003	0.3	1.099 ± 0.021	1.9
3	0.990 ± 0.004	0.4	1.025 ± 0.026	2.5
4	1.013 ± 0.009	0.9	1.010 ± 0.042	4.2
5	1.032 ± 0.007	0.7	1.068 ± 0.047	4.4
6	1.040 ± 0.008	0.8	1.060 ± 0.028	2.7
mean ±SD	1.009 ± 0.024		1.059 ± 0.035	
CV (%)	2.3		3.3	

SD, standard deviation; RSD, relative standard deviation; CV, coefficient of variation.

The matrix effect was evaluated by plotting the concentration-dependent responses recorded from the analytes (cholesterol and 4ß-OH) in a biological matrix to those recorded from the analytes in the surrogate matrix solution. A regression line was constructed. Peak area ratios (standard response normalized by internal standard) were plotted; those obtained from the extraction of biological matrix on the *y*-axis, and those obtained from the analysis of pure solution on the *x*-axis. Deviation of the slope from unity is indicative of absolute matrix effect. However, even more important is the evaluation of the relative matrix effect, which is the comparison of matrix effect values between different lots of biofluids. Six different lots were tested. The variability of standard line slopes in different lots of biofluids expressed as coefficient of variation, CV (%) serves as a good indicator of relative matrix effect.[47] The precision value should not exceed 4% for the method to be considered reliable and free from relative matrix effect. Table 5 represents the standard line slopes for each lot, the mean slope line and the precision value obtained for cholesterol and 4ß-OH.
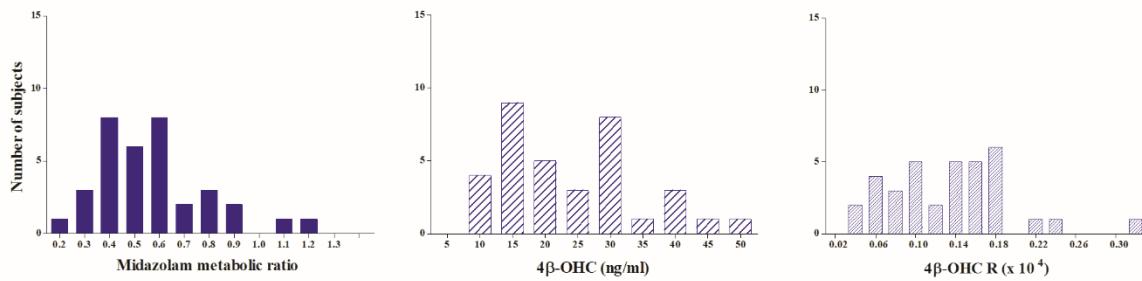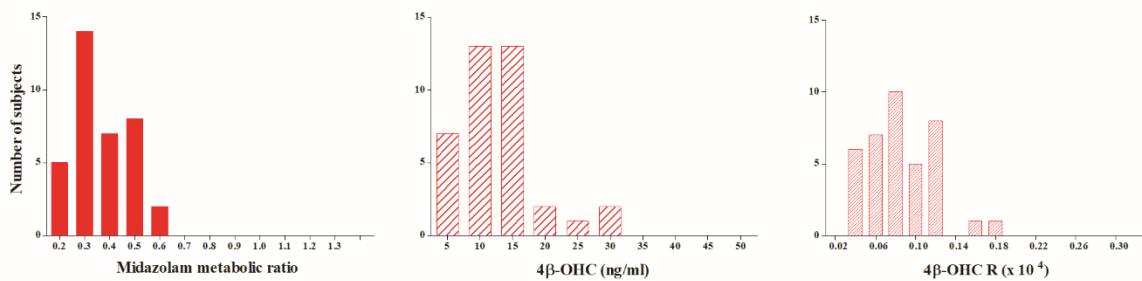
Figure 1. Distribution of phenotypic markers of CYP3A activity using the metabolic ratio of midazolam (1-OH-midazolam/midazolam), plasma concentrations of 4β-OHC and the 4β-OHC ratio normalized by cholesterol levels in (a) non-diabetic control participants and (b) in patients with T2D.
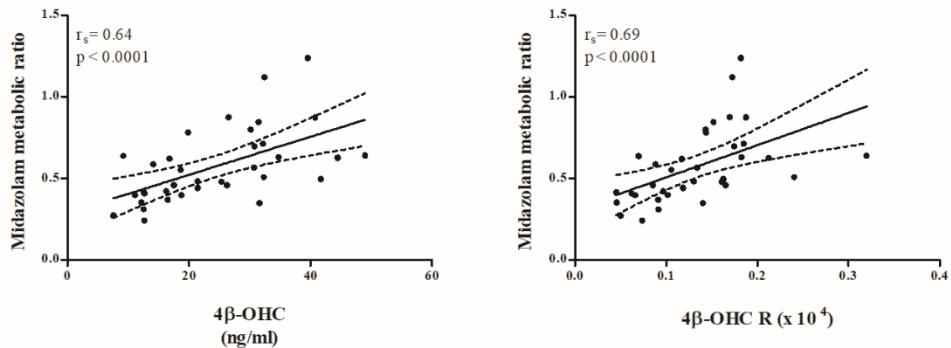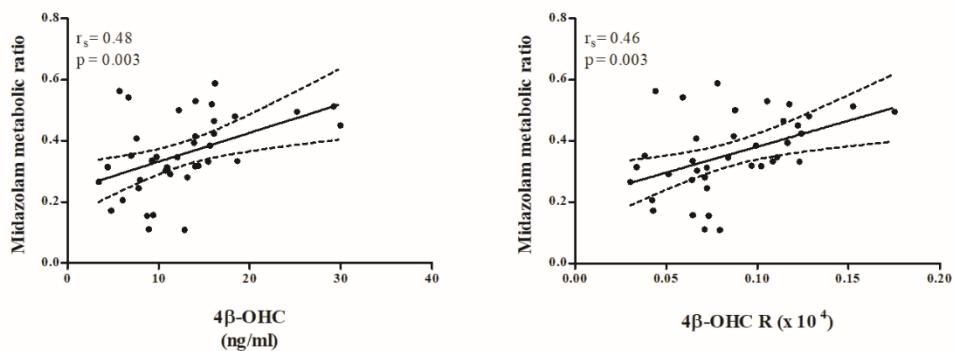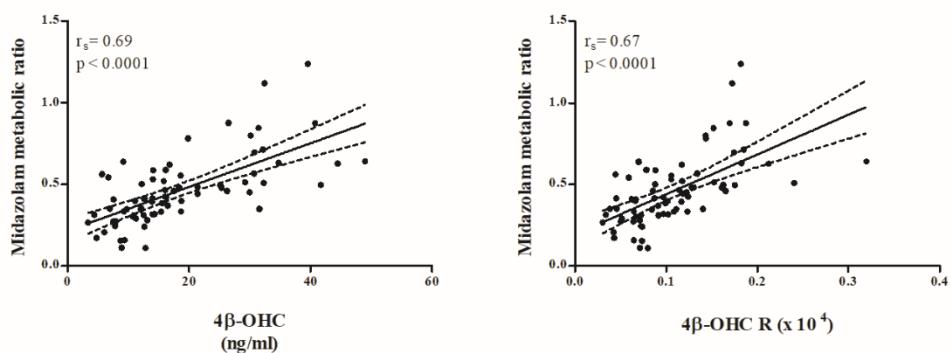
Figure 2. Correlation (r_s) between midazolam metabolic ratio calculated as AUC-metabolite/AUCsubstrate over 8 hours and both cholesterol-based metrics, *i.e.*4β-OHC plasma concentrations and 4β-OHC ratio normalized by the cholesterol concentrations in (a) non-diabetic participants (n=35); (b) patients with T2D (n=38); and (c) all study participants (n=73). Linear regression is significant with correlation coefficient of (a) $r_s = 0.69$ (p<0.0001) and (b) $r_s = 0.67$ (p<0.0001).

SUPPLEMENTARY MATERIALS

Figure S1. Typical extracted ion chromatograms for the 4β-OHC product ion m/z 385.4 →
[97.1 + 109.1] of a blank sample (red line), an extract of human plasma fortified at
the LLOQ (blue line) and a human plasma sample (green line).
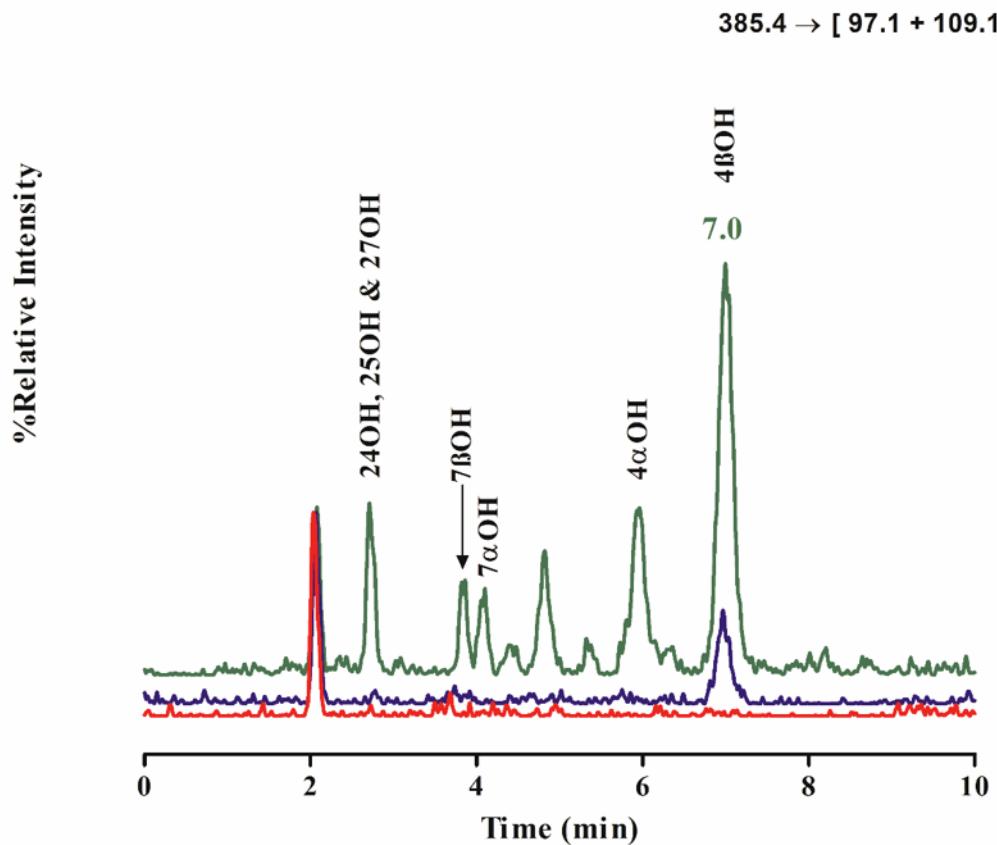
2.2.3. Discussion

Nous avons démontré que le 4β-hydroxycholestérol est un marqueur valide de l'activité du CYP3A dans nos populations non diabétique et diabétique. En effet, les concentrations plasmatiques du 4β-hydroxycholestérol seul ou normalisé pour les concentrations totales du cholestérol corrélaient fortement avec le ratio métabolique du midazolam lequel est la référence la plus utilisée pour déterminer le phénotype d'activité du CYP3A. Ces résultats suggèrent donc que le 4β-hydroxycholestérol pourrait être considéré comme un biomarqueur endogène de l'activité du CYP3A valide, pratique et applicable en clinique chez des patients.

Plusieurs articles ont rapporté l'utilité du 4β-hydroxycholestérol comme biomarqueur endogène en rapportant seulement la variation de ses concentrations plasmatiques après un traitement avec des inhibiteurs ou inducteurs connus du CYP3A. Bien que pertinent dans le contexte d'études d'interactions médicamenteuses, ces études ne démontrent pas l'habileté du 4β-hydroxycholestérol à refléter l'activité intrinsèque du CYP3A. Pour valider le 4β-hydroxycholestérol comme biomarqueur endogène de l'activité basale du CYP3A, ses concentrations devraient corréler avec un substrat-marqueur connu et validé comme le midazolam. Plusieurs études n'incluent pas de données comparatives d'un marqueur phénotypique de référence en plus d'être généralement réalisées auprès de sujets sains.[334-336] Notre étude utilisait un marqueur phénotypique de référence, ainsi qu'une population contrôle et des patients atteints de DT2. Cela nous a permis de montrer des corrélations significatives entre les valeurs phénotypiques du CYP3A obtenues avec le midazolam et les concentrations du 4β-hydroxycholestérol ou son ratio normalisé pour le cholestérol total (r = 0,64 et 0,69, respectivement).

Quelques études ont déjà évalué le biomarqueur endogène de l'activité du CYP3A, le 4β-hydroxycholestérol, dans des populations de sujets sains. Bien que notre population non diabétique fût constituée d'individus avec des caractéristiques démographiques variables, et même des sujets recevant des médicaments, notre étude a montré des corrélations supérieures à d'autres études publiées précédemment entre les niveaux du 4β-hydroxycholestérol ou de son ratio normalisé pour cholestérol et les valeurs phénotypiques du midazolam.[334-336] Shin *et al.* (2013) a déterminé la clairance du midazolam administré par voie intraveineuse, ainsi que les concentrations du 4β-hydroxycholestérol chez 24 hommes sains et leurs résultats démontrent une corrélation de $r^2 = 0,381 (p < 0.001)$.[336] Bjorkhem-Bergman *et al.* (2013) a rapporté une corrélation de 0.29 entre la clairance orale du midazolam sur 10 heures et les concentrations du 4β-hydroxycholestérol normalisées pour cholestérol chez 24 sujets sains d'origine caucasienne (p<0,01).[334] Tomalik *et al.* (2009) a déterminé des corrélations significatives entre les concentrations plasmatiques de 4β-hydroxycholestérol et la clairance du midazolam à la suite d'une administration par voie intraveineuse ou orale ($r_s = 0,24$ et $0,27$, respectivement) lorsque leurs 5 phases d'étude étaient considérées conjointement. Cependant, lorsque leurs cinq cohortes sont considérées séparément, leurs résultats semblent variables.[335] Dans une autre étude réalisée avec des sujets sains (n=51), Woolsey *et al.* (2016) n'a mesuré aucune corrélation significative entre les valeurs pour midazolam et le 4β-hydroxycholestérol.[146] En revanche, Woolsey *et al.* a opté pour une stratégie d'échantillonnage restreinte à un seul temps de prélèvement pour le midazolam afin de déterminer les indices phénotypiques de référence.[146]

Plusieurs études ont évalué le 4β-hydroxycholestérol comme biomarqueur endogène de l'activité du CYP3A en présence d'inhibiteurs ou inducteurs dans des cohortes de patients

présentant diverses pathologies.[140] Cependant, très peu ont évalué l'utilité du 4β-hydroxycholestérol pour mesurer l'activité intrinsèque du CYP3A en présence de maladies à l'aide d'un comparateur valide. Il a été démontré que certaines conditions pathologiques influencent l'activité des CYP450s, particulièrement en présence d'inflammation.[155,236,337] Dans notre population de patients atteints du DT2, nous avons précédemment mesuré une diminution de l'activité du CYP3A en utilisant le midazolam comme substrat-marqueur validé. Il est intéressant de noter que les niveaux du 4β-hydroxycholestérol et de son ratio normalisé pour le cholestérol total ont aussi révélé une corrélation avec les niveaux de marqueurs inflammatoires ($p \leq 0,0002$).

Il a été suggéré que la concentration plasmatique du 4β-hydroxycholestérol normalisée pour le cholestérol total pourrait être davantage pratique pour les populations avec des niveaux variables de cholestérol ou chez les patients prenant des hypolipémiants.[335,338] Cependant, de nombreuses études ont démontré que les niveaux de cholestérol n'influençaient que légèrement (<10%) les concentrations du 4β-hydroxycholestérol et que ses concentrations plasmatiques reflétaient aussi bien l'activité du CYP3A que son ratio normalisé pour le cholestérol total.[339-342] Bien que les niveaux de cholestérol étaient variables dans notre population (0.85 à 2.68 mg/ml) et qu'environ la moitié de nos sujets prenaient des statines ou ézétimibe, les concentrations du 4β-hydroxycholestérol corrélaient aussi bien que le ratio normalisé pour le cholestérol total avec les données du midazolam. Aussi, le coefficient de corrélation de rang de Spearman entre les concentrations plasmatiques du 4β-hydroxycholestérol et les ratios du 4β-hydroxycholestérol normalisés pour le cholestérol total était $r_s = 0,93$. Ce haut coefficient de corrélation dans notre population confirme que les niveaux de cholestérols ne semblent pas

affecter le potentiel phénotypique du 4β-hydroxycholestérol. Ceci est un avantage pratique puisque seul le 4β-hydroxycholestérol aurait besoin d'être quantifié dans les échantillons plasmatiques réduisant ainsi le coût et le défi analytique que représente la grande différence d'abondance relative entre le cholestérol (mg/ml) comparé à 4β-hydroxycholestérol (ng/ml).

Une autre biomarqueur endogène proposé de l'activité du CYP3A est le ratio urinaire du 6-hydroxycortisol/cortisol. Bien que celui-ci soit non-invasif, une collecte urinaire d'au moins 24 h serait recommandé et il est rapporté que le ratio 6-hydroxycortisol/cortisol présente une grande variabilité intra-individuelle dû en partie à des variations diurnes.[334,343] Ces caractéristiques constituent donc une limite importante à son utilisation en clinique et à son évaluation selon notre protocole.

L'utilisation d'un biomarqueur endogène de l'activité du CYP3A présenterait de nombreux avantages en clinique. Notre devis expérimental nous a permis d'évaluer le 4β-OHC dans une cohorte de patients atteints du DT2 en utilisant des donnés d'un substrat-marqueur de référence, le midazolam, et d'un groupe témoin de sujets non diabétiques.

2.3. Manuscrit 4

Manuscrit 4: « A pilot study towards the impact of type 2 diabetes on the expression and activities of drug metabolizing enzymes and transporters in human duodenum. »

- Gravel S, Panzini B, Belanger F, Turgeon J, Michaud V. *Int. J. Mol. Sci.* 2019.

2.3.1. Introduction

Suivant l'administration orale de notre *cocktail* de substrats-marqueurs des CYP450s, nous avons noté une modulation au niveau systémique de l'activité des CYP450s par le DT2 (manuscrit 2).[231,344]. Le foie et l'intestin sont deux organes riches en enzymes du métabolisme et transporteurs de médicaments. Ainsi, en plus du foie, l'intestin joue un rôle considérable dans la variabilité de réponse aux médicaments.[1,2] L'intestin comme organe du métabolisme des médicaments est de plus en plus étudié. La modulation des CYP450s, par certaines pathologies par exemple, peut être tissu spécifique. Notre groupe a d'ailleurs démontré précédemment que le diabète affectait l'expression et l'activité des CYP450s de façon tissu- et isoforme-spécifique dans un modèle de souris DT2.[273] (appendice 2) Bien que quelques études rapportent un effet du diabète sur les CYP450s intestinaux chez l'animal, très peu de données sur l'effet du DT2 sur les CYP450s au niveau intestinal chez l'humain sont disponibles. Ainsi, ce deuxième volet de mon doctorat permet l'étude de l'impact du DT2 localement au niveau de l'intestin chez l'humain, soit plus précisément au niveau du duodénum. En plus de l'expression de 10 isoformes des CYP450s et 3 transporteurs, l'expression de 2 carboxylestérases (CES-1 et CES-2) a été évalué au niveau du duodénum dans cet article. Les carboxylestérases sont des enzymes du métabolisme grandement impliquées dans les réactions d'hydrolyse au niveau du premier passage intestinal, ce qui leur confère un rôle important notamment dans la bioactivation de

médicaments prodrogues.[345] Les carboxylestérases sont présentes au niveau de plusieurs organes et l'isoforme majoritaire au niveau du petit intestin chez l'humain est le CES-2.[345]

Hypothèse : Le DT2 pourrait influencer l'expression et l'activité de diverses enzymes du métabolisme et transporteurs des médicaments au niveau intestinal chez l'humain.

Objectifs :

- L'objectif primaire de cette étude était de déterminer si l'expression et l'activité des CYP450s intestinaux est modulée par le DT2 de manière tissu- et isoforme-dépendant à partir de biopsies duodénales obtenues chez des patients avec le DT2 versus des sujets non diabétiques.

- Comme objectif secondaire, nous avons investigué l'impact du DT2 sur l'expression relative d'ARNm des enzymes de métabolisme carboxylesterases et de transporteurs dans le duodénum chez l'humain. De plus, l'influence de marqueurs inflammatoires, de polymorphismes génétiques des CYP450s et de variables démographiques a été examinée.

2.3.2. **Manuscrit 4:** « A pilot study towards the impact of type 2 diabetes on the expression and activities of drug metabolizing enzymes and transporters in human duodenum. »

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ABSTRACT

To characterize effects of type 2 diabetes (T2D) on mRNA expression levels for 10 Cytochromes P450 (CYP450s), two carboxylesterases, and three drug transporters (ABCB1, ABCG2, SLCO2B1) in human duodenal biopsies. To compare drug metabolizing enzyme activities of four CYP450 isoenzymes in duodenal biopsies from patients with or without T2D. mRNA levels were quantified (RT-qPCR) in human duodenal biopsies obtained from patients with (n = 20) or without (n = 16) T2D undergoing a scheduled gastro-intestinal endoscopy. CYP450 activities were determined following incubation of biopsy homogenates with probe substrates for CYP2B6 (bupropion), CYP2C9 (tolbutamide), CYP2J2 (ebastine), and CYP3A4/5 (midazolam). Covariables related to inflammation, T2D, demographic, and genetics were investigated. T2D had no major effects on mRNA levels of all enzymes and transporters assessed. Formation rates of metabolites (pmoles mg protein-1 min-1) determined by LC-MS/MS for CYP2C9 (0.48 ± 0.26 vs. 0.41 ± 0.12), CYP2J2 (2.16 ± 1.70 vs. 1.69 ± 0.93), and CYP3A (5.25 ± 3.72 vs. 5.02 ± 4.76) were not different between biopsies obtained from individuals with or without T2D ($p > 0.05$). No CYP2B6 specific activity was measured. TNF-α levels were higher in T2D patients but did not correlate with any changes in mRNA expression levels for drug metabolizing enzymes or transporters in the duodenum. T2D did not modulate expression or activity of tested drug metabolizing enzymes and transporters in the human duodenum. Previously reported changes in drug oral clearances in patients with T2D could be due to a tissue-specific disease modulation occurring in the liver and/or in other parts of the intestines.

Key words: Drug metabolism, Cytochromes P450, Intestine, Type II Diabetes Mellitus.

What is already known about this subject:

- A chronic inflammatory status such as T2D could modulate CYP450 activities.

- Altered CYP450 activities have been shown in T2D animal models in an isoform specific and model-dependent manner.

- Very few data are available on the impact of T2D on human CYP450 activities. Data on intestinal CYP450s are practically inexistent.

What this study adds:

- The expression and activity of major CYP450s in human duodenum are not modulated by T2D.

- The previously demonstrated impact of T2D on drug clearance would be due to a tissue specific modulation of CYP450s.

- A better understanding of T2D influence on drug clearance could reduce intersubject variability in drug response.

INTRODUCTION

Type 2 diabetes (T2D) is the most common form of diabetes mellitus worldwide and its global prevalence is increasing yearly [1]. Patients with T2D often require multiple drugs to treat their numerous comorbidities. They also present a highly variable response to drugs: some T2D patients appear resistant to drugs while others are more sensitive (*e.g.* warfarin, clopidogrel, cyclosporine, tacrolimus, antihypertensive agents) [2-7]. The exact mechanism underlying this interindividual variability in drug response is still unknown.

Intersubject variability in drug response is largely explained by drug pharmacokinetics and pharmacodynamics (PK-PD). First pass elimination of orally administered drugs involves both gastro-intestinal and hepatic-mediated metabolism and transport. In addition to the liver, the small intestine could be a major contributor to pre-systemic drug-metabolism and excretion (through efflux transporters).

Cytochrome P450s (CYP450s) constitute a superfamily of enzymes contributing largely to the metabolism of drugs. CYP450s are highly expressed in tissues involved in first pass metabolism such as the intestines and liver. CYP450 expression and activity profiles are tissue specific and obviously, extra-hepatic tissues differ from the liver [8-13]. Indeed, various expression profiles for drug metabolizing enzymes and transporters have been observed along the intestinal tract [14-17]. There is a general consensus that the most highly expressed CYP450s in human intestines are CYP2C and CYP3A subfamilies [18].

CYP450 metabolic activity can be regulated by numerous factors including genetic makeup, environmental factors, concomitant medications and inflammation [19-25]. For instance, inflammatory mediators, such as cytokines, can modulate CYP450-dependent drug metabolism [20, 26-29]. T2D pathophysiology is associated with a chronic active inflammatory status and

patients with T2D also present an important prevalence of metabolic syndrome that is in turn linked to a status of low-chronic inflammation [30-32].

Using a diet-induced obesity model of T2D in mice, our laboratory has demonstrated that T2D can affect CYP450 expression and activities in an isoenzyme and tissue-specific manner [12]. In a clinical case-control pharmacokinetic study, our preliminary results showed that patients with T2D presented a decreased metabolic activity for CYP2B6, CYP2C19 and CYP3As following the oral administration of a cocktail of probe drugs [33, 34]. However, very few reports (if any) are available on the impact of T2D on human intestinal CYP450s. In animal models (mice and rats), expression of cyp3a in the small intestine was shown to be altered by diet-induced or streptozotocin-induced diabetes [35-37].

Our aim was to investigate whether intestinal CYP450 activities are modulated by T2D in an isoform-specific manner by assessing major CYP450 expression and activities in human duodenal biopsies from patients with or without T2D. As a secondary objective, the impact of T2D on other drug metabolizing enzymes and transporters was examined. Additionally, the influence of inflammation, genetic, demographic and T2D-related variables on major CYP450 activities was determined.

METHODS

Subjects. Study protocol (Trial #12.386) was approved by the ethic review board of the CHUM research centre (CRCHUM, Montreal, Canada). This trial was carried out in compliance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines. Written informed consent was obtained from all participants prior to any initiation of study procedure.

A total of 36 participants referred to the CHUM gastroenterology department for a scheduled gastrointestinal endoscopy were recruited to constitute two study groups; a group of 16 subjects classified as non-diabetic volunteers according to their medical histories, physical examinations and relevant laboratory tests and a second group of 20 patients diagnosed with T2D. All patients were required to be ≥18 years old and to abstain from grapefruit juice within two weeks before the digestive endoscopy. Exclusion criteria included use of CYP450 inhibitors or inducers, patients with altered renal or hepatic functions, presence of an important inflammatory condition or a posteriori diagnosis of duodenitis.

Study design and sample collection. On the morning of the gastrointestinal endoscopy, participants were admitted at the CHUM after an overnight fast and restrained from all medication (except for insulin) until procedures were over or as instructed by the gastroenterologist. Prior to procedures, five blood samples were drawn in Vacutainers® tubes (Becton-Dickinson). Two 5 ml blood samples collected in SST II vials were sent to the CHUM biochemistry laboratory for insulin and glucose measurements and three separate blood samples collected in Vacutainers® with EDTA were processed as follows: one tube was sent to the CHUM biochemistry laboratory to measure glycated haemoglobin and the other two blood samples were sent to the research laboratory to determine genotypes of studied CYP450s and to quantify pro-inflammatory cytokine levels (IFN-γ, IL-1β, IL-6 and TNF-α). During the intervention, 8-10 biopsies (about 2-4 mg/specimen) were sampled in the small intestine from the second part of the duodenum downstream to the hepatopancreatic ampulla. Two biopsies were preserved for the conventional histologic analyses, two other biopsies were snap-frozen in liquid nitrogen and preserved at -80°C for mRNA analysis and six biopsies were conserved on ice in sterile normal saline and transferred immediately to the CRCHUM laboratory to serve for

CYP450 activity analyses. Patients were discharged after the sample collection and gastrointestinal endoscopy.

Determination of Intestinal CYP450 activities. Upon reception of the biopsies in sterile normal saline at the laboratory facilities, biopsies were transferred into tubes containing 3 ml of a solution (pH 7.4) composed of TRIS 50 mM; KCl 150 mM; EDTA 1 mM, benzamidine 1 mM, and 50 ug mL^{-1} aprotinin (glycerol 10%) similar to previously described [38-40]. Tissue was then homogenized on ice. The resulting preparation was centrifuged at 4°C and 10,000 g for 20 min and supernatant (S9 fraction) was snap-frozen in liquid nitrogen and kept at -80°C until determination of CYP450 activities.

On study day, the resulting supernatants were thawed on ice and incubated with CYP450 probes: 600 µM bupropion (CYP2B6), 400 µM tolbutamide (CYP2C9), 5 µM ebastine (CYP2J2) and 10 µM midazolam (CYP3A4/5). Specificity of the probe substrates in the intestinal biopsies was evaluated using selective inhibitors: 10 µM ticlopidine (CYP2B6), 10 µM sulfaphenazole (CYP2C9), 150 µM astemizole (CYP2J2) and 5 µM ketoconazole (CYP3A4/5). Following incubation, the formation rate of metabolites was determined by measuring their concentrations using a sensitive and precise LC-MSMS detection method [12, 41]. Activity was normalized for total protein content measured using the Pierce® BCA Protein Assay Kit (ThermoFisher Scientific Inc., USA). Details on chemicals, incubation procedures and detection methods have been reported elsewhere [12].

Quantification of Intestinal CYP450 mRNAs and CYP450 genotype analysis. Standard methods were used and described in the Appendix 1(Method details).

Determination of Proinflammatory cytokine levels. Blood samples for the quantification of proinflammatory cytokines were kept on ice and rapidly sent to the research laboratory to be centrifuged at 1,100 g (10 min at 4°C) within 1 hour of sampling. Plasma was then aliquoted and stored at -80°C until use. Levels of inflammatory markers INF-γ, IL-1β, IL-6 and TNF-α were quantified by electrochemiluminescence immunoassays using the V-PLEX Proinflammatory Panel 1 Human Kit, QuickPlex SQ120 Imager and WORKBENCH software (MSD®, Rockville, MD).

Statistical analysis. To control for sex, age and genotype metabolizer status, we ran linear regression models with diabetes status as a dichotomous predictor of phenotypic activity and mRNA expression levels of studied CYP450s and other drug metabolizing enzymes or transporters using R Statistical Software version 3.5.1 (Foundation for Statistical Computing, Vienna, Austria). GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used to perform spearman rank correlation coefficient analyses with two-sided p-value and $\alpha<0.05$ to test the impact of continuous covariables (insulinemia, glycemia, HbA1c, HoMA-IR, HoMA-β, age, BMI, time since diagnostic and pro-inflammatory cytokine levels) on CYP450 activities and mRNA expression levels. Finally, the impact of discrete covariables (genotype, gender and time since diagnostic categories) on CYP450 activities and expression were evaluated using Mann-Whitney or Kruskall-Willis analyses on GraphPad Prism 5 (GraphPad Software, La Jolla, CA). When relevant, adjustment for false discovery rate using Benjamini-Hochberg multiple correction procedures were applied.

RESULTS

As presented in Table 1, study population was composed of 16 non-diabetic participants and 20 patients with T2D. Individuals with T2D had a slightly higher BMI compared to patients without T2D (p = 0.03). Biomarkers of T2D (including insulin levels, glycemia, HbA1C and HOMA-IR) and drugs used in this condition were all significantly higher in the T2D group, as expected per protocol inclusion criteria. Most of the participants enrolled in our study were Caucasians (n=29), 2 subjects were Blacks and one Asian (data was missing for 4 individuals).

As illustrated in Figure 1A and 1B, the most expressed mRNAs in both study groups for drug metabolizing enzymes were CES-2, CYP2C9 and CYP3A. Figure 1C and 1D show that the most abundant drug transporters in the duodenum for non-T2D and T2D groups was ABCG2, followed by OATP2B1 and ABCB1.

Expression profile for all drug metabolizing enzymes and transporters was similar between the two study groups (Figure 1). As presented in Table 2, T2D had no effect on mRNA levels for all studied drug metabolizing enzymes (CYP450s and CES) (p-value > 0.05), although a tendency was observed with increased CYP2C9 mRNAs (p=0.051) in patients with T2D. When adjusting for age and gender in the multivariate regression model analyses, influence of diabetes on expression levels of studied drug metabolizing enzymes remained not significant (adjusted p-value > 0.05; Table 2). The expression profile for one drug transporter, namely OATP2B1, seemed to differ between individuals with T2D compared to non-diabetic patients independently of age and gender. As shown in Table 2, levels of mRNA transcripts for OATP2B1 were slightly higher, about 20%, in patients with T2D than in the non-diabetic controls (p-value and adjusted p-value = 0.02). However, this could be of marginal clinical relevance. Illustrative graphs of

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mRNA transcripts levels for all drug metabolizing enzymes and transporters in all patients with T2D *vs* non-diabetic controls are displayed in supplementary Figure S1 (Appendix 2).

In this study, sampling of duodenal biopsies enabled the determination of activity levels for four important CYP450 isozymes, namely CYP2B6, CYP2C9, CYP2J2 and CYP3A4/5. Both study groups exhibited activities for CYP2C9 (hydroxytolbutamide), CYP2J2 (hydroxyebastine) and CYP3A4/5 (1'-hydroxymidazolam), but not for CYP2B6 (hydroxybupropion). No significant difference was measured for the formation rate of the various metabolites (mean $\pm$ SD) between individuals without T2D or patients with T2D for CYP2C9 (0.41 ± 0.12 *vs* 0.48 ± 0.26 pmoles mg protein^{-1} min^{-1}), CYP2J2 (1.69 ± 0.93 *vs* 2.16 ± 1.70 pmoles mg protein^{-1} min^{-1}) or CYP3A (5.02 ± 4.76 *vs* 5.25 ± 3.72 pmoles mg protein^{-1} min^{-1}) (p-value > 0.05; Figure 2). When controlling for age, gender and genotype metabolizer status with diabetic status as the dichotomous predictor in a linear regression model, no significant effect on activity levels of all studied isoforms was revealed. Moreover, no significant correlation was observed between relative mRNA levels and metabolic activity for CYP2C9, CYP2J2 and CYP3A4/5 (Appendix 3 Figure S2).

To confirm that the measured metabolic transformation of probe-substrates used was due to the targeted CYP450 pathway, *in vitro* incubations in the presence of specific inhibitors were performed. Activity levels for CYP2J2 and CYP3A in the presence of astemizole and ketoconazole were reduced by 89 ± 5 and 93 ± 5%, respectively. Formation rate of hydroxytolbutamide in the presence of sulfaphenazole was decreased to levels below or near level of quantification (5 nM hydroxytolbutamide). For CYP2B6, the trivial (although detectable) hydroxybupropion formation was not inhibited by the selective inhibitor ticlopidine.

This suggests that the metabolic activity observed is not specific and that CYP2B6 is not a major contributor to bupropion metabolism in the duodenum.

As reported in Table 3, mean ± SD levels of TNF-α cytokine were significantly (p=0.03) higher in blood samples from individuals with T2D (2.71 ± 1.25 pg ml^{-1}) compared to the non-diabetic patients (2.00 ± 0.36 pg ml^{-1}). However, no significant difference between non-diabetic and T2D patients was measured for the other inflammatory markers (IFN-γ, IL-1β and IL-6). We pursued our analysis by trying to measure a correlation between those cytokine levels with measured metabolic activities of CYP2C9, CYP2J2 and CYP3A. Results reported in supplementary Table S1 show no correlation between quantified pro-inflammatory cytokines and measured activities for the various CYP450 isoforms (Appendix 4).

Influence of numerous covariables on CYP2C9, CYP2J2 and CYP3A activities such as T2D-related covariables (insulinemia, glycemia, HbA1c and HOMA-IR) and demographic covariables (age and BMI) was tested. Only one significant correlation was found between HbA1c and hydroxytolbutamide formation rate ($r_s = 0.35$ and $p = 0.04$; Appendix 5 Table S2). Influence of gender on CYP2C9, CYP2J2 and CYP3A activities was considered and no significant effect was observed ($p = 0.8$, 0.3 and 0.4, respectively). Time since diagnostic of T2D could also be a covariable affecting development of comorbidities and inflammation which could in turn affect CYP450 activities. Hence, we divided our diabetic population into three groups (less than 5 years, 5 to 10 years and more than 10 years) and no statistically significant effect of time since diagnostic on metabolic activities was observed (Appendix 6 Table S3).

Lastly, a qualitative evaluation (because of the small number of subjects in some genotyping groups) of the influence of CYP450 genotypes on metabolic activity was performed for the

entire study population. Individuals homozygous for variants *CYP2C9*2* or *CYP2C9*3* presented lower mean values of metabolic activities than their wild type or heterozygotes counterparts. For *CYP2J2*, presence of a **7* variant reduced mean metabolic activity by almost 50% compared to wild type *CYP2J2*1/*1* (1.04 *vs* 2.07 pmoles mg protein^{-1} min^{-1}). Mean metabolite formation rates for individuals carrying one **22* allele for *CYP3A4* were decreased compared to wild type individuals. Finally, formation rate of 1'-hydroxymidazolam was slightly lower in non-expressers *vs* expressers of the *CYP3A5* gene (Appendix 7 Table S4).

DISCUSSION

In this pilot, we show that T2D has no major impact on mRNA expression levels of 10 major intestinal CYP450 isoforms, 2 carboxylesterases and 3 transporters as measured in duodenal biopsies obtained from patients with or without T2D. Furthermore, the metabolic activity of 3 important CYP450s namely, CYP2C9, CYP2J2 and CYP3As were not modulated significantly by T2D.

We measured significant mRNA amounts for multiple CYP450 isoforms with mRNAs of CYP2C9 and CYP3A4/5 being the most expressed. This is in agreement with several studies reporting mRNA levels or immune-quantified proteins of CYP450s in the human small intestine [16-18, 42-45]. High levels of mRNA for CES-2 in duodenal biopsies for both study groups were also detected in this study. It is known that CES are highly expressed in liver and intestines and particularly, CES-2 as the major isozyme in the small intestine [46-48].

Duodenal mRNA expression of all CYP450s and CES was similar between individuals with or without T2D. Very few studies have looked at the impact of T2D on CYP450 expression in human and if so, most studies focused on the liver. One study reported an increased protein

expression of CYP2E1 in human liver microsomes from diabetic patients although mRNA levels were similar to non-diabetic controls [49]. Another study in human investigated the impact of T2D on CYP2E1 [50]. This study reported that CYP2E1 mRNA measured in peripheral blood mononuclear cells was increased in patients with T2D compared to non-diabetic control [50]. In our study, relative mRNA levels CYP2E1 were very low in both study groups (<0.04%) and no difference was observed between the two groups. This result is in agreement with several studies reporting low or undetectable levels of CYP2E1 in the small intestines [18, 51-54]. CYP3A is another CYP450 family for which mRNA transcripts have been quantified in individuals with T2D. Again, although a decreased protein expression and metabolic activity for CYP3A in human liver microsomes from diabetic patients was observed, this change was not reflected in mRNA levels [49]. We also did not observe any effects of T2D on mRNA levels for CYP3A in duodenal biopsies.

Some studies report on the impact of T2D on CYP450 mRNA levels using animal models [12, 35, 55-58]. Available reports in mice models display highly variable results and most observations are on hepatic cyp450s although significant changes in mRNA levels for cyp1a, cyp2a, cyp2b, cyp2c, cyp2e and cyp3a were reported in the liver, kidney and lungs [12, 35, 55-58]. Results are often contradictory and no clear conclusion on the effects of diabetes on cyp450s mRNA levels can be drawn.

The presence of three drug-transporter mRNAs (ABCB1, ABCG2 and OATP2B1) in duodenum biopsies was detected and levels of OATP2B1 were slightly higher in the T2D group. Previous animal studies in mice showed no impact of diabetes on abcg2, Mrp2 and Mrp3 in the liver [56, 58]. Results pertaining to the influence of diabetes on ABCB1 are variable.

Interestingly, some data are available in the intestines, but one group reports a decrease in duodenal expression for a diabetic model in mice while another study shows an increase in intestinal expression using an obese rat model [59, 60].

CYP3A is involved in the metabolism of about 50% of all drugs and is highly expressed in both the liver and intestines [18, 61, 62]. A CYP3A metabolic activity was measured in the S9 fractions (5.15 pmoles of 1'-hydroxymidazolam mg protein^{-1} min^{-1}) prepared from duodenum biopsies of our subjects. Important CYP3A-dependent midazolam metabolism in human intestines has been reported previously in microsomal fractions although the contribution of intestinal CYP3As to midazolam metabolism is less than CYP3A intrinsic clearance in the liver by about 10-fold [63-65]. One study reported mean CYP3A activity of 230 pmoles of 1'-hydroxymidazolam mg protein^{-1} min^{-1} in pooled microsomes obtained from the entire human intestine [65]. Other studies reported significant CYP3A midazolam metabolism in duodenal homogenates from healthy subjects with a mean hydroxymidazolam formation rate of 67 to 446 pmoles mg protein^{-1} min^{-1} [38, 39, 66]. We recently found similar CYP3A metabolic activities in intestinal microsomes from human duodenum with 1-hydroxymidazolam formation ranging from 1-213 pmoles mg protein^{-1} min^{-1} (mean 69 ±75) [17].

In a diabetic mice model, intestinal CYP3A metabolic activity was shown to be increased compared to controls [35]. In human, we previously demonstrated a decreased oral clearance of the CYP3A probe-substrate midazolam in patients with T2D compared to non-diabetic subjects [34]. In this study we observed no effect of T2D on duodenal CYP3A activity *in vitro* (p=0.47). This suggests that the reduced oral clearance of midazolam that we observed previously in T2D patients could be explained by a tissue-specific modulation of CYP3A occurring most probably

in the liver. This hypothesis is supported by other studies demonstrating a decrease in 1'-hydroxymidazolam formation in human liver microsomes from patients with T2D [67]. Another explanation why no difference was found in this study can rely on the patient's pre-medical conditions: *i.e.* the gastro-intestinal endoscopy was medically indicated thus, the pre-medical conditions could have already induced a phenoconversion in their duodenum and the impact of other concomitant pathologies such as diabetes is blunted and could not be revealed.

CYP2C9 is an important isoform of the CYP450s both in the liver and in the small intestines [18, 62]. It is noteworthy that multiple oral antidiabetic drugs such as sulfonylureas, meglitinides and thiazolidinediones are metabolised by CYP2Cs [68, 69]. Significant metabolic activity for CYP2C9 in human intestines has been reported although it was found to be lower than CYP3A activities: our results are in agreement with these observations [5.15 pmoles of 1'-hydroxymidazolam mg protein^{-1} min^{-1} (CYP3A) *vs* 0.45 pmoles of hydroxytolbutamide mg protein^{-1} min^{-1} (CYP2C9)] [44, 65]. Using tolbutamide as a probe-substrate, two studies reported mean activity levels of 3 and 13 pmoles of hydroxytolbutamide mg protein^{-1} min^{-1} [17, 65]. These values are slightly higher than those measured in our biopsy S9 fractions which is a diluted matrix compared to microsomes. Additionally, biopsies were obtained from non-healthy non-diabetic and diabetic patients for whom a gastro-intestinal endoscopy was medically indicated. Our results suggest that T2D had no impact on CYP2C9 activity locally in the human duodenal biopsies (p=0.4). This finding is in agreement with a study that revealed no influence of diabetes on CYP2C activity in rats [70].

CYP2J2 expression and activity were investigated as this isoform has been reported to participate in the metabolic transformation of many drugs in addition to its important role in

arachidonic acid transformation [71-74]. In this study, we measured a relatively low mRNA expression level for CYP2J2 in the duodenum (0.4% of all CYP450s quantified). However, its expression was constant in all participants with a low interindividual variation. It has been reported before that CYP2J2 expression is constant throughout the entire gastrointestinal tract, but its low immune-quantified CYP protein (0.9 pmol mg^{-1}) raises doubt about its overall importance in first-pass metabolism of drugs [18, 75]. Despite its low expression, we measured significant levels of CYP2J2 specific activity in the duodenum using the probe-substrate ebastine, a selective probe for CYP2J2 over CYP3As [73, 76]. This is in agreement with 2 previous studies reporting metabolic activities in human intestinal microsomes or in human duodenal microsomes with activity levels respectively ranging from 0.009-0.076 nmol mg^{-1} min^{-1} and averaging 0.026 nmol mg^{-1} min^{-1}, respectively compared to the 0.0006-0.007 nmol mg^{-1} min^{-1} obtained for this study [17, 73].

In the last few years CYP2J2 has been studied for its possible implication in T2D pathophysiology and its role in comorbidities linked to its implication in arachidonic acid metabolism to epoxyeicosatrienoic acids (EETs). Indeed, the CYP2J2-EETs-sEH metabolic pathway has been shown in mice to have a beneficial effect on adiposity, non-alcoholic fatty liver disease, systemic inflammation and insulin resistance [77-79]. In two distinct studies, endothelial and cardiovascular tissue specific overexpression of CYP2J2 in diabetic mice reduced nephropathy and cardiomyopathy which are important diabetic comorbidities [80, 81]. In our study, levels of CYP2J2 activity in subjects affected with diabetes were similar to patients without metabolic syndromes.

CYP2B6 mRNA relative level of expression in the duodenum were low (0.6%) in both study groups. We tested CYP2B6 activity in duodenal biopsies from T2D and non-diabetic patients by incubating bupropion with duodenal biopsies homogenates in presence and absence of the CYP2B6 selective inhibitor ticlopidine. In the small intestine, no significant CYP2B6 specific activity was measured. This is in agreement with a previous study that showed that no hydroxybupropion, the CYP2B6 specific metabolite, was produced in intestines and that metabolism of bupropion in this extra-hepatic tissue seems to be through multiple carbonyl reductase enzymes [82].

Finally, CES-2 mRNA levels were detected in duodenal biopsies. High expression levels of CES-2 have been reported previously [46-48]. Among transporters studied, the most abundant drug transporters in the duodenum was ABCG2, followed by OATP2B1 and ABCB1. This finding is in agreement with other studies reporting that ABCG2 exhibited high expression levels in human duodenum [83, 84].

Although non-parametric tests did not show significant effects of covariables on expression or activity, influence of gender and age were controlled in the linear regression analysis models as those factors have been shown to influence liver activity and expression of CYP450s in an isoform specific manner [85-88]. Concerning modulation of intestinal CYP450s expression and activity by age and gender, not much is available. For CYP3A, evidence suggests that gender might only influence CYP3A in the liver, but not in the intestines [89]. In the linear regression models with diabetes as a dichotomous variable, gender seemed to influence significantly CYP2C19 duodenal mRNA levels with higher expression in female subjects ($\beta=1.28$, $p=0.01$). No influence of gender on CYP3A and all other tested enzymes and transporters expression or

activity was unveiled by the linear regression analysis models. This may suggest that modulation as a function of gender can be tissue, isoform and/or substrate-dependent.

Our data suggest that CYP450 activities were not modulated by T2D in the duodenum. This finding is based on a small number of subjects and an important intersubject variability was observed. Consequently, further investigations are needed to confirm our current findings and whether changes in reduced oral clearance observed clinically following oral drug administration in patients with T2D can be explained by a tissue-specific modulation occurring either in the liver or in other parts of the intestine.

This pilot, alongside with previously reported results on the impact of T2D on CYP450 expression and activities, supports the hypothesis that T2D influence drug metabolism via CYP450 modulation in a tissue and isoform-specific manner in human. This type of effect of T2D has been vastly explored in animal models but results remain highly variable and dependent on model used. Indeed, results of comprehensive studies showing the modulation of multiple CYP450s expression and activity in various hepatic and extra-hepatic tissues of animal models by T2D have been reported recently [12, 35, 55-57, 70, 90]. However, only sparse data are available in humans and knowledge on the impact of T2D on CYP450s has to be gained to understand the high interindividual response to treatment observed in this sub-population.

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COMPETING INTERESTS

The authors declared no conflict of interest.

CONTRIBUTIONS

S.G., V.M. and J.T. wrote the manuscript; V.M. designed the research; B.P. recruited patients and performed biopsies; S.G. and F.B. performed experiments; S.G., F.B. and V.M. analysed the data. V.M. obtained funding.

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Table 1.   Baseline demographic data and clinical characteristics of patients

Parameters	Non-T2D patients	Patients with T2D
No. of subjects	16	20
Sex: No. (%) M:F	7:9 (44:56)	8:12 (40:60)
Age (years)	57 ± 16	62 ± 11
BMI (kg m^{-2})	25.8 ± 5.7	30.2 ± 6.6*
Insulin (pmol L^{-1})	44.8 ± 26.2	188.2 ± 262.7*
Glycaemia (mmol L^{-1})	4.8 ± 0.5	7.1 ± 2.3*
HbA1C (%)	5.4 ± 0.4	7.2 ± 1.0*
HOMA-IR	1.4 ± 0.9	11.4 ± 25.8*
HOMA-β	98 ± 50	151 ± 145
Time since diagnostic (years)	NA	9.5 ± 5.8
Medication use, No. (%) of subjects		
Metformin	0	13 (65)*
Sulfonylurea	0	7 (35)*
DPP4-I	0	3 (15)
Insulin	0	5 (25)
Statins	2 (13)	9 (45)
ACEI	2 (13)	4 (20)
ARB	2 (13)	7 (35)
CCB	2 (13)	4 (20)
PPI	10 (63)	8 (40)
β-Blockers	2 (13)	4 (20)
Aspirin	4 (26)	8 (40)
other NSAID	3 (19)	1 (5)
Antidepressants	1 (6)	5 (25)

Continuous variables are presented as mean ± SD

*Demographic parameters are significantly different between study groups (p<0.05)

NA, not applicable; Non-T2D, non-diabetic patient group; T2D, patients with a diagnostic of Type 2 diabetes group.

BMI, body mass index; HbA1C, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β, homeostatic model assessment of bêta cells function; DPP4-I, dipeptidyl peptidase-4 inhibitors; ACEI, angiotensin-converting-enzyme inhibitors; ARB, angiotensin II receptor blockers; CCB, calcium channel blockers; PPI, proton pump inhibitors; NSAID, non-steroidal anti-inflammatory drugs.

Table 2. Relative mRNA transcripts for drug metabolizing enzymes and transporters in duodenal biopsy homogenates from non-T2D participants and patients with T2D.

	Non-T2D patients (n = 15)	Patients with T2D (n = 20)	p-value	Adjusted p-value[a]
Drug Metabolizing Enzymes				
CES-1	2.5 (1.3 – 3.7)	2.8 (2.1 – 3.8)	0.4	0.5
CES-2	2.1 (2.0 – 2.6)	2.3 (2.1 – 2.6)	0.8	0.9
CYP1A1	6.0 (1.5 – 15.2)	6.2 (3.3 – 20.2)	0.5	0.4
CYP2B6	4.1 (1.9 – 4.7)	4.8 (3.4 – 8.9)	0.3	0.2
CYP2C8	3.8 (1.8 – 6.2)	2.7 (1.4 – 4.4)	0.5	0.3
CYP2C9	9.0 (6.3 – 13.0)	11.9 (8.9 – 14.7)	0.051	0.09
CYP2C19	4.4 (3.3 – 5.2)	4.3 (3.4 – 5.6)	0.7	0.7
CYP2D6	2.4 (1.5 – 3.6)	2.9 (1.6 – 5.3)	0.1	0.1
CYP2E1	2.2 (0.9 – 6.8)	1.6 (1.2 – 2.6)	0.3	0.2
CYP2J2	1.8 (1.2 – 2.1)	2.0 (1.5 – 2.5)	0.3	0.3
CYP3A4	4.1 (2.9 – 5.9)	4.6 (3.5 – 7.1)	0.5	0.6
CYP3A5	3.9 (3.0 – 19.4)	4.2 (3.5 – 8.1)	0.3	0.3
Drug Transporters				
ABCB1	1.5 (1.1 – 2.0)	1.6 (1.2 – 1.9)	0.7	0.5
ABCG2	2.7 (2.3 – 3.6)	3.2 (2.8 – 4.1)	0.2	0.2
OATP2B1	0.7 (0.6 – 0.9)	0.9 (0.8 – 0.1)	0.02*	0.02*

Results are expressed as median (interquartile range) of N-fold differences in drug metabolizing enzyme and transporter genes relative to the average expression of housekeeping genes and calibrator ($2^{-\Delta\Delta CT}$). Each experiment was performed three times in triplicates.

[a]Adjusted p-values from linear regression models analyses with diabetes status as a dichotomous predictor with sex and age as controls.

*p<0.05

Non-T2D, non-diabetic control patients group; T2D, patients with Type 2 diabetes group.

Table 3.   Proinflammatory cytokine plasma levels in non-diabetic patients (Non-T2D) and patients with T2D

Proinflammatory cytokines	Non-T2D patients (n = 16)	Patients with T2D (n = 20)	p-value
IFN-γ	3.80 ± 2.67	3.41 ± 2.15	0.98
IL-1β	0.07 ± 0.07	0.09 ± 0.10	0.86
IL-6	0.99 ± 0.49	1.07 ± 0.94	0.35
TNF-α	2.00 ± 0.36	2.71 ± 1.25	0.03*

Plasma concentrations (pg ml^{-1}) of proinflammatory cytokines are presented as mean $\pm$ SD

*p<0.05

Non-T2D, non-diabetic control patients group; T2D, patients with type 2 diabetes group; IFN-γ, interferon-gamma; IL-1β, interleukine-1bêta; IL-6, interleukine-6; TNF-α, tumour necrosis factor alpha.
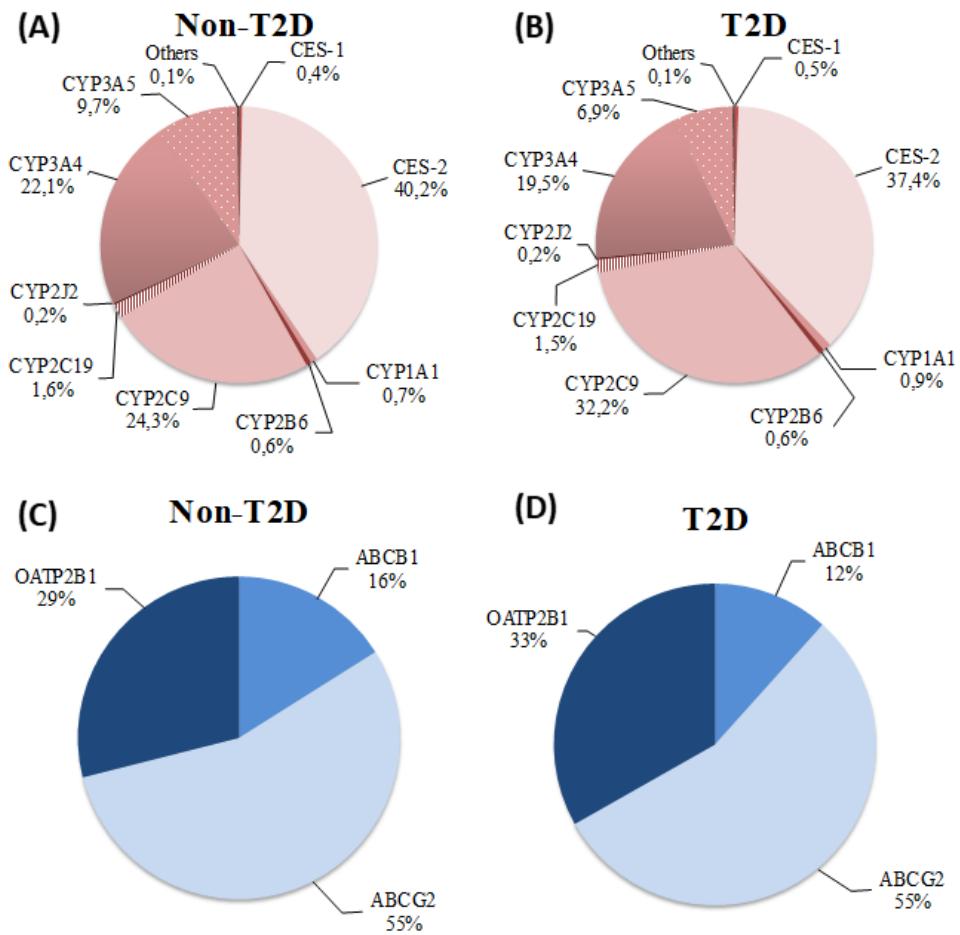
Figure 1. Drug metabolizing enzymes and transporters relative mRNA expression levels. Total mRNA transcripts ($2^{-\Delta CT}$) for each drug metabolizing enzymes (CYP450s and CES) and drug-transporters are displayed as expressed in human duodenal biopsies according to study group: (A) relative mRNA expression of drug metabolizing enzymes in non-T2D patients (n=15); (B) relative mRNA expression of drug metabolizing enzymes in T2D patients (n=20); (C) relative mRNA expression of drug-transporters in non-T2D patients (n=15); (D) relative mRNA expression of drug transporters in T2D patients (n=20). CYP450 mRNA transcript with a relative contribution >0.2 % are illustrated, and "others" have a relative contribution ≤0.07%. Others include the following isoforms: CYP2C8, CYP2D6 and CYP2E1.
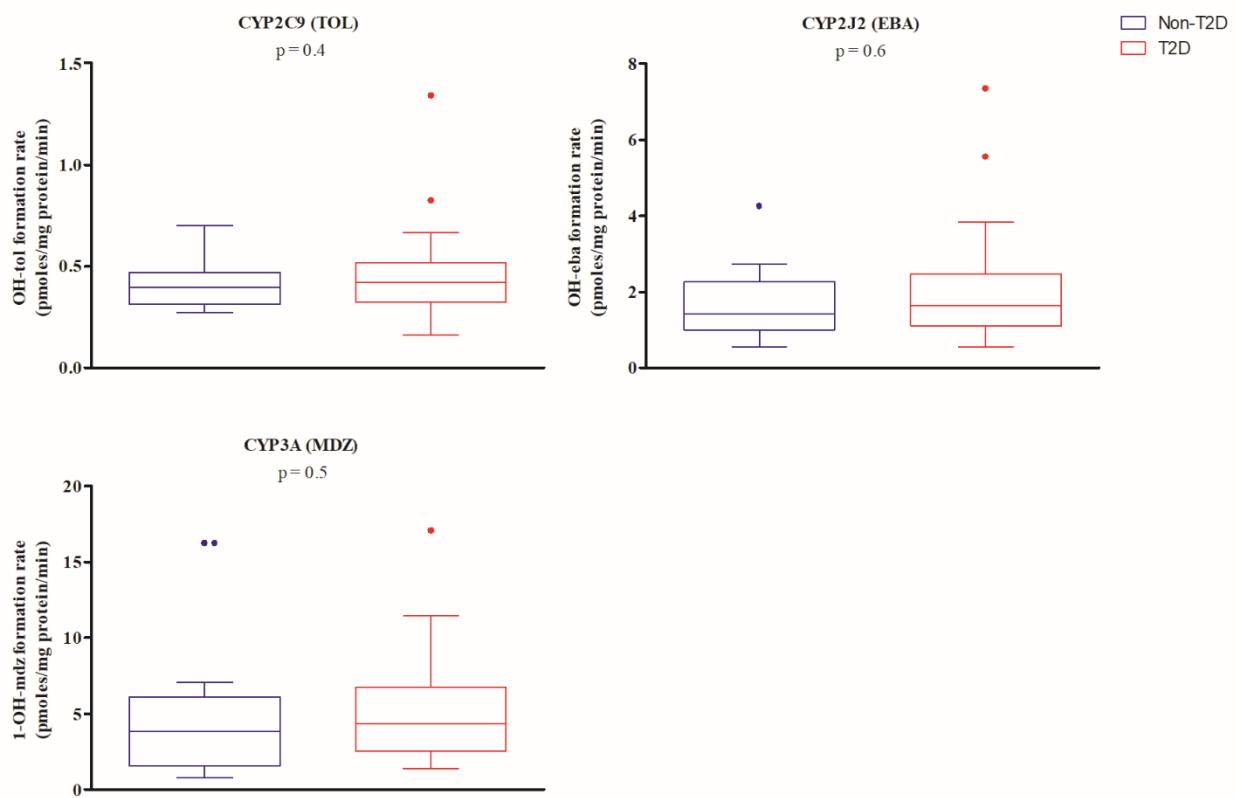
Figure 2. Duodenal biopsy homogenate (S9 fraction) activities. Rate of pathway-specific metabolite formation (pmoles mg protein^{-1} min^{-1}) for CYP2C9 (Tolbutamide (TOL) → Hydroxytolbutamide (OH-tol)), CYP2J2 (Ebastine (EBA) → Hydroxyebastine (OH-eba)) and CYP3A (Midazolam (MDZ) → 1'-Hydroxymidazolam (1-OH-mdz)) in non-diabetic patients (non-T2D) and patients with T2D are presented as box plots with Tukey whiskers.

APPENDICES

Appendix I: Method details

Quantification of Intestinal CYP450 mRNAs. For each participant, two biopsies ($\approx$10 mg of tissues) were homogenized in 1 mL of TRIzol® and incubated for 5 min at room temperature. Chloroform (200 µl) was added, the mixture shaken for 15 sec and then, centrifuged at 16,000 g for 30 min at 4°C. The aqueous supernatant (500 µl) was transferred and ethanol 70% was added (1:1 v/v). RNA was extracted using the Qiagen kit (RNeasy Mini kit; Qiagen Sciences, MD, USA) according to the manufacturer's recommendations. RNA concentration and quality were assessed by spectrometry. Total RNA (2 µg) from each sample was used for reverse transcription as described previously and resulting cDNA was kept at -80°C until use.[12] Real-time quantitative PCR was performed using TaqMan® probe and primer sets from Applied Biosystem (Foster, CA, USA) on a QuantStudio 6 Flex System (Life Technologies Inc., Burlington, ON, Canada) as detailed elsewhere.[12] The analysed drug metabolizing enzymes and transporters were: CYP1A1 (Hs01054796_g1), CYP2B6 (Hs04183483_g1), CYP2C8 (Hs00946140_g1), CYP2C9 (Hs00426397_m1), CYP2C19 (Hs00426380_m1), CYP2D6 (Hs02576167_m1), CYP2E1 (Hs00559368_m1), CYP2J2 (Hs00951113_m1), CYP3A4 (Hs00604506_m1), CYP3A5 (Hs01070905_m1) CES-1 (Hs00275607_m1), CES-2 (Hs01077945_m1) ABCB1 (Hs00184500)_m1) ABCG2 (Hs01053790_m1) OATP2B1 (Hs01030343_m1). GAPDH (Mm99999915_g1) and NUP214 (Hs01090093_m1) were used as housekeeping genes and villin (Hs00200229_m1) as a calibrator. The relative quantification of various gene expression was calculated with the comparative CT method using the formula $2^{-\Delta CT}$.[38] All measured mRNA levels were within the quantifiable range (CT values <35). In

addition, mRNA levels associated with the expression of each CYP450 isoenzymes between our two study groups were determined using a calibrator and the following formula: $2^{-\Delta\Delta CT}$.[38,39]

CYP450 genotype analysis. Blood samples for genotyping were kept at room temperature and DNA was extracted from leukocytes according to standard procedures within seven days using the GenElute™ Blood Genomic DNA kit (Sigma Aldrich, Oakville, Can). Resulting purified genomic DNAs were stored at -20°C until genotyping procedures for major CYP450 isoforms were performed. Variants for the isoenzymes *CYP2B6*, *CYP2C9*, *CYP2J2* and *CYP3A4/5* were detected using the Taqman® qRT-PCR SNP Genotyping Assay (Life Technologies, Burlington, Can). The PCR assay was performed and analysed using the QuantStudio™ 6 Flex System (Life Technologies, Burlington, Can). The SNP Genotyping assay was completed using specific probes for all SNPs (Life Technologies, Burlington, Can). Tested SNPs for *CYP2B6* were **5* (rs3211371) and **9* (rs3745274) using specific probes C_30634242_40 and C_7817765_60, respectively. Two SNPs for *CYP2C9* were considered using specific probes: *CYP2C9*2* (rs1799853; C_25625805_10) and *CYP2C9*3* (rs1057910; C_27104892_10). Presence of *CYP2J2*6* (rs72547598) and *CYP2J2*7* (rs890293) were verified using specific probes C_27859821_10 and C_9581699_80. The tested SNP for the isoform *CYP3A4* was *CYP3A4*22* (rs35599367) with specific probe C_59013445_10. For *CYP3A5*, presence of two SNPs were tested; *CYP3A5*3* (rs776746) and *CYP3A5*6* (rs10264272). The specific probes used were C_26201809_30 and C_30203950_10, respectively.

A
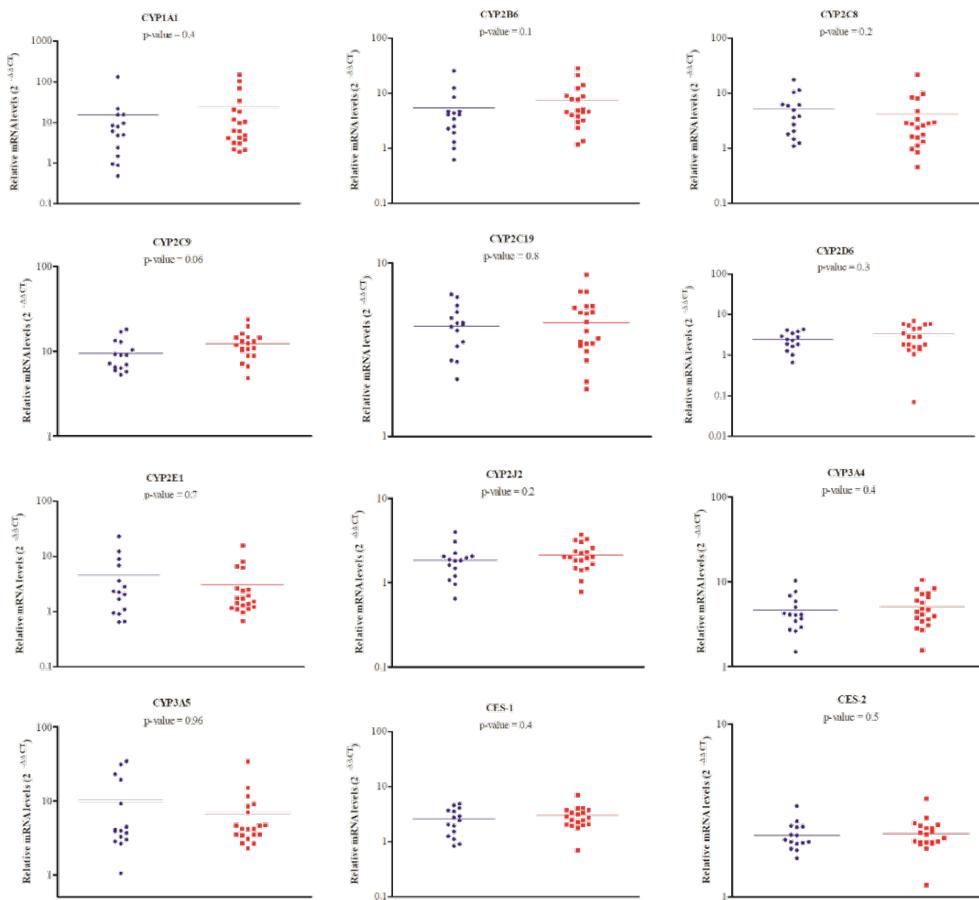
B
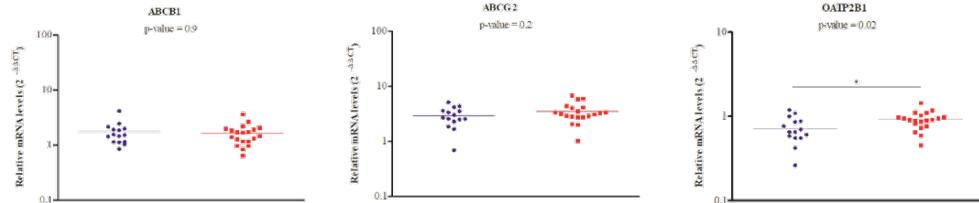
Figure S1. Quantified mRNA transcript levels expressed as N-fold differences relative to the average expression of housekeeping genes and calibrator ($2^{-\Delta\Delta CT}$) for (**A**) all drug metabolizing enzyme proteins and (**B**) all drug transporters in patients with T2D (red squares ■) versus non-diabetic controls (blue circles ●). Each experiment was performed three times in triplicates, means are displayed for all individuals and Mann Whitney t-test was performed on overall mean values.

Appendix III.
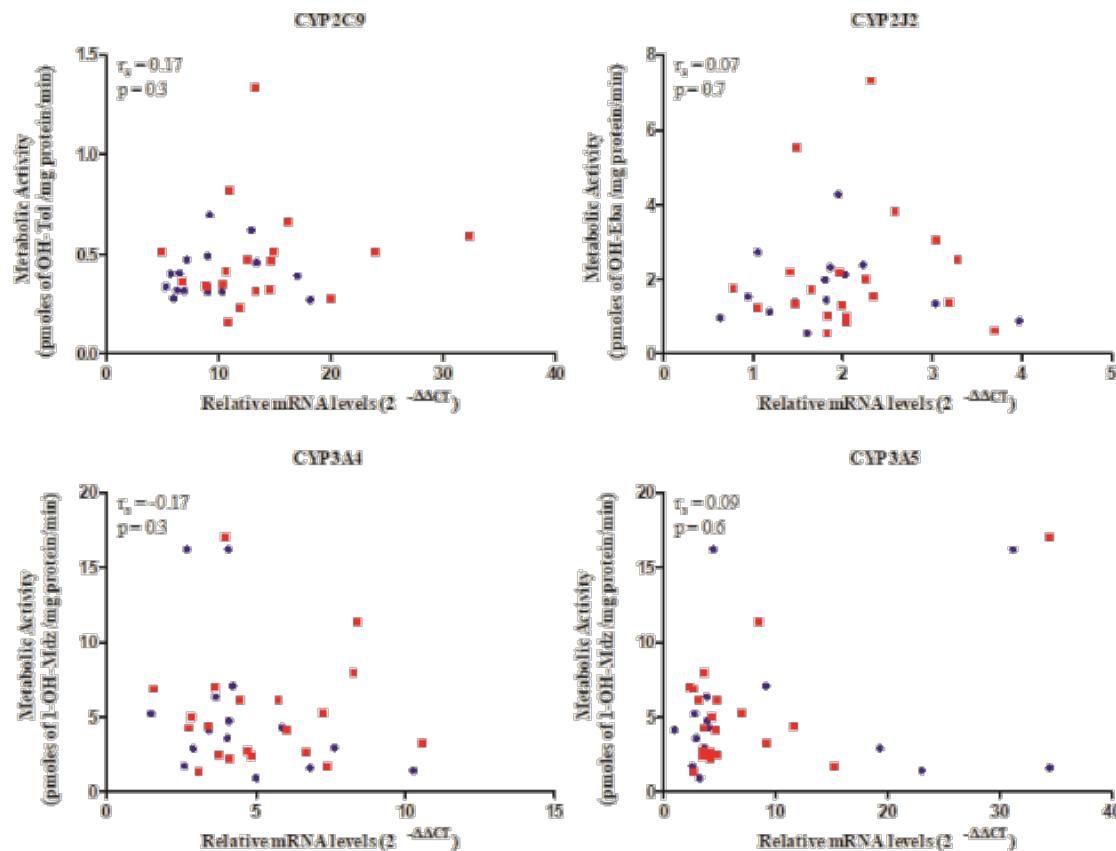
Figure S2. Correlations between mRNA expression levels as N-fold differences relative to the average expression of housekeeping genes and calibrator ($2^{-\Delta\Delta CT}$) and metabolic activities expressed as metabolite formation rates (pmoles mg protein^{-1} min^{-1}) in both study groups for CYP2C9 (OH-Tol), CYP2J2 (OH-Eba), CYP3A4 and CYP3A5 (1-OH-Mdz). Patients with T2D are displayed with red squares (■) and non-diabetic controls are blue circles (●). Spearman's rank correlation coefficient (r_s) and respective p-values are provided on each graph.

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Appendix IV.

Table S1.   Correlation between proinflammatory cytokine levels and CYP450 activities

Isozymes	Proinflammatory cytokines	Correlation coefficient (r_s)	p-value
CYP2C9	IFN-γ	- 0.09	0.6
	IL-1β	- 0.06	0.7
	IL-6	0.10	0.6
	TNF-α	0.12	0.5
CYP2J2	IFN-γ	- 0.10	0.6
	IL-1β	0.21	0.2
	IL-6	- 0.04	0.8
	TNF-α	0.01	0.9
CYP3A	IFN-γ	- 0.19	0.3
	IL-1β	0.10	0.6
	IL-6	- 0.19	0.3
	TNF-α	0.05	0.8

r_s, Spearman's rank correlation coefficient; IFN-γ, interferon-gamma; IL-1β, interleukine-1 beta; IL-6, interleukine-6; TNF-α, tumour necrosis factor alpha

Appendix V.

Table S2. Correlation between CYP450 activities and T2D-related and demographic covariables.

Isozymes	Covariables	Correlation coefficient (r_s)	p-value
CYP2C9	Glycemia	0.32	0.06
	HbA1c	0.35	0.04
	HOMA-IR	0.07	0.67
	Insulinemia	0.02	0.90
	Age	0.14	0.43
	BMI	0.12	0.48
CYP2J2	Glycemia	0.03	0.86
	HbA1c	0.05	0.77
	HOMA-IR	0.09	0.59
	Insulinemia	0.13	0.44
	Age	0.01	0.97
	BMI	- 0.07	0.67
CYP3A	Glycemia	0.18	0.30
	HbA1c	0.12	0.50
	HOMA-IR	0.24	0.15
	Insulinemia	0.29	0.09
	Age	- 0.04	0.82
	BMI	- 0.12	0.47

r_s; Spearman's rank correlation coefficient
Correlation of glycemia (mmol L^{-1}), HbA1c (% glycated haemoglobin), HOMA-IR (homeostatic model assessment of insulin resistance), insulinemia (pmol L^{-1}), Age (years) and BMI (body mass index in kg $m^{2 \, -1}$) with CYP450s metabolic activity (rates of pathway-specific metabolite formation in pmoles mg protein^{-1} min^{-1}) for CYP2C9 (Tolbutamide → Hydroxytolbutamide), CYP2J2 (Ebastine → Hydroxyebastine) and CYP3A (Midazolam → 1'-Hydroxymidazolam)

Appendix VI.

Table S3.   Influence of time since diagnostic of T2D on metabolic activity of CYP2C9, CYP2J2 and CYP3A.

Time since diagnostic (years)	Metabolic activity (pmoles mg protein^{-1} min^{-1})	p-value
CYP2C9		
< 5 (n=4)	0.37 ± 0.10	
5 to 10 (n=6)	0.47 ± 0.20	0.5
> 10 (n=9)	0.53 ± 0.34	
CYP2J2		
< 5 (n=4)	1.41 ± 0.63	
5 to 10 (n=7)	2.63 ± 2.20	0.5
> 10 (n=9)	2.13 ± 1.61	
CYP3A		
< 5 (n=4)	3.75 ± 1.61	
5 to 10 (n=7)	5.43 ± 5.32	0.4
> 10 (n=9)	5.79 ± 3.03	

Values of metabolic activities are presented as mean ± SD
p-values for Kruskall-Wallis test are reported
Influence of time since diagnostic (years) on CYP450s metabolic activity (rates of pathway-specific metabolite formation in pmoles mg protein^{-1} min^{-1}) for CYP2C9 (Tolbutamide → Hydroxytolbutamide), CYP2J2 (Ebastine → Hydroxyebastine) and CYP3A (Midazolam → 1'-Hydroxymidazolam)

Appendix VII.

Table S4. Qualitative analysis of the influence of genetic variants on metabolic activity in overall study population.

Genetic Variant	Metabolic activity (pmoles mg protein^{-1} min^{-1})
CYP2C9	
CYP2C9 *1/*1 (n=24)	0.46 ± 0.24
CYP2C9 *1/*2 (n=6)	0.47 ± 0.17
CYP2C9 *1/*3 (n=3)	0.38 ± 0.09
CYP2C9 *2/*2 and **3/*3* (n=2)	0.32 ± 0.06
CYP2J2	
CYP2J2 *1/*1 (n=32)	2.07 ± 1.45
CYP2J2 *1/*7 (n=4)	1.04 ± 0.40
CYP3A4	
CYP3A4 *1/*1 (n=33)	5.35 ± 4.28
CYP3A4 *1/*22 (n=3)	2.97 ± 0.32
CYP3A5	
Expressers: **1/*1, *1/*3* and **1/*6* (n=6)	6.01 ± 5.38
Non-expressers: **3/*3* (n=30)	4.98 ± 3.95

No statistical analysis was performed due to the small number of subjects with variant alleles.

Reported metabolic activities are rates for pathway-specific metabolite formation (pmoles mg protein^{-1} min^{-1}) for CYP2C9 (Tolbutamide → Hydroxytolbutamide), CYP2J2 (Ebastine → Hydroxyebastine) and CYP3A (Midazolam → 1'-Hydroxymidazolam)

2.3.3. Discussion

Les résultats de cette étude suggèrent que le DT2 n'affecte pas significativement les niveaux d'expression d'ARNm des dix isoformes des CYP450s (1A1, 2B6, 2C8-9-19, 2D6, 2E1, 2J2, 3A4-5), des deux carboxylestérases (CES-1 et CES-2) et des trois transporteurs (ABCB1, ABCG2 et OATP2B1) mesurés dans des biopsies duodénales. La présence de DT2 n'a pas influencé les niveaux d'activité des CYP450s au niveau duodénal. Les activités métaboliques observées à partir des biopsies duodénales pour le CYP2C9 (tolbutamide), le CYP2J2 (ébastine) et le CYP3A (midazolam) étaient semblables chez les sujets avec ou sans DT2. En résumé, bien que notre étude fût limitée par le nombre de participants et une grande variabilité inter-individuelle, le DT2 ne modulerait pas l'expression et l'activité des enzymes du métabolisme des médicaments et transporteurs testés au niveau duodénal chez l'humain. Davantage de recherche serait nécessaire afin de confirmer nos résultats et déterminer si les changements de la clairance orale via les CYP450s observés précédemment en présence du DT2 pourraient être expliqués par un effet tissu-spécifique de la pathologie au niveau du foie et/ou dans différentes portions intestinales.

De nombreux articles documentent l'expression en ARNm ou en protéines des CYP450s au niveau de l'intestin grêle chez l'humain.[80,125,346-350] Tel qu'attendu, les isoformes des CYP450s présentant l'expression en ARNm la plus importante pour nos deux groupes à l'étude étaient CYP3A et CYP2C9. De plus, des niveaux élevés de l'ARNm du CES-2 ont été mesurés dans les biopsies obtenues chez les sujets non diabétiques et les patients atteints du DT2. Au niveau de l'intestin grêle, le CES-2 a d'ailleurs été rapporté comme l'isoforme majeure des carboxylestérases, famille d'enzymes du métabolisme hautement exprimée au niveau du foie et

des intestins.[345,351,352] Tel que rapporté précédemment, le transporteur présentant les plus hauts niveaux d'expression dans nos biopsies duodénales était le ABCG2.[353,354]

Peu d'études ont évalué l'influence du DT2 sur l'expression en ARNm des enzymes du métabolisme et transporteur des médicaments chez l'humain, et la plupart ont porté sur les effets au niveau du foie. Bien que les études sur l'influence du DT2 sur l'expression en ARNm des CYP450s chez l'humain soient rares, plusieurs études ont été réalisées dans des modèles animaux.[267,270,272,273,275,355] Dans des modèles du diabète chez la souris, les résultats de l'influence de la maladie sur l'expression en ARNm des CYP450s sont variables. Bien que la plupart des observations soient au niveau hépatique, des différences significatives des niveaux d'ARNm chez la souris en présence du diabète ont été rapportées pour les cyp1a, cyp2a, cyp2b, cyp2c, cyp2e et cyp3a au foie, aux reins et aux poumons.[267,270,272,273,275,355] Chez la souris, aucun effet du diabète sur l'expression de l'ARNm des transporteurs abcg2, Mrp2 and Mrp3 au niveau hépatique n'a été rapporté.[267,272] Les résultats se rapportant à l'ABCB1 sont variables. Son expression en ARNm au niveau des intestins dans des modèles murins du diabète sont disponibles, mais rapportent des résultats contradictoires. Un groupe a observé une diminution de son expression duodénale dans un modèle du diabète chez la souris, et un autre groupe a rapporté une augmentation de son expression au niveau intestinal dans un modèle de rat obèse.[356,357]

Tel qu'il a été rapporté dans la littérature, nos niveaux d'ARNm du CYP2E1 au duodénum sont très bas (<0.04%) dans notre population à l'étude et aucun effet du DT2 n'a pu être mesuré.[80,358-361] Il a été rapporté qu'au niveau hépatique le diabète augmenterait l'expression de la protéine du CYP2E1, mais cette augmentation n'était pas accompagnée par une différence

significative dans les niveaux de son ARNm.[362] Par contre, une autre étude a rapporté des niveaux d'ARNm du CYP2E1 significativement plus élevés dans les cellules mononucléaires du sang périphérique chez des patients atteints du DT2 comparés à des sujets non diabétiques.[291] L'expression d'ARNm et les niveaux d'activité des isoformes CYP2C9 et CYP3A au niveau intestinal ont été plus largement étudiés. Des niveaux spécifiques et significatifs d'activité des isoformes CYP2C9, CYP2J2 et CYP3A4 dans des intestins humains ont été rapportés dans plusieurs études et par notre groupe précisément au duodénum.[193,350,363-368] Toutefois, les données sur l'influence du DT2 au niveau intestinal restent rares. Bien que nous n'ayons mesuré aucun effet du DT2 sur l'activité du CYP3A au niveau duodénal chez l'humain, une étude dans un modèle du diabète chez la souris avait montré une diminution de l'activité métabolique du CYP3A au niveau intestinal par rapport au contrôle.[275] En accord avec une étude réalisée chez le rat, nous n'avons mesuré aucun effet significatif du DT2 sur l'activité métabolique du CYP2C9 au niveau duodénal chez l'humain.[279] Malgré l'absence d'effet du DT2 sur l'activité du CYP2J2 dans notre étude, il était intéressant d'évaluer l'activité du CYP2J2 chez des sujets diabétiques. En effet, en plus de son rôle dans le métabolisme des médicaments, le CYP2J2 serait possiblement impliqué dans la physiopathologie du DT2 et le développement de ses comorbidités en lien avec le métabolisme de l'acide arachidonique, un substrat du CYP2J2.[187,189-191,193,369]

Dans notre étude, aucun effet du DT2 sur les CYP450s dans des fractions S9 de biopsies duodénales chez l'humain n'a été mesuré. Toutefois, les niveaux d'activité que nous avons mesurés étaient faibles pour nos deux groupes à l'étude. Les biopsies duodénales de notre groupe témoin étaient obtenues de sujets non diabétiques, mais présentant des symptômes cliniques qui nécessitent une endoscopie gastro-intestinale. Cette condition médicale pourrait

donc déjà avoir induit une phénoconversion au duodénum. Ainsi, l'impact des maladies concomitantes comme le diabète serait confondu et pourrait ne pas être déterminé par notre protocole. En résumé, bien que l'effet du DT2 sur les CYP450s ait été largement étudié à l'aide de modèles animaux, les résultats sont hautement variables et semblent dépendre du modèle utilisé.[270,272-275,279,355] Cependant, très peu de données sont disponibles chez l'humain, notamment au niveau intestinal, et davantage de recherches cliniques sur l'influence du DT2 sur les CYP450s sont nécessaires afin de mieux caractériser et comprendre la grande variabilité interindividuelle de réponse aux médicaments observée chez les diabétiques en clinique.

Section 3 : Conclusion

De nombreux facteurs intrinsèques et extrinsèques peuvent influencer la variabilité interindividuelle dans la réponse aux médicaments. L'objectif de ma thèse était de mieux comprendre les mécanismes sous-jacents à la variabilité inter-individuelle dans la réponse aux médicaments observée dans une sous-population de patients atteints de DT2 lesquels nécessitent fréquemment une polypharmacie. Une meilleure compréhension de ces mécanismes permettra éventuellement une optimisation de la pharmacothérapie chez ces patients en minimisant les réactions indésirables aux médicaments et optimisant l'efficacité des traitements. De manière générale, un déterminant majeur de la réponse aux médicaments est sa pharmacocinétique qui est entre autres déterminée par le métabolisme des médicaments. Mon projet de doctorat s'intéressait donc précisément au système enzymatique majeur impliqué dans la biotransformation des médicaments, les CYP450s. Mes recherches ont permis d'évaluer l'impact d'une condition pathologique, le DT2, sur l'activité métabolique des CYP450s en deux volets : une évaluation clinique du métabolisme systémique et une quantification *in vitro* du métabolisme au niveau de l'intestin. En parallèle à l'évaluation de l'impact du DT2 sur l'activité des CYP450s, le protocole de l'étude clinique nous a permis d'évaluer la validité d'un biomarqueur endogène du CYP3A dans notre population non diabétique et diabétique.

Le volet clinique consistait en une étude de pharmacocinétique qui visait à évaluer l'activité métabolique de différentes isoformes des CYP450s en utilisant un *cocktail* de substrats-marqueurs en fonction du statut diabétique. Cette étude m'a permis de montrer que les patients avec le DT2 présenteraient une clairance systémique réduite via les isoformes CYP2B6, CYP2C19 et CYP3A d'environ 46%, 45% et 38%, respectivement. L'effet du DT2 observé sur les niveaux d'activité des CYP1A2 et CYP2C9 serait expliquer par un effet du sexe et de l'âge.

Aucune différence significative des niveaux d'activité n'a été observée pour les isoformes CYP2D6 et CYP2E1.

Bien que la variabilité de l'activité du CYP2B6 soit surtout étudiée par rapport aux polymorphismes génétiques, des modèles du diabète chez la souris ont montré une diminution d'expression et d'activité du cyp2b.[267,272,273] À notre connaissance, nous sommes les premiers à avoir testé et démontré l'effet du DT2 sur l'activité du CYP2B6 chez l'humain. En plus du DT2, nous avons montré que les niveaux des marqueurs inflammatoires IFN-γ et TNF-α sont associés significativement à la diminution observée de l'activité du CYP2C19. En accord avec nos résultats, le rôle de l'inflammation dans la modulation de l'activité du CYP2C19 avait été démontré *in vitro* et *in vivo* pour d'autres statuts pathologiques que le DT2.[62,236,304,312] Le CYP3A est l'isoforme des CYP450s la plus impliquée dans le métabolisme des médicaments. Quelques résultats sur l'impact du DT2 sur l'activité du CYP3A sont disponibles.[281,282,295] La réduction de l'activité du CYP3A que nous avons mesuré chez nos sujets diabétiques est en accord avec les résultats disponibles dans la littérature qui rapportait une diminution de l'élimination du nisoldipine et de la cyclosporine chez les sujets diabétiques.[281,295] Notre étude appuie ces observations par l'utilisation du midazolam, un substrat-marqueur *in vivo* validé de l'activité du CYP3A, et ce dans un contexte strict d'étude de pharmacocinétique. Le degré de diminution du métabolisme du midazolam d'environ 40% que nous avons mesuré dans notre population diabétique s'accorde bien avec l'impact du DT2 mesuré en clinique. En effet, il a été rapporté précédemment que les niveaux de la protéine CYP3A4 étaient environ 1,6-fois moindre dans les microsomes de foies provenant de patients avec le DT2 versus des non diabétiques.[282]

Cette étude clinique nous a aussi permis de vérifier en objectif secondaire la validité du 4β-hydroxycholestérol comme biomarqueur endogène de l'activité du CYP3A dans notre population contrôle, puis dans notre population avec le DT2. Nous avons démontré que les concentrations plasmatiques de 4β-hydroxycholestérol, ainsi que les concentrations plasmatiques de 4β-hydroxycholestérol normalisées pour le cholestérol total, corrélaient significativement avec le ratio métabolique du midazolam pour notre population non diabétique (r_s=0,64; r_s=0,69, $p < 0,0001$) et diabétique (r_s=0,48; r_s=0,46, $p = 0,003$). Plusieurs études ont évalué le 4β-hydroxycholestérol comme biomarqueurs endogènes de l'activité du CYP3A en présence d'inhibiteurs ou inducteurs pour différentes populations de patients.[140] Cependant, très peu ont évalués l'utilité du 4β-hydroxycholestérol pour caractériser l'activité intrinsèque du CYP3A dans différentes populations de patients comme les diabétiques en utilisant un comparateur validé tel le midazolam. Le besoin d'identifier une technique rapide et non invasive pour évaluer l'activité du CYP3A chez les patients présente un grand intérêt, particulièrement chez les diabétiques qui sont sujets à être polymédicamentés et qui tendent à montrer une réponse aux traitements hautement variable en clinique.[258-260,262]

L'effet du DT2 sur l'activité des CYP450s serait, en plus d'être isoforme-spécifique, tissu-dépendant. En effet, au laboratoire, en utilisant un modèle de souris diabétique induit par la diète nous avons montré un effet du DT2 isoforme- et tissu-spécifique.[273] En plus du foie, l'intestin joue un rôle majeur dans le métabolisme des médicaments tel que discuté dans l'introduction. En effet, l'intestin peut contribuer à la variabilité dans la réponse aux médicaments au niveau du premier passage entéro-hépatique pour les médicaments administrés

par voie orale. De plus, dans des modèles animaux (rats et souris), il a été rapporté que l'expression du cyp3a dans l'intestin grêle était altérée par le diabète.[271,275,370]

Le volet *in vitro* de mes travaux nous a donc permis d'évaluer au niveau de l'intestin chez l'humain l'influence du DT2 sur l'expression de plusieurs CYP450s, carboxylestérases et transporteur, ainsi que sur l'activité des CYP2B6, CYP2C9, CYP2J2 et CYP3A. À partir de biopsies duodénales obtenues chez des patients avec DT2 et chez des non diabétiques, aucun impact significatif du DT2 n'a été observé sur l'expression d'ARNm des CYP450s (1A1, 2B6, 2C8-9-19, 2D6, 2E1, 2J2, 3A4-5), des carboxylestérases (CES-1 et CES-2) et transporteurs (ABCB1, ABCG2 et OATP2B1) testés. Également, aucune différence d'activité pour le CYP2C9 (hydroxytolbutamide), le CYP2J2 (hydroxyebastine) et le CYP3A4/5 (1'-hydroxymidazolam) n'a été mesuré à partir des biopsies duodénales. Aucune activité CYP2B6 spécifique n'a été détectée au niveau du duodénum. En considérant le statut diabétique, l'âge, le sexe et le statut génotypique des CYP450s d'intérêt dans un modèle de régression linéaire, aucun effet significatif sur les niveaux d'activité des isoformes étudiés n'a été détecté. Cependant, les niveaux d'activité mesurés dans notre étude étaient faibles. Les conditions médicales présentes chez nos participants, autre que le diabète, qui nécessitaient une endoscopie gastro-intestinale pourraient avoir influencé les CYP450s.

En outre, mes travaux de doctorat proposent une étude détaillée de l'influence du DT2 sur les CYP450s chez l'humain dans l'optique de mieux comprendre la variabilité dans la réponse aux médicaments observée chez cette sous-population de patients. Avec le nombre de diabétiques qui est en constante augmentation à travers le monde, l'intérêt de mieux comprendre cette variabilité est primordial. Les patients atteints du DT2 présentent souvent plusieurs

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comorbidités et complications pour lesquelles des médicaments autres qu'antidiabétiques doivent être administrés. Une grande proportion des adultes diabétiques souffre d'hypertension artérielle, ce qui augmente significativement le risque qu'ils subissent des complications cardiovasculaires. La prise en charge pharmacologique de l'hypertension artérielle chez les patients atteints du DT2 inclut l'utilisation de bloqueurs de canaux calciques tel le nisoldipine.[371] Hors, tel que mentionné précédemment, la disposition du nisoldipine est altérée chez les patients atteints du DT2.[295] Des évidences, dont nos résultats, suggèrent que ceci serait dû à une diminution de son métabolisme par le CYP3A chez les individus diabétiques.[295,372] Le diabète est aussi une des principales causes d'insuffisance rénale en phase terminale. Les receveurs d'une transplantation rénale reçoivent des immunosuppresseurs comme la cyclosporine ou le tacrolimus qui sont des substrats du CYP3A. Chez les receveurs de greffes du rein atteints du diabète, les concentrations des métabolites de la cyclosporine mesurées étaient moindre que chez des greffés non diabétiques.[281] De plus des niveaux plus élevés de tacrolimus ont été associés à la présence du diabète chez les receveurs d'une transplantation rénale.[294] En accord avec nos résultats, ces observations suggèrent une diminution du métabolisme par CYP3A chez les sujets atteints du DT2.[281,294,372] Tout comme pour l'antiplaquettaire clopidogrel pour lequel la présence du DT2 affecterait l'efficacité, le statut diabétique a été intégré à l'algorithme du calcul de la dose initiale pour l'anticoagulant warfarine qui présente une fenêtre thérapeutique étroite.[259,293] Le risque de développer de l'athérosclérose est de 2 à 4 fois plus élevé chez des individus diabétiques.[373] Pour prévenir le risque cardiovasculaire, les patients atteints du DT2 reçoivent souvent un hypocholestérolémiant comme des statines. Or, il a été mesuré que la clairance de l'atorvastatine lactone était significativement diminuée chez les individus atteints du diabète reflétant une diminution de l'activité du CYP3A et les prédisposant ainsi à

d'éventuelle toxicité.[296] La pertinence clinique de cette diminution du métabolisme reste à confirmer dans le cadre d'études avec d'importantes populations et serait surtout pertinente dans des cas ou les patients présentent plusieurs comorbidités et reçoivent plusieurs médicaments.

À notre connaissance, nous sommes les premiers à avoir caractérisé simultanément *in vivo* chez l'humain l'effet du DT2 sur la capacité à éliminer les médicaments via plusieurs isoformes des CYP450s grâce à l'utilisation d'un *cocktail* de substrats-marqueurs connus. Nos résultats apportent donc des éléments de connaissance additionnels qui visent à mieux comprendre la variabilité de réponse aux médicaments observée chez les patients diabétiques. D'approfondir nos connaissances de l'impact du DT2 sur l'activité des CYP450s pourrait entres autres permettre d'optimiser le régime pharmacologique des patients diabétiques qui requièrent souvent une polypharmacie. Ultimement, une application en pratique clinique permettrait d'augmenter l'efficacité des médicaments ou de réduire leurs effets indésirables dans cette population à risque.

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Annexes

Annexe 1 : Article de la méthode de détection par LC-MS/MS

Highly sensitive LC–MS/MS methods for the determination of seven human CYP450 activities using small oral doses of probe-drugs in human

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ABSTRACT

Cocktails composed of several Cytochrome P450 (CYP450)-selective probe drugs have been shown of value to characterize *in vivo* drug-metabolism activities. Our objective was to develop and validate highly sensitive and selective LC–MS/MS assays allowing the determination of seven major human CYP450 isoenzyme activities following administration of low oral doses of a modified CYP450 probe-drug cocktail in patients. The seven-drug cocktail was composed of caffeine, bupropion, tolbutamide, omeprazole, dextromethorphan, midazolam (all administered concomitantly) and chlorzoxazone (administered separately) to phenotype for CYP1A2, 2B6, 2C9, 2C19, 2D6, 3A4/5 and 2E1, respectively. Serial plasma and urine samples were collected over an 8 h period. The probe-drugs and their respective metabolites were measured in both human plasma and urine, except for omeprazole (plasma only) and chlorzoxazone (urine only). Samples were analyzed by high performance liquid chromatography with heated electrospray ionization tandem mass spectrometry (HPLC-HESI-MS/MS) using a Phenomenex Luna PFP (2) analytical column (3 μm PFP(2) 150 × 3 mm) for chromatographic separation. Optimal detection was achieved based on 3 different analytical methods; (1) isocratic elution with a mobile phase consisting of acetonitrile and water both fortified with 0.01% formic acid for the analysis of bupropion, tolbutamide, chlorzoxazone and their respective metabolites; (2) isocratic elution with a mobile phase composed of acetonitrile and ammonium formate (pH 3; 10 mM) for omeprazole, dextromethorphan, midazolam and their metabolites; (3) for caffeine and paraxanthine, gradient elution using acetonitrile and 0.01% formic acid in water was used. All calibration functions were linear for all probe drugs and metabolites in both matrices over wide analytical ranges. The main advantages of our methods are the use of specific probe drugs available in most countries, the administration of small doses of probe drugs, small volume of plasma required for the analyses and simple and rapid extraction procedures. The methods met all requirements of specificity, sensitivity, linearity, precision and accuracy and stability generally accepted in bioanalytical chemistry. Determination of CYP450 phenotype in patients will permit characterization of their capacities to metabolize drugs through CYP450 under specific conditions at a definite time. This tool will be highly clinically relevant since wide intersubject variability observed in drug response is largely explained by variation in drug metabolism; it will be particularly useful in polymedicated patients with multiple comorbidities. So far, our CYP450 cocktail assays have been successfully applied to phenotype CYP450 activities in patients.

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1. Introduction

The Cytochrome P450 (CYP450) enzymatic system constitutes the most important pathway for drug metabolism and elimination in humans. Nowadays, 57 different isoenzymes have been characterized in the human body and classified in different families and subfamilies [1,2]. The most important families involved in drug metabolism are the CYP1, CYP2 and CYP3. The CYP3A

and particularly the CYP3A4/5 are major isoenzymes implicated in the metabolism of about 50% of drugs (*e.g.* calcium channel blockers, statins, anesthetic, immunosuppressive, benzodiazepine) [3,4]. The CYP2D, CYP2C, and CYP1A subfamilies are also implicated in the metabolism of many drugs, and particularly their major isoenzymes namely, CYP2D6 (*e.g.* dextromethorphan, β-blockers, antiarrhythmic agents) [3,5], CYP2C9 (*e.g.* nonsteroidal anti-inflammatory drugs, S-warfarin, losartan) [3,6] and CYP2C19 (*e.g.* tricyclic antidepressants, proton-pump inhibitors, R-warfarin, clopidrogrel) [3,5], and CYP1A2 (*e.g.* caffeine, theophylline, mexiletine) [7]. To a lesser extent, the CYP2E1 isoenzyme takes part in the metabolism of some drugs such as chlorzoxazone, acetaminophen or inhalation anesthetics [3,8]. Finally, the interest in CYP2B6 metabolism has increased recently as its role in the metabolism of substrates (*e.g.* bupropion, cyclophosphamide, ifosfamide, methadone) has been demonstrated [9,10].

Wide inter-individual differences in expression or activity of CYP450 contribute to variations in both clearance and efficacy/toxicity of several drugs. Phenotyping measures provide information on the real-time activities of CYP450 and provide clinically relevant information as they reflect the combination of factors such as genetic, drug–drug interactions, environmental and endogenous factors including diseases [11–16]. Thus, a phenotyping measure using selective CYP450 probes is a valuable tool to determine *in vivo* CYP450 activities.

In vivo methods to characterize subject's phenotype may involve either the administration of single probe drugs, one at a time, on numerous occasions or a mixed phenotyping approach based on a cocktail method where multiple CYP450-probe drugs are administered simultaneously. The major advantage of the cocktail approach is to provide information on several CYP450 activities in a single experiment. However, drugs used in these cocktails must be specific for individual CYP450 isoenzymes and should not interact with each other. Also, in order to use such cocktails in a clinical setting, *i.e.* in patients with several co-morbidities, probe drugs should be given at low oral doses to minimize drug exposure and ensure patient's safety.

Numerous cocktails composed of several CYP450-selective probe drugs have already been described in the literature to study *in vivo* drug-metabolism activities [17–31]. Although previous published cocktails such as Inje, Karolinska, Cooperstown 5 + 1 or Pittsburg cocktail have shown value they also suffer from many limitations [24,26–29,31]. For instance, they often comprise probe drugs which are not or no longer available in several countries. Furthermore, they have been validated using high performance liquid chromatography methods with limited detection sensitivity. This led to the utilization of high doses of probe substrates.

In recent years, the use of liquid chromatography/tandem mass spectrometry (LC–MS/MS) has become a preferred approach to measure drugs and metabolites in biological fluids due to its very high selectivity and sensitivity [17–23,25,30]. The great sensitivity of LC–MS/MS permits the use of small blood volumes in patients while its specificity allows measurement of metabolites that often co-elute.

We describe herein the development and validation of three sensitive and specific LC–MS/MS assays allowing the determination of major human CYP450 isoenzyme activities following administration of a drug cocktail in healthy volunteers and patients. This seven-drug cocktail is composed of caffeine (CAF), bupropion (BUP), tolbutamide (TOL), omeprazole (OME), dextromethorphan (DM), midazolam (MDZ) and chlorzoxazone (CZX) (which is administered separately) to phenotype for CYP1A2, 2B6, 2C9, 2C19, 2D6, 3A4/5 and 2E1, respectively (Fig. 1). Our objective was to develop highly sensitive, robust and fast assays to determine CYP450 phenotypes in a real clinical setting using small blood volumes and low oral doses of mixed probe drugs in order to minimize risk of side effects.

2. Materials and methods

2.1. Chemicals and reagents

Caffeine (CAF), chlorzoxazone (CZX), dextromethorphan (DM) hydrobromide monohydrate, dextrorphan (DX) tartrate and tolbutamide (TOL) were obtained from Sigma Aldrich (St. Louis, MO, USA). Midazolam (MDZ), α-hydroxymidazolam (α-OH-MDZ), $^2H_4$-MDZ maleate and $^2H_4$-α-OH-MDZ were bought from Cerilliant (Round Rock, TX, USA). Bupropion (BUP) hydrochloride, hydroxybupropion (OH-BUP), paraxanthine (PXT), 6-hydroxychlorzoxazone (OH-CZX), 4-hydroxymidazolam (4-OH-MDZ), omeprazole (OME), 5-hydroxyomeprazole (OH-OME) sodium salt, 5-O-desmethyl omeprazole (DES-OME), omeprazole sulfone (OME-SULF), hydroxytolbutamide (OH-TOL), 4-carboxytolbutamide (COOH-TOL), $^2H_9$-BUP hydrochloride, $^2H_6$-OH-BUP, $^2H_9$-CAF, $^2H_3$-PXT, $^2H_3$-CZX-4,6,7, $^2H_3$-DM, $^2H_3$-DX tartrate salt, $^2H_3$-OME, $^2H_3$-5-OH-OME sodium salt, $^2H_3$-5-O-DES-OME, $^2H_3$-OME-SULF, $^2H_9$-TOL, $^2H_9$-OH-TOL, $^2H_9$-4-COOH-TOL, and $^2H_5$-4-OH-MDZ methanoate were purchased from Toronto Research Chemicals (Toronto, ON, CA). ^{15}N-$^2H_2$-6-OH-CZX was bought from TLC Pharmaceutical Standards (Vaughant, ON, Canada). LC–MS grade water was purchased from EMD Millipore (Billerica, MA, USA) and LC–MS grade acetonitrile, methanol and ammonium formate Optima® LC/MS were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid, dimethyl sulfoxide (DMSO) and β-glucuronidase/sulfatase from Helix pomatia type HP-2 were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium hydroxide was obtained from RICCA chemical (Arlington, TX, USA). Blank male human plasma with potassium EDTA as anticoagulant was obtained from BioreclamationIVT (Westbury, NY, USA). Human plasma and urine were stored at −80 °C and −20 °C, respectively.

2.2. Stock solutions

Stock solutions for calibration standards in plasma were prepared by accurately weighing and dissolving the compounds at 5 mg/mL for CAF and 1 mg/mL for PXT, DM and DX in water; at 2.5 mg/mL for TOL in 0.1 N sodium hydroxide and at 1 mg/mL for DES-OME in DMSO. BUP, OH-BUP, OH-TOL, COOH-TOL, OME, OH-OME, OME-SULF and 4-OH-MDZ were prepared in methanol at 1 mg/mL and certified solutions at 1 mg/mL were used for MDZ and α-OH-MDZ. Similar stock solutions were used for calibration standards in urine except for BUP and OH-BUP which were both prepared at 2 mg/mL and for DX which was prepared at 10 mg/mL. All internal standard stock solutions were prepared at 1 mg/mL in methanol except for $^2H_9$-CAF, $^2H_3$-PXT and $^2H_3$-DES-OME which were prepared in DMSO. An internal standard mix solution, containing all isotopically labeled internal standards, was prepared in methanol by diluting each stock solution to obtain relevant concentrations of each compound. All stock solutions were stored at 4 °C except for OME, its metabolites and their respective internal standards which were stored at −80 °C due to instability. All stock solutions were stable at least eight months at 4 °C or −80 °C (OME, OME metabolites and internal standards).

2.3. Chromatographic conditions

Chromatographic separation was achieved on a Phenomenex Luna 3 μm PFP (2) 150 × 3 mm column with a Phenomenex Security Guard Cartridge PFP 4 × 2 mm (Phenomenex, Torrance, CA, USA).
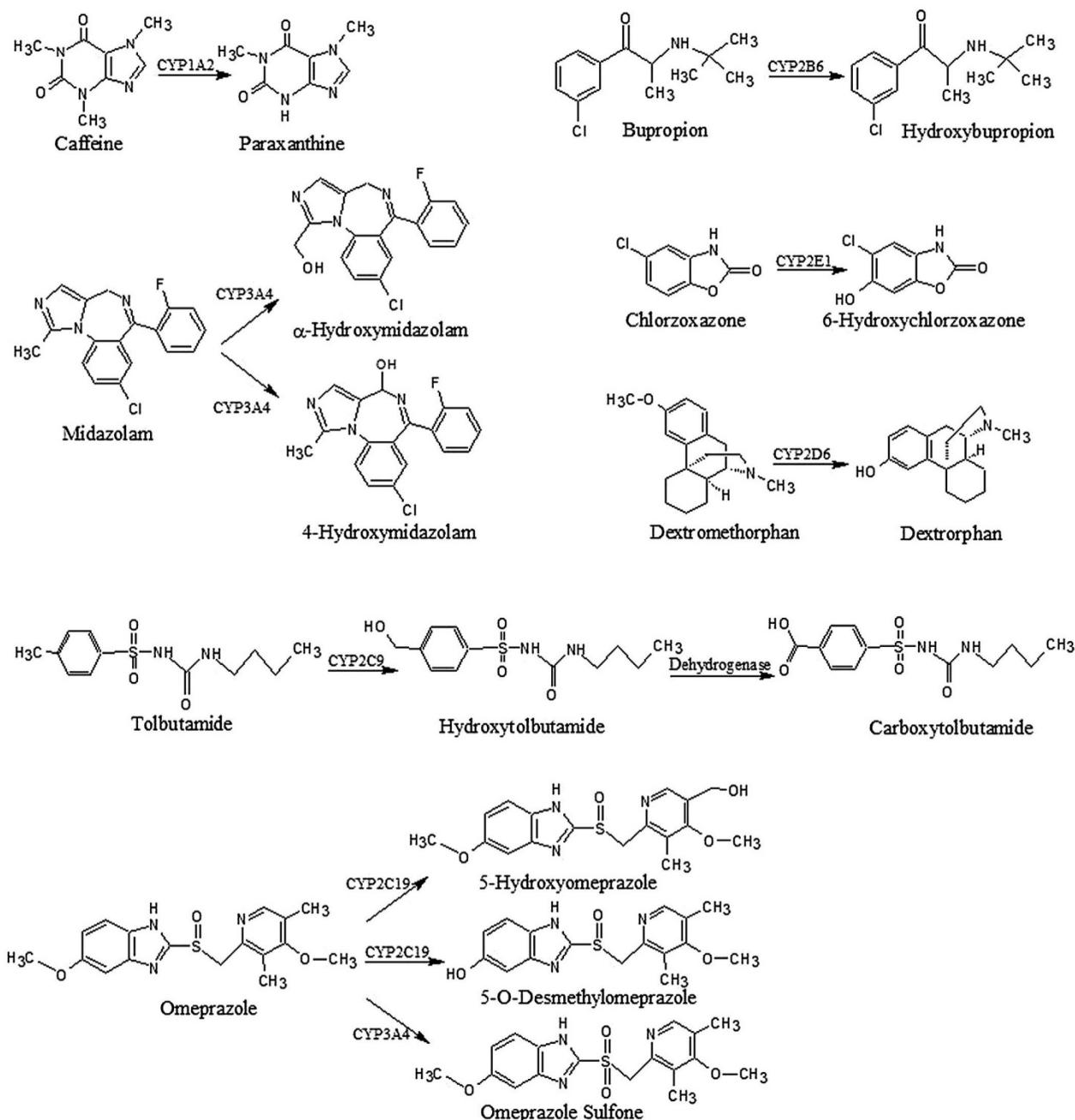
Fig. 1. Chemical structures of the probe drugs and their metabolites. This figure illustrate the major metabolic pathway for the selected specific probes.

For the analysis of OME, MDZ and DM in plasma and urine, elution was made possible using an isocratic mobile phase consisting of acetonitrile and ammonium formate in water (pH 3; 10 mM) (40:60; v/v). The flow rate was fixed at 350 μL/min and column temperature was set at 40 °C. Five microliters (5 μL) of the extracted sample was injected and the total run time was set at 8 min.

For the analysis of TOL, BUP and CZX, elution was achieved by an isocratic mobile phases consisting of acetonitrile and water both fortified with 0.01% (v/v) formic acid (40:60, v/v). The flow rate was 300 μL/min and the column temperature was set at 50 °C. Injection volume was 2 μL (plasma) or 5 μL (urine) and the total run time was 11 min for TOL and BUP, and 5 min for CZX.

For the analysis of CAF, separation was achieved under a gradient program. The initial mobile phase conditions consisted of acetonitrile and 0.01% formic acid in water (20:80, v/v), and this ratio was maintained for a period of 0.5 min. From 0.5–3 min, a linear gradient was applied up to a ratio of 80:20 and this ratio was maintained for 2 min. At 5.1 min, the mobile phase composition was reverted to 20:80 and the column was allowed to equilibrate for 2 min for a total run time of 7 min. The flow rate was 300 μL/min, the column temperature was set at 50 °C and the injection volume was 5 μL.

Table 1

Mass spectrometry conditions: SRM transitions (precursor to product transitions), fragmentation conditions (CE, collision energy; RF, radio frequency) and polarity mode (3500 V, positive mode; −3000 V, negative mode) for probe drugs, metabolites and internal standards.

Analyte	Polarity	Precursor (m/z)	Product (m/z)	CE (eV)	RF (V)
Caffeine	Positive	195.1	138.3	20	58
$^2H_9$-Caffeine	Positive	204.1	144.1	20	58
Paraxanthine	Positive	181.1	124.1	20	58
$^2H_3$-Paraxanthine	Positive	184.1	127.2	20	58
Bupropion	Positive	240.1	184.1	13	39
$^2H_9$-Bupropion	Positive	249.2	185.1	13	39
Hydroxybupropion	Positive	238.1	139.1	24	100
$^2H_6$-Hydroxybupropion	Positive	244.2	139.1	24	100
Dextromethorphan	Positive	272.2	215.1	24	60
$^2H_3$-Dextromethorphan	Positive	275.2	215.1	24	65
Dextrorphan	Positive	258.2	157.2	38	63
$^2H_3$-Dextrorphan	Positive	261.2	157.2	38	65
Midazolam	Positive	326.1	291.1	27	86
$^2H_4$-Midazolam	Positive	330.1	295.2	27	96
α-OH-Midazolam	Positive	342.1	203.1	27	73
$^2H_4$-α-OH-Midazolam	Positive	346.0	202.3	27	71
4-OH-Midazolam	Positive	342.1	234.0	23	68
$^2H_5$-4-OH Midazolam	Positive	347.3	235.1	23	62
Omeprazole	Positive	346.1	136.2	33	54
$^2H_3$-Omeprazole	Positive	349.1	136.1	33	46
Hydroxyomeprazole	Positive	362.1	152.2	34	56
$^2H_3$-Hydroxyomeprazole	Positive	365.2	152.1	34	49
Omeprazole Sulfone	Positive	362.1	298.1	19	75
$^2H_3$-Omeprazole Sulfone	Positive	365.2	301.2	19	70
5′-O-Desmethyl omeprazole	Positive	332.1	136.2	33	47
$^2H_3$-5′-O-Desmethyl omeprazole	Positive	335.2	138.1	33	44
Tolbutamide	Negative	269.1	169.9	17	67
$^2H_9$-Tolbutamide	Negative	278.2	170.0	17	77
Hydroxytolbutamide	Negative	285.1	185.9	19	71
$^2H_9$-Hydroxytolbutamide	Negative	294.2	186.2	19	83
4-Carboxytolbutamide	Negative	299.1	200.1	17	64
$^2H_9$-4-Carboxytolbutamide	Negative	308.1	200.0	17	73
Chlorzoxazone	Negative	167.7	132.2	20	103
$^2H_3$-Chlorzoxazone	Negative	170.8	134.1	20	101
6-Hydroxychlorzoxazone	Negative	183.7	120.2	20	56
$^{15}N^2H_2$-6-Hydroxychlorzoxazone	Negative	188.8	123.1	20	65

2.4. Mass spectrometer's conditions

A Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer (San Jose, CA, USA) was interfaced with the Thermo Scientific Ultimate 3000 XRS UHPLC system (San Jose, CA, USA) using pneumatic assisted heated electrospray ion source (HESI). In order to optimize ionization conditions and MS/MS parameters, standard solutions of all compounds were infused into the mass spectrometer. The following parameters were obtained: nitrogen was used for sheath, auxiliary and sweep gases and was set at 50, 15 and 5 arbitrary units; the HESI electrode was set at −3000 V in negative mode and 3500 V in positive mode; vaporizer and capillary temperatures were set at 400 °C and 350 °C, respectively; argon was used as collision gas at a pressure of 2.5 mTorr. The protonated molecular precursor ion [M+H]$^+$ for CAF, PXT, BUP, OH-BUP, DM, DX, MDZ, α-OH-MDZ, 4-OH-MDZ, OME, OH-OME, DES-OME and OME-SULF were at m/z 195.1, 181.1, 240.1, 238.1, 272.2, 258.2, 326.1, 342.1, 342.1, 346.1, 362.1, 332.1 and 362.1, respectively. The deprotonated molecular precursor ion [M-H]$^-$ for TOL, OH-TOL, COOH-TOL, CZX and OH-CZX were at m/z 269.1, 285.1, 299.1, 167.7 and 183.7, respectively. The SRM transitions, collisions energies and RF voltages and product ion spectrums of probe drugs are reported in Table 1 and Supplemental Fig. 1, respectively.

2.5. Calibration standards and quality control samples

Three series of working solutions were prepared by diluting the stock solutions in different solvents: (a) BUP, OH-BUP, MDZ, α-OH-MDZ, 4-OH-MDZ, OH-TOL, COOH-TOL, OME, OH-OME, OME-SULF, DES-OME, CZX and OH-CZX were diluted in methanol; (b) CAF,

PXT, DM and DX were diluted in water and (c) TOL was diluted alone in water, since its alkaline content caused drug precipitation upon aqueous dilution with other working solutions. Calibration standards and quality control samples were prepared by fortifying blank (drug-free) human plasma or human urine with the working solutions to enable concentrations spanning relevant analytical ranges (Table 2).

2.6. Plasma samples

Plasma standard and quality control preparations were performed with two different sources of plasma: (a) human drug-free plasma with EDTA (purchased from BioreclamationIVT) was used for BUP, DM, TOL, MDZ and OME; (b) for CAF, due to variability in baseline levels of both CAF and PXT, multiple donors were tested and CAF analyte free plasma was obtained from a male donor who withheld consumption of any caffeine, tea or chocolate products for a period of at least 10 days. LC–MS/MS analyses were performed to confirm the absence of CAF/PXT in plasma and urine matrices (data not shown).

Using a protein precipitation as sample preparation technique, BUP, DM, TOL, MDZ, OME and their respective metabolites were isolated from human plasma. The sample preparation was performed at 4 °C. Internal standard solution (750 μL: 250 ng/mL of $^2H9$-TOL; 25 ng/mL of $^2H9$-OH-TOL and $^2H9$-COOH-TOL; 20 ng/mL of $^2H3$-OME, $^2H3$-OH-OME, $^2H3$-DES-OME and $^2H3$-OME-SULF; 10 ng/mL of $^2H9$-BUP and $^2H9$-OH-BUP; 5 ng/mL of $^2H4$-MDZ, $^2H4$-α-OH-MDZ and $^2H5$-4-OH-MDZ; 1.5 ng/mL of $^2H3$-DM and $^2H3$-DX in methanol) was added to a 200 μL aliquot of plasma sample. The sample was vortexed for approximately 5 s and let stand for a period

Table 2
Summary of calibration range, correlation coefficient (mean ± SD), LLOQ and recovery at low and high concentrations for probe drugs and metabolites in human plasma and urine. Recovery results expressed as mean of percentage of reference sample.

Analyte	Matrix	Calibration range (ng/ml)	$R^2 \pm$ SD	LLOQ (ng/ml)	Recovery LOQ (%)	Recovery HOQ (%)
Caffeine	Plasma	10–10000	0.9987 ± 0.0016	10	107.6	104.6
	Urine	30–10000	0.9983 ± 0.0009	30	95.6	106.7
Paraxanthine	Plasma	10–10000	0.9997 ± 0.0002	10	101.9	101.4
	Urine	30–10000	0.9988 ± 0.0006	30	89.2	105.4
Bupropion	Plasma	0.5–500	0.9989 ± 0.0011	0.5	107.2	92.3
	Urine	6–2000	0.9982 ± 0.0009	6	102.1	114.5
Hydroxybupropion	Plasma	1–1000	0.9989 ± 0.0009	1	108.8	102.7
	Urine	6–2000	0.9982 ± 0.0014	6	83.4	115.3
Dextromethorphan	Plasma	0.1–100	0.9995 ± 0.0002	0.1	84.5	87.8
	Urine	15–5000	0.9988 ± 0.0013	15	89.5	103.0
Dextrorphan	Plasma	0.1–100	0.9991 ± 0.0006	0.1	101.5	91.2
	Urine	600–200000	0.9993 ± 0.0004	600	90.6	103.1
Midazolam	Plasma	0.1–100	0.9995 ± 0.0002	0.1	89.5	93.7
	Urine	0.3–100	0.9984 ± 0.0010	0.3	81.7	114.4
α-Hydroxymidazolam	Plasma	0.1–100	0.9995 ± 0.0004	0.1	88.4	93.7
	Urine	6–2000	0.9983 ± 0.0010	6	85.9	114.6
4-Hydroxymidazolam	Plasma	0.1–100	0.9989 ± 0.0005	0.1	86.0	89.3
	Urine	6–2000	0.9979 ± 0.0015	6	83.8	113.5
Omeprazole	Plasma	1–1000	0.9995 ± 0.0003	1	91.8	93.6
Hydroxyomeprazole	Plasma	1–1000	0.9993 ± 0.0003	1	93.0	93.5
Des-O-methylomeprazole	Plasma	1–1000	0.9995 ± 0.0003	1	90.2	91.3
Omeprazole-Sulfone	Plasma	1–1000	0.9994 ± 0.0003	1	92.9	94.3
Tolbutamide	Plasma	50–50000	0.9992 ± 0.0004	50	104.0	106.8
	Urine	30–10000	0.9990 ± 0.0004	30	104.0	103.4
Hydroxytolbutamide	Plasma	5–5000	0.9989 ± 0.0007	5	107.8	100.5
	Urine	30–10000	0.9982 ± 0.0010	30	82.6	114.9
Carboxytolbutamide	Plasma	5–5000	0.9986 ± 0.0014	5	110.5	102.4
	Urine	30–10000	0.9983 ± 0.0012	30	83.8	113.3
Chlorzoxazone	Urine	3–1000	0.9983 ± 0.0006	3	91.1	114.4
6-Hydroxychlorzoxazone	Urine	30–10000	0.9972 ± 0.0017	30	94.4	111.5

of 10 min at 4 °C, then centrifuged at 16,000*g* for 10 min. For BUP, TOL and their metabolites, 100 μL was immediately transferred to injection vial for analysis. The remainder of the supernatant, approximately 750 μL, was transferred into a clean 16 × 100 mm borosilicate tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dried extract was re-suspended with 100 μL of methanol-water (25:75, v/v) solution and transferred to an injection vial for analysis of DM, MDZ, OME and their metabolites.

For the analysis of CAF, a protein precipitation technique was also used to isolate the analyte and metabolite from human plasma. Internal standard solution (500 μL: 150 ng/mL of ^{2}H$_9$-CAF and ^{2}H$_3$-PXT in methanol) was added to a 50 μL aliquot of plasma sample. The sample was vortexed for approximately 5 s and let stand for a period of 10 min, then centrifuged at 16,000*g* for 10 min. The supernatant was transferred to an injection vial for analysis. Sample preparation procedures are shown in Supplemental Fig. 2 (plasma).

2.7. Urine samples

OME and its metabolites rapidly degrade (less than 4 h) in an acidic urine milieu (pH 4.5–7.5) [32,33] and thus, were not measured in human urine (data not shown). Furthermore, since CZX was given separately, three different urine preparations were performed: (a) CAF, DM, MDZ and TOL, after enzymatic deconjugation of urinary-excreted metabolite glucuronides, (b) BUP without exposure to enzymatic deconjugation, and (c) CZX after enzymatic deconjugation of urinary-excreted metabolite glucuronides.

Pretreatment of samples for the analysis of DM, TOL, MDZ and CAF samples was performed as follows: 25 μL of ammonium acetate (pH 5; 100 mM) buffer and 125 units of β-glucuronidase/sulfatase were added to a 25 μL aliquot of urine samples. The samples were incubated at 37 °C overnight. The internal standard mix solution (500 μL: 50 ng/mL of ^{2}H9-TOL, ^{2}H9-OH-TOL, ^{2}H9-COOH-TOL, ^{2}H4-MDZ, ^{2}H4-α-OH-MDZ, ^{2}H5-4-OH-MD, ^{2}H3-DM and ^{2}H3-DX; 30 ng/mL of ^{2}H9-CAF and ^{2}H3-PXT in methanol-water (25:75, v/v)) was added to an aliquot of 25 μL of the pretreated sample. Samples were vortexed approximately 5 s and transferred to injection vials for LC–MS/MS analyses.

Determination of BUP levels was performed without exposure to β-glucuronidase/sulfatase enzymes due to its instability at room temperature [34,35]. Samples were diluted 1:1 with ammonium acetate (pH 5; 100 mM) buffer. Five hundred (500 μL) microliters of the same internal standard mix solution (10 ng/mL of ^{2}H9-BUP and ^{2}H9-OH-BUP in methanol-water (25:75, v/v)) was added to an aliquot of 25 μL of diluted sample. Samples were vortexed, centrifuged and transferred to injection vials for analysis.

For CZX, samples were pretreated by adding 50 μL of ammonium acetate buffer (pH 5; 100 mM) and 500 units of β-glucuronidase/sulfatase to a 100 μL aliquot of urine samples. Samples were incubated at 37 °C overnight. A simple dilution was used to isolate CZX and its metabolite from urine. Two hundred and fifty (250 μL) microliters of internal standard mix containing 20 ng/mL of ^{2}H$_3$-CZX and 600 ng/mL of ^{15}N^{2}H$_2$-6-OH-CZX in methanol was added to pretreated urine samples. Samples were vortexed approximately 5 s and centrifuged at 16,000*g* for 2 min. The supernatant was transferred to injection vials for analysis.

Sample preparation procedures are shown in Supplemental Fig. 3 (urine).

2.8. Application to a clinical project

These validated methods were applied to a clinical study approved by the Centre hospitalier de l'Université de Montreal (CHUM) institutional research ethic committee. Informed consent was obtained from healthy volunteers and patients before enrollment in the study. Subjects received, after an overnight fast, an oral dose of the CYP450 probe cocktail consisting of 100 mg CAF, 100 mg BUP, 30 mg DX, 2 mg MDZ, 20 mg OME and 250 mg TOL. Serial blood samples were drawn immediately before (t = 0 h) and at 0.3, 0.6, 1, 1.5, 2, 3, 4, 6 and 8 h post-dose and urine was collected during an 8 h-period. At bedtime of the study day, a 250 mg oral dose of CZX (Acetazone® containing chlorzoxazone and acetaminophen) was taken alone and urine collected overnight. CZX is administered alone during the evening due to possible drug–drug interactions; CZX can inhibit first pass metabolism of oral MDZ in the gastrointestinal tract [36]. Moreover, the CZX formulation used in this cocktail contains acetaminophen and it is well known that acetaminophen is metabolized by CYP1A2 as well as CAF [37]. Plasma and urine samples were stored at −80 °C and −20 °C, respectively until analysis. Samples were processed as described above (Supplemental Figs. 2 and 3). Pharmacokinetic parameters such as metabolic clearance and phenotypic index based on plasma and/or urine samples were derived to estimate activities of individual CYP450 [17,23–25,30].

3. Results

These methods were validated according to U.S Food and Drug Administration guidelines for bioanalytical methods validation [38]. All probe-drugs tested in our CYP450 cocktail were evaluated for selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, and stability (freeze and thaw cycles, short term, long term, stock and auto sampler stabilities). Probe drugs and their metabolites can be measured in human plasma and urine, except for OME and CZX which were analyzed in human plasma and urine only, respectively.

3.1. Linearity

For all probe drugs and metabolites, assays were linear over a wide range (Table 2). A linear regression (weighted 1/concentration) was judged to produce the best fit for the concentration-detector relationship for all analytes and metabolites in both plasma and urine. The coefficients of correlation (R^2) were greater than 0.9956 for all compounds in all batches during validation.

3.2. Sensitivity and selectivity

Selectivity of the assays were investigated by processing and analyzing six independent blank (drug-free) samples using the previously described isolation procedures (except for CAF where solely a specific donor was used due to the variability in baseline levels of CAF and PXT). Each blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification (LLOQ). During all experimentation, no interference was observed at the mass transition and retention times for all compounds monitored (except CAF and PXT). The LLOQ concentration was chosen based on the literatures reported concentrations for the administered doses [19,22,24,25,39–42]. The LLOQ varied between 0.1–50 ng/mL in human plasma and between 0.3–600 ng/mL in human urine (Table 2). Precisions were better than 10.3% and accuracies were

in the 98.0–116.7% range. The LLOQ precision and accuracy statistical results are shown in Table 3 (plasma) and Table 4 (urine). Fig. 2 shows representative chromatograms of the overlay of processed blank plasma and the LLOQ standards in plasma for all substrates and metabolites, except for CZX which the overlay chromatogram obtained from a processed urine sample is shown.

3.3. Precision and accuracy

The intra-day precision and accuracy was assessed by replicate analysis (n = 6) of QC samples at three different concentrations (low, mid and high) within an analytical run whereas the inter-day precision and accuracy was assessed by replicate analysis (n = 6) of QC samples at three different concentrations (low, mid and high) within three different analytical runs for a total of 18 measurements. The intra-batch precision was evaluated as relative standard deviation (RSD), and the accuracy as relative error (RE). Precisions were better than 8.7% and accuracies were in the 90.6–110.2% range. The intra and inter batch precision and accuracy statistical results are shown in Table 3 (plasma) and Table 4 (urine).

3.4. Recovery and matrix effect

The extraction recoveries were determined for human plasma and urine at low and high concentrations (LQC and HQC). The recovery was determined by comparing pre-processed and fortified quality control samples to those of post-isolation and fortified quality control samples (representing 100% recovery). Recoveries were between 84.5% and 110.5% in plasma and between 81.7% and 115.3% in urine. Recoveries for all compounds are reported in Table 2.

Evaluation of a matrix effect was assessed using six different matrix lots fortified at LLOQ and high quality control concentrations for all analytes (not performed for CAF and PXT due to high variability in baseline levels) in both matrices and their concentrations were back calculated against the calibration curve. No difference was observed between the different lots assayed as shown in Table 5 (plasma) and Table 6 (urine).

3.5. Stability

Stability experiments were performed in order to demonstrate whether or not all compounds were stable under typical sample analysis and storage conditions in both plasma and urine. Stability results were evaluated by back calculating the concentration of stability samples (low, mid and high concentrations) against freshly prepared calibration curves. Stability data was deemed acceptable if the %CV of the replicate (n = 6) determinations did not exceed 15% and the mean accuracy value was within ±15% of the nominal value. Stability has been tested for freeze and thaw, short-term, autosampler, stock solution and long term stability. As experimentally verified (data not shown) and previously mentioned [34,35], BUP was found unstable in human plasma or urine when processed at room temperature. Thus, all stability testing were performed at 4 °C except for CZX which was evaluated at room temperature since it was analyzed separately.

Freeze and thaw stability was evaluated by performing 4 freeze (−80 °C) and thaw (4 °C or ambient temperature for CZX) cycles. In short-term stability experiments, the quality control samples were kept at 4 °C (or ambient temperature for CZX) over 24 h period. For long term stability, quality control samples were stored at −80 °C for a period of at least 40 days and the stability data was obtained against a freshly prepared calibration curve. To evaluate the autosampler stability, quality control samples were maintained in the autosampler for a minimum period of 3 days and the stability data was obtained against a freshly prepared calibration curve. All probe drugs and metabolites were stable at least 3 days in the

Table 3

Summary of intra-day (six replicate per concentration) and inter-day (three individuals runs) precision and accuracy of quality control samples for CAF, BUP, DM, MDZ, OME, TOL and their metabolites in human plasma. Results are expressed as concentration mean ± SD; RSD, relative standard deviation; RE, relative error.

|	Concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
		Mean ± SD (ng/mL)	RSD (%)	RE (%)	Mean ± SD (ng/mL)	RSD (%)	RE (%)
CAF	10	10.3 ± 0.8	7.5	2.6	10.2 ± 0.6	5.9	2.0
	30	30.3 ± 1.0	3.4	1.1	29.8 ± 1.6	5.3	−0.8
	1250	1243.8 ± 8.8	0.7	−0.5	1317.0 ± 70.6	5.4	5.4
	6000	6578.9 ± 125.7	1.9	9.6	6441.1 ± 286.9	4.5	7.4
PXT	10	11.6 ± 0.2	1.3	16.2	10.7 ± 0.9	8.6	2.1
	30	31.0 ± 1.0	3.1	3.5	30.0 ± 1.4	4.8	0.0
	1250	1236.0 ± 12.8	1.0	−1.1	1324.8 ± 66.1	5.0	6.0
	6000	6373.8 ± 69.4	1.1	6.2	6385.9 ± 89.5	1.4	6.4
BUP	0.5	0.5 ± 0.03	5.1	3.5	0.5 ± 0.04	6.8	2.1
	1.5	1.5 ± 0.1	5.0	−2.5	1.5 ± 0.1	6.0	−0.7
	62.5	60.5 ± 2.2	3.6	−3.1	63.4 ± 2.7	4.2	1.5
	300	298.7 ± 2.4	0.8	−0.4	314.6 ± 15.4	4.9	4.9
OH-BUP	1	1.2 ± 0.02	1.8	15.6	1.2 ± 0.07	6.0	16.4
	3	3.0 ± 0.2	5.2	1.4	3.1 ± 0.1	4.2	4.3
	125	125.6 ± 1.9	1.5	0.5	122.3 ± 8.7	7.1	−2.2
	600	638.4 ± 11.9	1.9	6.4	635.1 ± 26.8	4.2	5.8
DM	0.1	0.1 ± 0.01	2.0	12.7	0.1 ± 0.01	3.9	10.1
	0.3	0.3 ± 0.01	2.2	−6.1	0.3 ± 0.02	7.9	−3.1
	12.5	12.1 ± 0.1	0.6	97.1	12.6 ± 0.4	3.1	0.5
	60	58.6 ± 0.7	1.2	−2.3	60.1 ± 1.2	2.0	0.1
DX	0.1	0.1 ± 0.01	3.9	10.5	0.1 ± 0.01	5.2	11.4
	0.3	0.3 ± 0.01	2.0	−5.8	0.3 ± 0.01	2.8	−3.5
	12.5	12.4 ± 0.1	0.9	−0.8	12.6 ± 0.3	2.2	0.5
	60	59.0 ± 0.7	1.2	−1.7	61.0 ± 3.5	5.7	1.7
MDZ	0.1	0.1 ± 0.01	4.7	7.3	0.1 ± 0.01	5.8	8.2
	0.3	0.3 ± 0.02	6.0	−1.4	0.3 ± 0.01	3.9	−2.4
	12.5	12.7 ± 0.3	2.0	1.6	12.4 ± 0.4	3.1	−1.2
	60	62.8 ± 1.3	2.1	4.6	60.5 ± 2.0	3.4	0.9
α-OH-MDZ	0.1	0.1 ± 0.01	10.2	6.2	0.1 ± 0.01	7.2	7.1
	0.3	0.3 ± 0.01	2.4	−5.4	0.3 ± 0.02	7.9	−1.1
	12.5	12.2 ± 0.3	2.6	−2.3	12.3 ± 0.2	1.8	−1.5
	60	59.3 ± 0.7	1.2	−1.1	62.6 ± 3.0	4.8	4.4
4-OH-MDZ	0.1	0.1 ± 0.01	9.6	11.9	0.1 ± 0.01	6.5	13.8
	0.3	0.3 ± 0.01	3.5	4.4	0.3 ± 0.03	8.7	6.4
	12.5	12.4 ± 0.3	2.4	−0.6	12.6 ± 0.4	3.5	0.5
	60	65.9 ± 1.5	2.3	9.9	63.2 ± 2.9	4.6	5.4
OME	1	1.1 ± 0.03	2.8	7.3	1.0 ± 0.05	4.8	2.7
	3	2.8 ± 0.1	2.7	−7.2	2.8 ± 0.1	2.7	−8.3
	125	131.3 ± 1.0	0.8	5.0	130.0 ± 4.1	3.2	4.0
	600	604.3 ± 5.4	0.9	0.7	616.6 ± 12.6	2.0	2.8
OH-OME	1	1.1 ± 0.05	5.0	5.4	1.0 ± 0.05	4.4	4.7
	3	2.7 ± 0.1	2.5	−9.4	2.8 ± 0.1	3.7	−8.1
	125	132.2 ± 1.5	1.1	5.7	130.0 ± 4.4	3.4	4.1
	600	609.4 ± 4.1	0.7	1.6	617.9 ± 15.2	2.5	3.0
DES-OME	1	1.2 ± 0.02	1.5	16.7	1.1 ± 0.05	4.3	11.3
	3	2.8 ± 0.1	3.5	−8.3	2.7 ± 0.1	2.7	−8.7
	125	128.0 ± 1.2	0.9	2.4	126.9 ± 4.8	3.8	1.5
	600	601.4 ± 5.6	0.9	0.2	610.4 ± 13.9	2.3	1.7
OME-SULF	1	1.0 ± 0.03	2.7	−2.0	1.0 ± 0.03	2.8	−1.2
	3	2.8 ± 0.1	2.2	−5.8	2.8 ± 0.1	2.1	−5.7
	125	132.6 ± 1.4	1.1	6.1	129.8 ± 3.9	3.0	3.9
	600	603.3 ± 5.4	0.9	0.5	610.3 ± 11.0	1.8	1.7
TOL	50	58.8 ± 1.0	1.7	17.6	58.3 ± 5.6	9.6	16.7
	150	149.9 ± 3.4	2.2	−0.1	151.0 ± 5.8	3.8	0.6
	6250	6165.7 ± 71.0	1.2	−1.3	6205.8 ± 165.2	2.7	−0.7
	30000	30292.2 ± 411.2	1.4	1.0	30982.3 ± 747.4	2.4	3.3
OH-TOL	5	5.7 ± 0.3	5.2	13.4	5.3 ± 0.4	8.4	6.2
	15	14.6 ± 0.7	4.7	−2.7	14.9 ± 0.9	5.9	−0.5
	625	614.5 ± 13.6	2.2	−1.7	631.4 ± 22.6	3.6	1.0
	3000	3137.3 ± 89.6	2.9	4.6	3181.3 ± 115.6	3.6	6.0
COOH-TOL	5	5.8 ± 0.2	3.4	15.0	5.4 ± 0.6	10.3	8.7
	15	15.8 ± 0.5	3.4	5.5	15.6 ± 0.8	5.2	3.8
	625	624.5 ± 10.0	1.6	−0.1	626.4 ± 29.4	4.7	0.2
	3000	3110.4 ± 63.0	2.0	3.7	3159.0 ± 166.7	5.3	5.3
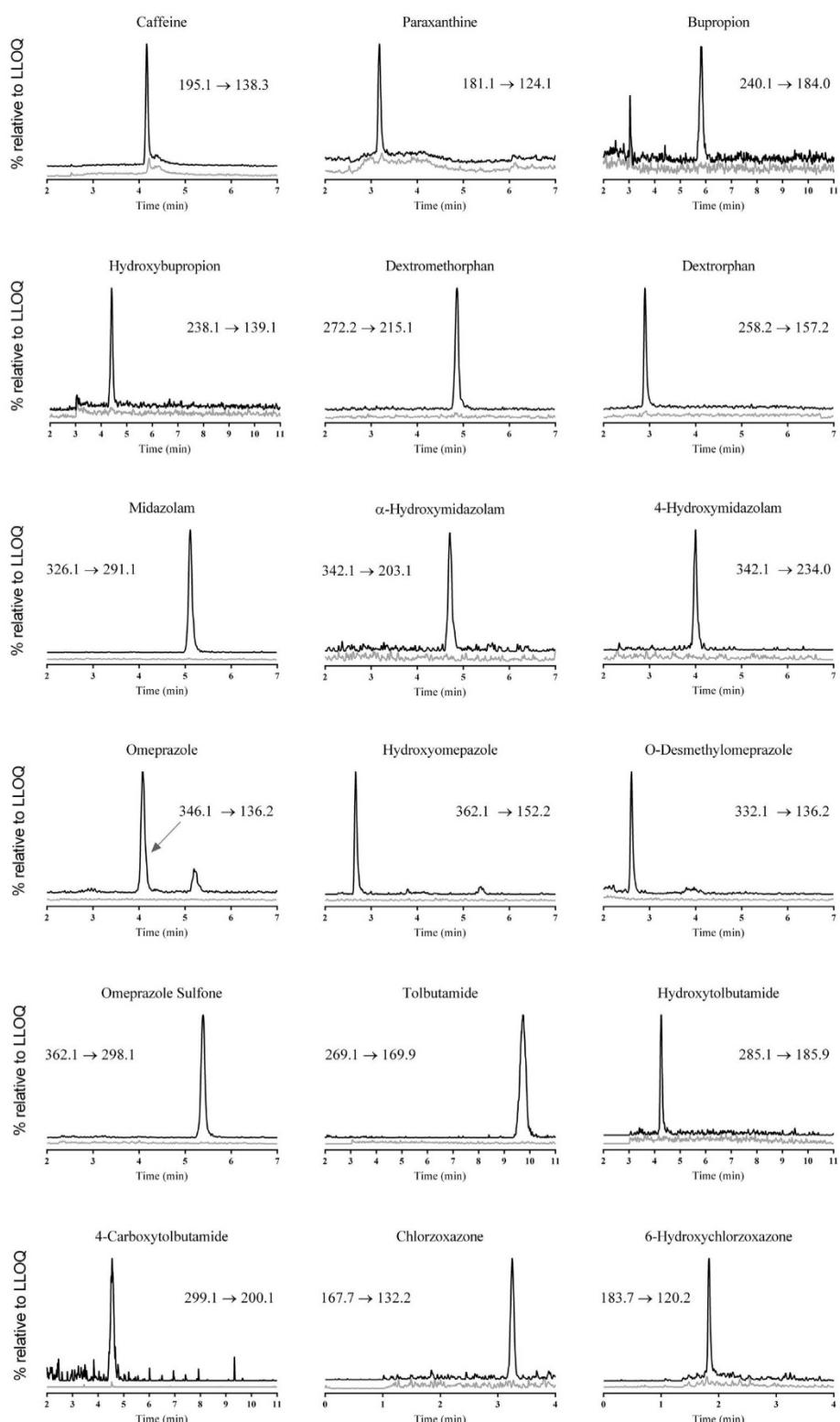
Fig. 2. Representative chromatograms of an extracted LLOQ human plasma spiked with probes drugs and their metabolites and an extracted control (blank) human plasma or urine (CZX).

x

Table 4

Summary of intra-day (six replicates per concentration) and inter-day (three individual runs) precision and accuracy of quality control samples for CAF, BUP, DM, MDZ, TOL, CZX and their metabolites in human urine. Results are expressed as concentration mean ± SD; RSD, relative standard deviation; RE, relative error.

	Concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
		Mean ± SD (ng/mL)	RSD (%)	RE (%)	Mean ± SD (ng/mL)	RSD (%)	RE (%)
CAF	30	34.1 ± 2.0	5.7	13.6	34.5 ± 1.8	2.5	15.1
	90	88.9 ± 5.0	5.7	−1.2	89.6 ± 6.2	6.9	−0.4
	1250	1229.2 ± 45.2	3.7	−1.7	1255.7 ± 48.2	3.8	0.5
	6000	6234.6 ± 199.1	3.2	3.9	6202.7 ± 349.6	5.6	3.4
PXT	30	32.3 ± 2.3	7.0	7.8	33.7 ± 2.2	6.4	12.4
	90	84.3 ± 3.5	4.2	−6.4	86.8 ± 5.4	6.2	−3.6
	1250	1218.6 ± 41.6	3.4	−2.5	1248.6 ± 47.1	3.8	−0.1
	6000	6170.7 ± 168.2	2.7	2.8	6148.2 ± 296.8	4.8	2.5
BUP	6	6.9 ± 0.3	4.2	14.5	6.8 ± 0.4	6.3	13.0
	18	17.7 ± 0.3	1.7	−1.8	18.0 ± 0.7	3.6	0.1
	250	233.5 ± 4.9	2.1	−6.6	246.2 ± 19.7	8.0	−1.5
	1200	1221.6 ± 35.7	2.9	1.8	1282.1 ± 72.1	5.6	6.8
OH-BUP	6	6.5 ± 0.3	4.0	7.8	6.8 ± 0.3	5.1	14.1
	18	17.1 ± 0.8	4.8	−5.3	17.1 ± 0.9	5.0	−5.0
	250	244.5 ± 10.5	4.3	−2.2	238.2 ± 15.6	6.6	−4.7
	1200	1206.6 ± 47.3	3.9	0.5	1248.9 ± 79.0	6.3	4.1
DM	15	15.8 ± 0.5	3.1	5.6	16.5 ± 0.8	4.7	9.7
	45	44.8 ± 2.4	5.6	−0.5	44.6 ± 2.0	4.5	−0.8
	625	662.4 ± 23.5	3.5	6.0	650.4 ± 34.8	5.4	4.1
	3000	3187.6 ± 162.0	5.1	6.3	3141.8 ± 150.0	4.8	4.7
DX (μg/mL)	0.6	0.6 ± 0.02	3.0	93.2	0.6 ± 0.03	5.2	−1.7
	1.8	1.9 ± 0.1	6.6	2.8	1.8 ± 0.1	5.3	1.2
	25	27.6 ± 0.7	2.6	10.2	27.0 ± 1.1	3.9	7.9
	120	128.4 ± 3.8	3.0	7.0	126.0 ± 5.2	4.1	5.0
MDZ	0.3	0.3 ± 0.02	6.6	11.7	0.4 ± 0.02	6.4	15.8
	0.9	0.9 ± 0.05	5.3	−1.9	0.9 ± 0.06	6.6	−4.9
	12.5	12.3 ± 0.4	2.9	−1.8	11.9 ± 0.8	6.5	−5.0
	60	59.9 ± 2.5	4.2	−0.2	62.5 ± 3.7	5.9	4.2
α-OH-MDZ	6	6.2 ± 0.4	6.8	103.5	6.7 ± 0.5	7.1	11.1
	18	17.6 ± 1.1	6.4	−2.1	17.0 ± 1.2	7.3	−5.7
	250	241.5 ± 11.1	4.6	−3.4	234.8 ± 14.0	6.0	−6.1
	1200	1203.8 ± 62.8	5.2	0.3	1240.9 ± 65.4	5.3	3.4
4-OH-MDZ	6	6.6 ± 0.6	9.5	10.1	6.9 ± 0.6	8.2	14.8
	18	17.6 ± 1.2	6.8	−2.5	17.6 ± 1.1	6.0	−2.1
	250	239.5 ± 7.9	3.3	−4.2	237.5 ± 10.8	4.5	−5.0
	1200	1206.6 ± 34.6	2.9	0.6	1256.0 ± 80.1	6.4	4.7
TOL	30	35.2 ± 1.6	4.7	17.2	35.0 ± 1.5	4.1	16.6
	90	89.2 ± 3.5	3.9	−0.9	92.6 ± 5.3	5.7	2.9
	1250	1199.4 ± 46.8	3.9	−4.0	1212.3 ± 46.3	3.8	−3.0
	6000	6023.9 ± 297.2	4.9	0.4	6171.1 ± 310.2	5.0	2.9
OH-TOL	30	33.5 ± 1.1	3.4	11.6	35.0 ± 1.7	4.8	16.7
	90	87.0 ± 4.9	5.6	−3.3	85.8 ± 4.0	4.7	−4.6
	1250	1195.3 ± 48.2	4.0	−4.4	1167.1 ± 65.4	5.6	−6.6
	6000	5957.7 ± 250.1	4.2	−0.7	6208.8 ± 410.6	6.6	3.5
COOH-TOL	30	30.2 ± 1.9	6.4	0.7	35.6 ± 4.6	8.3	11.9
	90	86.2 ± 3.6	4.2	−4.2	85.9 ± 4.6	5.3	−4.6
	1250	1204.7 ± 49.5	4.1	−3.6	1173.3 ± 63.6	5.4	−6.1
	6000	5853.3 ± 229.3	3.9	−2.4	6094.4 ± 371.5	6.1	1.6
CZX	3	3.4 ± 0.1	3.7	13.3	3.3 ± 0.3	7.5	11.8
	9	8.9 ± 0.4	4.7	−1.6	8.9 ± 0.5	5.8	−0.8
	250	244.6 ± 7.8	3.2	−2.1	256.4 ± 11.7	4.5	2.6
	700	717.1 ± 12.0	1.7	2.4	716.3 ± 31.8	4.4	2.3
6-OH-CZX							
	30	34.2 ± 3.3	9.6	13.9	32.9 ± 3.1	9.4	9.6
	90	91.8 ± 4.1	4.5	2.0	87.7 ± 0.5	7.5	−2.6
	2500	2497.1 ± 73.0	2.9	−0.1	2449.8 ± 11.7	7.0	−2.0
	7000	7051.5 ± 186.6	2.6	0.7	6814.0 ± 31.8	7.9	−2.7

autosampler (data not shown). For the stock stability, stock solutions were stored at 4 °C except OME and its metabolite which were stored at −80 °C and were compared to fresh stock solutions. All stock solutions were stable at least 8 months at 4 °C or −80 °C (OME) (data not shown). All compounds met the acceptance criteria and were stable for at least four freeze/thaw cycles, 24 h short term and over 40 days long term. Stability results are reported in Table 7 (plasma) and Table 8 (urine).

Table 5
Summary of matrix effect in human plasma for BUP, DM, MDZ, OME, TOL and their metabolites. Results are expressed as percent nominal ± SD, n = 6.

	Concentration	%Nominal (mean ± SD, n = 6)					
	(ng/mL)	Matrice 1	Matrice 2	Matrice 3	Matrice 4	Matrice 5	Matrice 6
BUP	0.5	97.1 ± 12.9	90.4 ± 3.9	100.0 ± 15.4	91.9 ± 10.5	101.7 ± 11.7	97.4 ± 8.4
	300	98.9 ± 2.1	95.5 ± 2.5	93.8 ± 1.3	95.3 ± 2.0	99.6 ± 2.8	98.1 ± 5.4
OH-BUP	1	111.4 ± 6.0	101.9 ± 7.5	108.5 ± 3.9	113.1 ± 6.2	118.6 ± 8.9	119.5 ± 2.5
	600	103.7 ± 1.3	102.0 ± 1.0	99.3 ± 2.1	101.6 ± 2.4	107.1 ± 3.4	104.7 ± 4.1
DM	0.1	110.4 ± 4.0	109.9 ± 5.1	106.8 ± 3.1	112.9 ± 4.2	108.0 ± 4.3	107.3 ± 3.3
	60	105.6 ± 0.9	103.2 ± 1.0	101.2 ± 0.7	103.5 ± 1.4	104.3 ± 0.4	102.4 ± 0.9
DX	0.1	108.8 ± 4.6	104.3 ± 5.6	107.6 ± 3.4	114.2 ± 5.1	105.5 ± 5.0	109.8 ± 4.2
	60	105.0 ± 1.1	101.0 ± 1.7	100.1 ± 1.3	102.7 ± 1.4	102.8 ± 1.1	102.5 ± 1.6
MDZ	0.1	99.9 ± 1.9	94.5 ± 1.8	99.5 ± 2.7	98.9 ± 5.2	106.4 ± 2.1	103.8 ± 3.5
	60	105.2 ± 1.9	101.7 ± 2.0	99.3 ± 1.5	101.2 ± 1.3	107.2 ± 1.8	108.3 ± 2.0
α-OH-MDZ	0.1	105.0 ± 7.0	104.6 ± 11.7	110.0 ± 9.7	99.4 ± 7.9	116.0 ± 5.3	110.7 ± 6.0
	60	104.5 ± 2.0	101.3 ± 1.5	98.6 ± 2.3	100.0 ± 2.3	106.5 ± 2.2	106.7 ± 2.5
4-OH-MDZ	0.1	113.2 ± 5.1	109.4 ± 11.0	118.7 ± 6.2	107.4 ± 7.4	106.6 ± 4.3	110.6 ± 4.5
	60	105.3 ± 2.2	100.8 ± 2.3	98.4 ± 2.2	101.5 ± 3.1	107.0 ± 3.7	107.4 ± 3.4
OME	1	101.1 ± 1.4	100.7 ± 3.2	107.2 ± 2.3	102.3 ± 3.7	114.6 ± 3.0	109.9 ± 2.1
	600	107.5 ± 1.6	106.2 ± 0.9	103.5 ± 1.0	105.3 ± 1.4	111.8 ± 1.1	111.6 ± 1.7
OH-OME	1	110.6 ± 3.9	107.9 ± 3.4	115.7 ± 4.0	111.8 ± 2.6	114.7 ± 3.2	117.0 ± 5.2
	600	106.7 ± 1.3	105.9 ± 1.3	103.1 ± 1.4	105.0 ± 2.1	111.8 ± 2.8	111.8 ± 1.8
DES-OME	1	99.8 ± 4.2	98.2 ± 2.5	102.4 ± 3.7	103.1 ± 6.5	96.5 ± 3.6	93.6 ± 3.0
	600	110.5 ± 1.0	110.4 ± 1.5	106.4 ± 1.5	107.0 ± 2.1	109.3 ± 1.3	112.5 ± 1.1
OME-SULF	1	94.6 ± 4.5	93.1 ± 2.7	100.0 ± 2.4	94.9 ± 1.2	101.7 ± 2.4	103.0 ± 1.5
	600	104.4 ± 0.8	101.9 ± 1.7	98.4 ± 1.4	100.5 ± 1.1	106.3 ± 1.4	106.4 ± 1.1
TOL	50	119.3 ± 5.2	110.8 ± 1.9	115.0 ± 1.5	112.3 ± 1.2	114.3 ± 2.5	115.1 ± 1.2
	30000	100.7 ± 1.5	96.2 ± 1.4	96.5 ± 1.3	99.3 ± 1.6	97.9 ± 2.2	99.0 ± 3.3
OH-TOL	5	101.8 ± 8.1	98.7 ± 8.0	104.7 ± 6.7	99.4 ± 5.4	107.8 ± 6.4	106.4 ± 9.1
	3000	105.4 ± 2.9	103.8 ± 1.1	100.6 ± 2.1	104.2 ± 2.8	108.1 ± 4.0	103.3 ± 9.9
COOH-TOL	5	100.1 ± 10.2	102.0 ± 7.2	100.3 ± 6.6	102.1 ± 4.4	112.4 ± 14.1	108.0 ± 9.8
	3000	104.9 ± 0.6	101.8 ± 1.3	98.2 ± 1.2	101.2 ± 1.9	105.9 ± 1.6	104.9 ± 2.2

Table 6
Summary of matrix effect in human urine for BUP, DM, MDZ, TOL, CZX and their metabolites. Results are expressed as percent nominal ± SD, n = 6.

	Concentration	%Nominal (mean ± SD, n = 6)					
	(ng/mL)	Matrice 1	Matrice 2	Matrice 3	Matrice 4	Matrice 5	Matrice 6
BUP	6	100.1 ± 5.5	108.4 ± 3.3	109.9 ± 4.0	106.0 ± 2.9	112.1 ± 4.4	103.7 ± 2.6
	1200	107.3 ± 4.1	111.2 ± 2.5	112.3 ± 4.0	112.6 ± 8.3	113.4 ± 6.2	108.8 ± 3.5
OH-BUP	6	118.3 ± 3.9	117.2 ± 4.1	117.0 ± 3.9	115.4 ± 4.0	113.7 ± 3.6	119.1 ± 3.5
	1200	110.1 ± 3.1	88.7 ± 6.4	101.7 ± 8.7	101.6 ± 2.9	105.7 ± 3.0	106.3 ± 3.1
DM	15	109.1 ± 1.3	105.0 ± 3.5	107.4 ± 2.8	107.7 ± 3.1	101.6 ± 3.3	106.6 ± 2.5
	3000	107.1 ± 5.5	104.4 ± 3.1	105.6 ± 8.8	106.0 ± 3.0	102.8 ± 2.4	105.6 ± 3.2
DX (μg/ml)	0.6	95.6 ± 3.1	91.7 ± 4.8	92.7 ± 3.4	92.9 ± 2.6	88.8 ± 4.9	93.3 ± 2.2
	120	108.4 ± 5.4	104.9 ± 3.8	106.7 ± 9.1	106.7 ± 2.9	102.6 ± 2.3	106.6 ± 2.7
MDZ	0.3	113.3 ± 4.3	107.5 ± 6.8	118.5 ± 11.5	113.8 ± 10.7	109.5 ± 6.1	113.7 ± 8.2
	60	111.9 ± 3.9	99.0 ± 2.3	100.7 ± 8.8	100.6 ± 4.1	103.2 ± 2.8	103.8 ± 4.2
α-OH-MDZ	6	114.1 ± 6.1	112.9 ± 6.8	113.2 ± 4.7	116.5 ± 4.7	111.8 ± 7.2	117.0 ± 3.1
	1200	109.0 ± 4.6	98.9 ± 2.4	99.2 ± 8.4	99.7 ± 3.8	103.5 ± 3.9	103.0 ± 4.4
4-OH-MDZ	6	118.6 ± 4.9	119.9 ± 3.3	118.9 ± 7.3	117.2 ± 3.7	112.5 ± 4.2	110.7 ± 3.4
	1200	114.3 ± 3.7	98.8 ± 2.0	103.0 ± 10.9	106.0 ± 6.7	107.4 ± 3.8	108.8 ± 3.3
TOL	30	117.0 ± 3.3	112.0 ± 6.0	115.0 ± 4.1	113.8 ± 3.0	109.5 ± 2.8	114.3 ± 1.8
	6000	105.9 ± 6.2	104.7 ± 2.9	103.2 ± 9.5	101.9 ± 3.1	103.8 ± 2.9	103.1 ± 2.4
OH-TOL	30	109.1 ± 8.4	111.4 ± 2.6	117.2 ± 4.3	118.9 ± 2.9	115.2 ± 5.7	119.4 ± 1.2
	6000	110.3 ± 3.7	95.0 ± 5.1	101.8 ± 9.1	101.4 ± 2.8	104.5 ± 3.4	106.2 ± 3.0
COOH-TOL	30	117.5 ± 7.0	114.6 ± 5.9	118.4 ± 2.1	118.0 ± 4.6	111.5 ± 5.2	118.9 ± 4.5
	6000	108.4 ± 3.2	114.0 ± 2.9	101.2 ± 8.9	100.3 ± 1.5	103.2 ± 2.2	105.0 ± 2.6
CZX	3	94.9 ± 6.0	97.1 ± 9.4	99.5 ± 10.8	96.2 ± 5.8	99.6 ± 7.7	102.5 ± 8.9
	700	95.1 ± 3.6	102.3 ± 5.8	109.2 ± 4.5	100.2 ± 2.5	98.5 ± 1.8	102.8 ± 3.3
OH-CZX	30	93.0 ± 6.7	96.7 ± 6.3	101.1 ± 17.1	100.5 ± 9.7	101.8 ± 17.8	106.1 ± 10.6
	7000	99.4 ± 7.1	99.0 ± 5.0	108.5 ± 5.0	100.4 ± 4.3	98.2 ± 3.1	106.7 ± 3.0

Table 7
Freeze and thaw (4 cycles), short-term (24 h) and long-term (40 days) stability results for CAF, BUP, DM, MDZ, OME, TOL and their metabolites in human plasma. Results are expressed as percent nominal ± SD, n = 6.

	Concentration (ng/mL)	Freeze/Thaw (4 cycles) %Nominal (mean ± SD, n = 6)	Short-term (24 h) %Nominal (mean ± SD, n = 6)	Long-term (40 days) %Nominal (mean ± SD, n = 6)
CAF	30.00	93.9 ± 1.6	97.8 ± 5.1	92.5 ± 4.4
	1250.00	96.3 ± 2.7	111.4 ± 1.3	87.2 ± 4.5
	6000.00	95.9 ± 2.2	107.0 ± 4.6	89.5 ± 3.9
PXT	30.00	92.6 ± 2.8	99.0 ± 5.7	98.8 ± 8.2
	1250.00	92.8 ± 4.2	113.5 ± 1.0	101.2 ± 7.7
	6000.00	96.3 ± 2.1	107.2 ± 5.2	96.9 ± 2.1
BUP	1	93.9 ± 3.3	86.7 ± 1.9	100.7 ± 6.0
	62.5	96.9 ± 2.0	90.6 ± 1.3	95.7 ± 3.8
	300	99.6 ± 0.9	93.7 ± 1.2	103.7 ± 1.6
OH-BUP	3	98.9 ± 2.9	91.2 ± 2.5	111.4 ± 5.3
	125	106.0 ± 1.8	90.8 ± 1.7	97.3 ± 4.5
	600.00	107.3 ± 2.3	95.3 ± 1.6	104.0 ± 4.5
DM	0.3	85.3 ± 3.7	86.3 ± 1.4	106.6 ± 9.4
	12.5	99.7 ± 1.6	91.7 ± 1.0	94.4 ± 5.6
	60	101.1 ± 1.0	94.4 ± 1.7	98.0 ± 2.4
DX	0.3	94.3 ± 1.1	91.4 ± 2.5	95.6 ± 5.7
	12.5	93.6 ± 1.9	94.5 ± 1.4	97.4 ± 10.4
	60.00	94.9 ± 1.4	95.9 ± 2.0	97.7 ± 4.2
MDZ	0.3	94.5 ± 1.1	88.4 ± 1.8	104.4 ± 7.7
	12.5	100.4 ± 1.5	91.8 ± 1.4	94.4 ± 4.3
	60	97.4 ± 1.1	96.2 ± 2.1	102.4 ± 2.6
α-OH-MDZ	0.3	88.8 ± 5.2	94.8 ± 3.8	103.1 ± 9.6
	12.5	95.5 ± 1.5	87.7 ± 1.2	93.5 ± 5.6
	600.00	96.1 ± 0.3	94.3 ± 1.5	104.0 ± 3.4
4-OH-MDZ	0.3	91.4 ± 5.0	95.9 ± 7.2	112.5 ± 7.6
	12.5	98.7 ± 2.3	92.1 ± 1.5	96.0 ± 4.6
	600.00	97.2 ± 2.2	97.0 ± 2.5	104.3 ± 3.3
OME	3	86.0 ± 2.3	91.7 ± 1.6	94.3 ± 7.4
	125	99.6 ± 0.8	103.0 ± 1.0	110.9 ± 6.0
	600	95.9 ± 0.5	103.5 ± 1.8	114.0 ± 3.8
OH-OME	3	86.9 ± 0.9	92.6 ± 2.6	107.4 ± 5.9
	125	97.9 ± 1.0	103.2 ± 1.6	114.0 ± 5.9
	600.00	92.3 ± 1.2	105.3 ± 1.7	113.6 ± 1.2
Des-OME	3.00	96.0 ± 3.7	93.7 ± 2.2	98.7 ± 10.4
	125.00	109.9 ± 1.5	103.6 ± 1.0	102.8 ± 5.3
	600.00	105.1 ± 2.1	107.3 ± 1.9	113.2 ± 3.2
OME-SULF	3.00	94.0 ± 1.2	90.4 ± 1.8	98.6 ± 2.9
	125.00	113.6 ± 2.0	95.3 ± 0.5	102.1 ± 4.5
	600.00	109.4 ± 1.1	95.7 ± 1.5	108.1 ± 6.3
TOL	150	93.9 ± 2.0	93.7 ± 1.8	98.3 ± 5.0
	6250	94.1 ± 2.1	92.0 ± 1.5	92.3 ± 3.8
	30000	94.9 ± 2.5	96.8 ± 1.8	99.7 ± 1.4
OH-TOL	15	96.8 ± 4.9	90.9 ± 3.8	100.0 ± 4.7
	625	105.3 ± 1.1	91.9 ± 1.5	98.6 ± 4.7
	3000.00	110.0 ± 1.1	96.4 ± 1.9	106.2 ± 2.1
COOH-TOL	15.00	89.0 ± 10.6	91.0 ± 3.6	100.8 ± 5.9
	625.00	102.2 ± 4.9	88.7 ± 1.4	95.9 ± 6.1
	3000.00	102.1 ± 8.2	95.0 ± 1.7	104.9 ± 2.4

3.6. Application of the method

These analytical methods were successfully applied to the analysis of human plasma and urine samples from a clinical study. The suitability of these analytical methods was confirmed by analyzing all probe drugs and their metabolites in human plasma and urine obtained in several polymedicated patients. Plasma concentration profiles of CAF, BUP, DM, MDZ, OME, TOL and their metabolites from one patient, following oral administration of the CYP450 cocktail, are shown in Fig. 3.

4. Discussion

Full validation of LC–MS/MS methods for the determination of CYP450 activities was carried out in compliance with FDA guidance for validation of bioanalytical methods. A phenotype determination for seven major isoenzymes in plasma and/or urine, namely CYP1A2, 2B6, 2C9, 2C19, 2D6, 3A4/5 and 2E1 can be achieved using these assays. Our optimized multiprobe cocktail assay utilizing low oral drug doses, simple extraction procedures and low volume of biological samples, has been successfully used to support sample analyses in polymedicated patients with several comorbidities. Our validated methods were found to be selective for all tested analytes

Table 8
Freeze and thaw (4 cycles), short-term (24 h) and long-term (40 days) stability results for CAF, BUP, DM, MDZ, TOL, CZX and their metabolites in human urine. Results are expressed as percent nominal ± SD, n = 6.

	Concentration (ng/mL)	Freeze/Thaw (4 cycles) %Nominal (mean ± SD, n = 6)	Short-term (24 h) %Nominal (mean ± SD, n = 6)	Long-term (40 days) %Nominal (mean ± SD, n = 6)
CAF	90	100.2 ± 5.0	100.5 ± 5.1	98.0 ± 9.8
	1250	96.0 ± 4.6	99.2 ± 2.5	93.1 ± 12.0
	6000	96.0 ± 2.8	101.4 ± 3.6	105.5 ± 6.6
PXT	90	97.1 ± 4.8	94.4 ± 7.0	99.5 ± 3.7
	1250	106.8 ± 5.8	109.7 ± 2.4	103.5 ± 1.6
	6000.00	99.6 ± 3.5	105.6 ± 3.2	102.7 ± 2.4
BUP	18	105.1 ± 5.0	96.4 ± 7.1	93.6 ± 2.5
	250	96.5 ± 5.1	102.2 ± 4.7	101.2 ± 1.7
	1200	111.2 ± 4.8	104.7 ± 7.1	110.8 ± 2.7
OH-BUP	18	97.5 ± 6.3	90.4 ± 6.2	97.0 ± 4.6
	250	95.4 ± 4.7	99.0 ± 3.3	98.9 ± 1.1
	1200.00	99.3 ± 2.4	106.1 ± 4.3	109.5 ± 2.0
MDZ	0.9	93.7 ± 5.4	94.0 ± 6.8	95.9 ± 7.4
	12.5	95.9 ± 4.2	98.2 ± 2.9	92.2 ± 2.2
	60	99.8 ± 2.1	106.3 ± 3.9	99.4 ± 1.5
α-OH-MDZ	18	94.0 ± 4.6	97.1 ± 7.7	92.2 ± 6.7
	250	95.6 ± 3.7	96.1 ± 4.0	92.7 ± 2.5
	1200.00	98.0 ± 2.2	103.6 ± 3.4	106.7 ± 4.4
4-OH-MDZ	18.00	99.5 ± 5.0	95.5 ± 7.6	90.4 ± 2.2
	250.00	99.8 ± 5.6	93.2 ± 3.3	85.6 ± 0.1
	1200.00	101.2 ± 2.1	99.2 ± 4.7	91.1 ± 1.1
DM	45	99.5 ± 5.1	98.1 ± 6.5	96.3 ± 3.5
	625	102.0 ± 3.7	104.4 ± 2.2	102.1 ± 3.3
	3000	98.7 ± 2.7	104.4 ± 5.8	103.2 ± 2.5
DX	1.8	99.1 ± 5.4	98.2 ± 7.6	102.1 ± 3.7
	25	105.9 ± 5.9	108.0 ± 2.3	106.0 ± 3.7
	120.00	99.3 ± 2.8	106.4 ± 4.4	103.5 ± 2.4
TOL	90	101.0 ± 6.7	103.4 ± 8.7	103.9 ± 5.2
	1250	97.9 ± 5.8	100.9 ± 2.7	98.3 ± 2.4
	6000	98.7 ± 2.7	104.7 ± 4.9	102.7 ± 3.5
OH-TOL	15	96.9 ± 5.4	88.8 ± 5.5	94.4 ± 2.7
	625	96.4 ± 5.4	98.8 ± 3.9	100.0 ± 2.0
	3000.00	99.4 ± 2.9	106.8 ± 5.1	109.4 ± 1.8
COOH-TOL	15.00	97.7 ± 5.6	92.6 ± 9.2	96.0 ± 3.2
	625.00	97.6 ± 4.8	98.6 ± 1.6	95.7 ± 1.8
	3000.00	98.3 ± 2.5	104.9 ± 3.4	105.2 ± 1.4
CZX	9	105.3 ± 3.5	101.4 ± 5.8	87.5 ± 2.4
	250	96.6 ± 3.5	100.6 ± 5.5	93.8 ± 3.5
	700	104.9 ± 3.8	104.4 ± 5.2	98.1 ± 1.8
6-OH-CZX	90	94.7 ± 9.0	99.5 ± 7.1	86.0 ± 0.5
	2500	90.8 ± 5.9	86.0 ± 7.7	89.7 ± 3.7
	7000.00	94.7 ± 6.0	92.1 ± 3.8	95.4 ± 2.2

and did not reveal interferences of matrix compounds or metabolites.

The most relevant CYP450 isoenzymes for drug metabolism are CYP3A4/5, CYP2D6, CYP2C9, CYP2C19, CYP2B6, CYP1A2 and CYP2E1 since they are heavily involved in the biotransformation of a large number of drugs. Therefore, most of previously developed probe cocktail methods were intended to target most of these isoenzymes although CYP2B6 and CYP2E1 were often not included [17–23,25]. Several multiprobe cocktail methods such as the Pittsburg cocktail, the Karolinska cocktail and the Cooperstown 5 + 1 cocktail were successfully used to phenotype and investigate drug–drug interactions *in vivo*; however, their sample bioanalyses were performed by HPLC, needed large volume of samples, lacked sensitivity or showed analytical interference between compounds [26,27,31]. The GW cocktail reported by Scott et al. [30] was the first validated cocktail using LC–MS/MS methods to phenotype for six isoenzymes and was composed of caffeine, diclofenac, mephenytoin, debrisoquine, chlorzoxazone and mida-

zolam as probes drugs. Mephenytoin and debrisoquin have been commonly used for phenotyping CYP2C19 and CYP2D6 activities, respectively. However, their administration is non-longer possible since these drugs were withdrawn from the market in most countries. Three cocktails described by Zhang et al. [22], Turpault et al. [23] and Yin et al. [25] have been also developed but, they were associated with some limitations including; lower detection sensitivity, use of higher doses of probe drugs increasing risk to adverse drug effects, and finally the assay methods were restricted to the analysis of probe drugs only hindering an accurate estimation of CYP450 phenotyping indices since specific metabolite measurements are often required. Many assays described in the literature were developed and validated in either plasma or in urine for specific CYP450 probes. Our methods are more versatile and will allow assessment of various pharmacokinetic parameters such as metabolic clearance and renal clearance.

Our methods (Supplemental Fig. 2) require only 50 μL of plasma for CAF and 200 μL for BUP, DM, MDZ, OME and TOL to assure a suf-
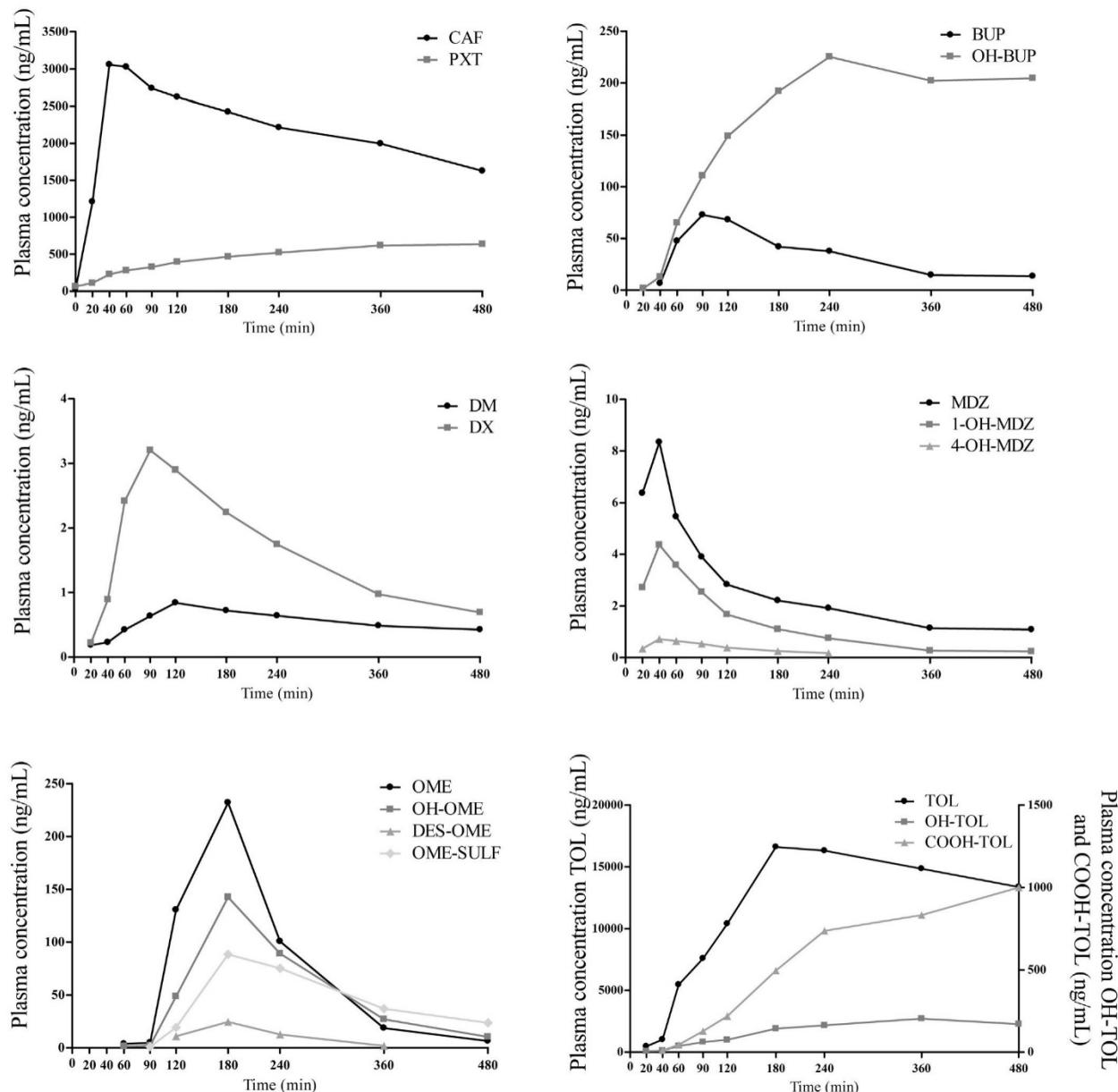
Fig. 3. Plasma concentration profiles of CAF, BUP, DM, MDZ, OME, TOL and their metabolites from one patient following the oral administration of our CYP450 cocktail.

ficient sensitivity compared to 300–1000 μL of plasma commonly used in most methods from literature [17,19–21,24,25,30]. Zhang et al. [22] developed an assay using only 50 μL of plasma. However, their method was limited to four probe drugs and did not measure any metabolites. Indeed, sample bioanalysis being less complex makes possible the use of smaller plasma volume while maintaining a good precision at low concentrations (LLOQ) for the probe drugs. The CYP450 cocktail described by Oh et al. used microdoses of 5 probe drugs [20]. Although their assay was associated with good sensitivity, it also required a higher volume of plasma than our methods (500 vs. 250 μL, respectively) and used propranolol as internal standard while isotopically labeled compounds are employed for all analytes in our methods. The use of labeled analytes as internal standards enhances the robustness of the method by minimizing possible matrix effects; this is an advantage partic-

ularly, while performing sample bioanalyses from polymedicated patients. De Andres et al. [18] and Wohlfarth et al. [19] have recently developed two methods using comparable oral doses as given in our cocktail and showed similar range of LLOQ. These methods were developed to phenotype for CYP1A2, 2C9, 2C19, 2D6 and 3A4 but did not measure probe drugs for CYP2B6 and CYP2E1 which are involved in the metabolism of approximately 7% and 3% of major commercial drugs, respectively [43].

Furthermore, our sample preparation uses a simple isolation procedure. Numerous CYP450 phenotyping methods employed extensive extraction procedures for sample preparation such as dual liquid extraction or solid phase extraction which are costly and time consuming [10,17–20,23–25,30]. A new method was recently reported by Tanaka et al. [17] for probe drug extraction i.e. "the Ostro Pass-Through Sample preparation" which showed

a significant improvement in bioanalysis compared to the use of protein precipitation solely. Extraction recoveries obtained using their method ranged between 66.4% and 116% which are quite comparable to the recoveries attained using our methods *i.e.* 81.7% and 115.3%. In addition, their method showed lower correlation coefficients (between 0.946 and 0.992). Therefore there is no added benefit in adding an extra step (filtration through a sorbent) with extra cost to the sample preparation process. Finally, the CYP450 phenotyping method reported by Stewart et al. [21] using UPLC–MS/MS raised many advantages compared to previous published methods in term of sensitivity, low doses of probe drugs, low blood volume and the rapidity and the simplicity of their extraction methods. Their assay allows the determination of five probe drugs to phenotype for CYP1A2, 2C9, 2C19, 2D6 and 2E1 in human plasma or urine. However, this cocktail approach displays two major limitations, *i.e.* the utilization of mephenytoin and debrisoquin as probes drugs to phenotype for CYP2C19 and 2D6 respectively, and it did not contain any probe drug to measure CYP3A4 activity which metabolizes approximately 30–50% of drugs used in clinic [43].

Our methods demonstrate many advantages compared to previously published methods for the evaluation of CYP450 activities by LC–MS/MS. These current assays offer an improvement in sensitivity allowing the detection of seven probe drugs and their respective metabolites in plasma and urine in polymedicated patients [23–25,30].The methods presented in this manuscript have been applied to a clinical study in 30 polymedicated patients to identify properly phenotypic CYP450 activities without induction of any drug side effects.

5. Conclusion

In this present work, we have developed and fully validated reliable, precise and sensitive assays which allow the determination of seven CYP450 probe drugs specifically CAF (CYP1A2), BUP (CYP2B6), DM (CYP2D6), MDZ (CYP3A4/3A5), OME (CYP2C19), TOL (CYP2C9) and CZX (CYP2E1) in human plasma and/or urine. Compared to the numerous cocktails and methods published in the literature, our methods present many advantages. The use of probe drugs available in most countries and their specificity towards CYP450 isoenzymes as well as analyses being performed by LC–MS/MS render our methods selective and easily applicable. The methods require a small volume of plasma and the sample preparation technique is simple, fast and inexpensive. The LLOQs are very low allowing the administration of small doses of probe drugs. The fundamental parameters such as accuracy, precision, linearity, selectivity, sensitivity, recovery, reproducibility and stability meet the criteria acceptance for all probe drugs and metabolites, confirming that these LC–MS/MS methods have been successfully developed and validated. The results for these methods validation, according to U.S. FDA guidelines for bioanalytical method validation, support that all clinical samples found in the validated concentration range can be measured with acceptable precision and accuracy. In addition to genotype, a better assessment of CYP450 phenotypes will enable the determination of interindividual variability in major CYP450, which are an important source of variation in drug pharmacokinetics and thus, in drug response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.12.006.

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Annexe 2 : Article sur les effets du diabète sur les CYP450s chez la souris

Article

Tissue Specific Modulation of cyp2c and cyp3a mRNA Levels and Activities by Diet-Induced Obesity in Mice: The Impact of Type 2 Diabetes on Drug Metabolizing Enzymes in Liver and Extra-Hepatic Tissues

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Abstract: Various diseases such as type 2 diabetes (T2D) may alter drug clearance. The objective of this study was to evaluate the effects of T2D on CYP450 expressions and activities using high-fat diet (HFD) as a model of obesity-dependent diabetes in C57BL6 mice. The cyp450 mRNA expression levels for 15 different isoforms were determined in the liver and extra-hepatic tissues (kidneys, lungs and heart) of HFD-treated animals ($n = 45$). Modulation of cyp450 metabolic activities by HFD was assessed using eight known substrates for specific human ortholog CYP450 isoforms: in vitro incubations were conducted with liver and extra-hepatic microsomes. Expression levels of cyp3a11 and cyp3a25 mRNA were decreased in the liver (>2–14-fold) and kidneys (>2-fold) of HFD groups which correlated with a significant reduction in midazolam metabolism (by 21- and 5-fold in hepatic and kidney microsomes, respectively, $p < 0.001$). HFD was associated with decreased activities of cyp2b and cyp2c subfamilies in all organs tested except in the kidneys (for tolbutamide). Other cyp450 hepatic activities were minimally or not affected by HFD. Taken together, our data suggest that substrate-dependent and tissue-dependent modulation of cyp450 metabolic capacities by early phases of T2D are observed, which could modulate drug disposition and pharmacological effects in various tissues.

Keywords: cytochromes P450; drug metabolism; mRNA; diet induced obesity; diabetes

1. Introduction

Type 2 diabetes (T2D) has become a worldwide public health concern as prevalence of the disease continues to rise [1]. In 2014, the American Diabetes Association reported that 29.1 million Americans, or 9.3% of the population, had T2D [2]. In addition to anti-diabetic drugs, T2D patients commonly require multiple drug therapies to treat a wide range of comorbidities such as hypertension, stroke, dyslipidemia, atherosclerosis and coronary artery disease [3]. Clinical practice reveals that T2D patients show highly variable pharmacokinetics and responses to several drugs used to treat T2D and its related comorbidities [4–7]. For instance, variable drug dosages and effects are observed for drugs such as clopidogrel, warfarin, cyclosporine and tacrolimus, as well as for anti-hypertensive and cholesterol

lowering drugs [8–14]. Hence, the treatment of co-morbidities in T2D is associated with variable drug response and unexpected toxicities [4,15–17].

Currently, information available on the underlying mechanisms responsible for this variability is uncertain. Patients with T2D have a high prevalence of metabolic syndrome (85% vs. 24% in general population), which is associated with a chronic low-grade inflammatory state [18–20]. Several reports showed that some inflammatory mediators may modulate expression levels and activities of numerous proteins including some isoenzymes of the cytochrome P450 (CYP450) superfamily [21–24]. In fact, interleukin-B (IL-1B), interleukin-6 (IL-6) and interferon-γ (INF-γ) have been associated with decreased expressions and activities of CYP450s, especially of CYP3A, in cultured human hepatocytes [25–27]. It is also known that pathophysiological changes resulting from obesity affect drug-metabolizing enzyme expressions and activities [28–30].

In humans, some studies have reported a decrease in CYP3A4 and an increase in CYP2E1 activities with obesity, while its effects on other isozymes remain uncertain [31–34]. For instance, Woolsey et al. have reported that CYP3A activity and CYP3A4 mRNA expression were reduced in humans and mice with nonalcoholic fatty liver disease [35]. However, results from animal studies are inconsistent from one study to the other [30,36–42]. These discrepancies could be explained by the type of diabetes being studied (i.e., T1D vs. T2D), and the strategy used to induce diabetes (i.e., genetically-modified animals vs. chemicals vs. diet). For instance, down-regulation of CYP1A2 and CYP3A1, and up-regulation of CYP3A2 are observed in Goto–Kakizaki rats (genetic model of non-obese T2D) [43]. Conversely, alloxan or streptozocin were associated with an increase expression of hepatic CYP1A2, CYP2B1/2, CYP3A1/23 and CYP2E1 in diabetic rats compared to controls [44–49]. No alteration in hepatic levels of cyp1a2 and cyp2e1 has been found in streptozocin-induced diabetic mice [41]. A study from Ghose et al. revealed a decrease of cyp3a activity, but unaffected cyp1a2 and cyp2e1 activities in high-fat diet (HFD) fed mice [38]. Finally, no difference was observed for cyp3a11 expression or activity in db/db mice (a genetic T2D mouse model) [37].

Several organs express various combinations of CYP450s and, thus, different patterns of CYP450 expression in tissues may be a key determinant of variability observed for drug response. Although extra-hepatic CYP450 activity is manifested to a lower magnitude compared to the liver [50], variability in tissue-specific metabolizing CYP450 enzymes may lead to variation in drug effects due to local metabolism in target organs. To date, there is a paucity of information on the influence of pathophysiological conditions such as T2D on the activity and expression of extra-hepatic CYP450s.

In this study, we sought to determine the effects of T2D on the expression and activities of hepatic and extra-hepatic CYP450 enzymes using the high fat-diet (HFD) diet-induced obesity (DIO) C57BL6 mouse as a T2D model [51]. DIO mice were stratified into two groups according to the effect of HFD on their body weight at the end of the treatment period: low-diet responders and high-diet responders. These two groups have been well characterized and correspond to the early diabetes situation observed in obese humans [51]. An extensive phenotyping characterization was conducted in many organs (liver, kidneys, lungs and heart) to assess the tissue-specific modulation of cyp450 expressions and activities.

2. Materials and Methods

2.1. Chemicals

Ebastine, hydroxyebastine, carboxyebastine, desalkylebastine, hydroxyebastine-d5, carebastine-d5, desalkylebastine-d5, bufuralol, hydroxybufuralol, hydroxybufuralol-d9, repaglinide, 2-despiperidyl-2-amino repaglinide (M1), 3′-hydroxyrepaglinide (M4), bupropion, hydroxubupropion, hydroxybupropion-d5, hydroxytolbutamide, carboxytolbutamide, 1′-hydroxytolbutaminde-d9, 4′-carboxytolbutamide-d9, 4-hydroxymidazolam, 4-hydroxymidazolam-d5, 6-hydroxychlorzoxazone and 12-hydroxydodecanoic-d20 acid were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Hydroxychlorzoxazone-d2 was obtained from TLC PharmaChem (Mississauga, ON, Canada). Midazolam and 1′-hydroxymidazolam-d4 were purchased from Cerilliant (Round Road,

TX, USA). Chlorzoxazone, tolbutamide, dodecanoic acid, β-Nicotinamide-Adenine Dinucleotide Phosphate (NADP), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), dimethyl sulfoxide (DMSO), trishydroxymethylaminomethame (TRIS), and phenylmethanesulfonyl (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethynediaminetetraacetic acid (EDTA) and dithiothreitol were obtained from Bishop (Burlington, ON, Canada) and from Gibco®, Life Technologies Ltd. (Eugene, OR, USA), respectively. All other chemicals used were commercially available and were of analytical grade.

2.2. Animals

Five-week-old male C57BL/6 mice (*n* = 45) were purchased from Charles River Laboratories (Montreal, QC, Canada). Animals were housed in a temperature-, light-, and humidity-controlled environment, they were housed 2 per cage and were maintained at an ambient temperature of 21 °C on a 12-h light/dark cycle with free access to water and food ad libitum. The obese diabetic mice were developed according to the experimental protocol described previously [51]. Briefly, one week after their arrival, mice (mean weight 20.0 ± 1.0 g) were fed with a high-fat diet (HFD) (Bio-Serv Diet #F3282, Frenchtown, NJ, USA, 60% fat by energy) or the standard normal diet (ND) (Teklad Global 18% protein diet; Harlan Teklad, Madison, WI, USA, 15% fat by energy) for 8 weeks. Body weight was measured weekly while blood glucose and insulin were determined at Week 8. After 8 weeks of diet, HFD mice were stratified according to their body weight and two groups were formed as follows; low responders to HFD (LDR) (<39.9 g) and high responders to HFD (HDR) (39.9–45 g). Peyot et al. have demonstrated that the LDR are less obese, develop intermediate severity of insulin resistance and have mild impairment in glycemia, while the HDR are more obese, insulin resistant, hyperinsulinemic and hyperglycemic [51]. Animals were sacrificed by cervical dislocation, and organs including heart, lungs, kidneys and liver were quickly excised, washed with cold TRIS 100 mM buffer (pH 7.4) and immersed in liquid nitrogen (−80 °C). Experimental protocols were approved by the institutional committee of animal protection and were carried out in accordance with the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

2.3. CYP450 mRNA Levels

2.3.1. Isolation of RNA and Preparation of cDNA

For each organ tested (using a pool of 3 mice/organ), about 100 mg of tissue was homogenized in 1 mL of Trizol and incubated for 5 min at room temperature. Chloroform (200 μL) was added, the mixture shaken for 15 s and then, centrifuged at $16,000 \times g$ for 30 min at 4 °C. The aqueous supernatant (500 μL) was transferred and ethanol 70% was added (1:1 *v/v*). RNA was extracted using the Qiagen kit (RNeasy Mini kit; Qiagen Sciences, MD, USA) according to the manufacturer's recommendations. RNA concentration and quality was assessed by spectrometry. Total RNA (2 μg) from each sample was used for reverse transcription. RNA, random primers (6 μg) and dNTP (25 mM) were preheated for 5 min at 65 °C. Then, 5X-first strand buffer, 80 units of RNAse inhibitor, DTT (0.01 M) and 400 units of Superscript II (Invitrogen, Carlsbad, CA, USA) were added to a final volume of 40 μL. Reverse transcription was carried out for 50 min at 42 °C and stopped by heating to 70 °C for 15 min (final RNA concentration 50 ng/μL). The resulting cDNA was frozen at −80 °C until analyzed.

2.3.2. RT-qPCR Analysis

Real-time quantitative PCR was performed using TaqMan® probe and primer sets from Applied Biosystem (Foster, CA, USA). The assay IDs for selected cyp450s were: cyp2b9 (Mm00657910_m1), cyp2b10 (Mm01972453_s1), cyp2c29 (Mm00725580_s1), cyp2c37 (Mm00833845_m1), cyp2c39 (Mm04207909_g1), cyp2c40 (Mm04204172_mH), cyp2d9 (Mm00651731_m1), cyp2d10 (Mm00731648_m1), cyp2d22 (Mm00530542_m1), cyp2e1 (Mm00491127_m1), cyp2j5 (Mm00487292_m1), cyp2j6 (Mm01268197_m1), cyp3a11 (Mm00731567_m1), cyp3a13 (Mm00484110_m1), cyp3a25

(Mm01209536_m1) and cyp4a10 (Mm01622743_g1). As reference genes, gapdh (Mm99999915_g1) and b2m (Mm00437762_m1) were used as housekeeping genes. cDNA was diluted to 10 ng/reaction, mixed with TaqMan® PCR Master Mix (10 μL) and amplified using cycling conditions as follows: 45 cycles consisting of 10 s at 95 °C and 45 s at 60 °C. Reactions were run in a QuantStudio 6 Flex System (Life Technologies Inc., Burlington, ON, Canada).

The relative quantification of various gene expressions was calculated to the comparative CT method using the formula $2^{-\Delta CT}$ [52,53]. Only CT values ≤ 35 were included in the analyses. Since CT values > 35 were not reliable and considered below the detection level of the assay, a CT value of 35 to 38 was defined not quantifiable (NQ) while a value of CT > 38 as not detectable (ND). For their part, mRNA levels associated with the expression of each isoenzyme under a specific diet condition (ND, LDR, HDR) were determined using a calibrator and the following formula $2^{-\Delta\Delta CT}$ [52]. The calibrator was prepared at the same mRNA concentration using a pool of RNA obtained for each tissue (Clontech A Takara, Bio Company, Mountain View, CA, USA). Determination of mRNA levels was performed in triplicate for each sample, and three independent experiments were repeated to confirm results.

2.4. In Vitro CYP450 Metabolism in Liver and Extra-Hepatic Organs

2.4.1. Preparation of Microsomes

Microsomes from liver, kidneys, lungs and heart were prepared according to our previously described methods with slight modifications [54]. Briefly, tissue (pools of organs from 3 mice) was homogenized in an ice-cold buffer consisting of 50 mM-150 mM-1 mM TRIS-KCL-EDTA buffer (liver and kidneys) or 100 mM-150 mM-1 mM PO$_4$-KCL-EDTA buffer (lungs and heart) and both buffers containing protease inhibitors namely, PMFS (0.01 mM) and DTT (0.5 mM). Microsomal subcellular fraction was prepared by centrifugation (10,000 $g \times$ 20 min, at 4 °C) followed by ultra-centrifugation (100,000 $g \times$ 90 min, at 4 °C). The microsomal pellets were resuspended in the same buffer (without PMSF and DTT), and frozen at -80 °C until in vitro metabolism experiments were performed. The protein concentration of the microsomes was determined by the Bradford method using bovine serum albumin as the standard.

2.4.2. Effects of the HFD on Hepatic and Extra-Hepatic cyp450 Activities

In order to investigate the effects of the HFD as a representative model of type 2 diabetes on cyp450 activities, in vitro incubations were performed in presence of various microsomes with several probe drugs of CYP450s including bupropion, repaglinide, tolbutamide, bufuralol, chlorzoxazone, ebastine, midazolam and dodecanoic acid, which were used as markers of the functional orthologs of human CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2J2, CYP2E1, CYP3A4/5 and CYP4A11, respectively. This study employed cocktails of probe substrates already accepted and validated to investigate the impact of diabetes on specific cyp450 activities. The production of each specific metabolite was quantified from substrate probes and variations of in vitro probes reactions can be inferred to affect all substrates metabolized by the same enzymes.

Assay conditions were previously optimized by standard incubations with probes (buffer, incubation period, protein contents and drug concentrations). All incubations were performed in triplicate. The incubation mixture containing microsomes [5 μL for liver (~20 mg proteins/mL) and 50 μL for kidney, lungs and heart microsomes (~7–14 mg proteins/mL)], NADPH-regenerating system solution (NADP 6.5 mM, G6P 16.5mM, MgCl$_2$ 5 mM and 0.2 U G6PD) and 100 mM phosphate buffer PO$_4$ (pH 7.4) or 100 mM TRIS buffer (pH 7.4, for tolbutamide and dodecanoic acid) were pre-incubated in a shaking bath for 10 min at 37 °C. Reaction was initiated by the addition of substrates (bupropion, tolbutamide, bufuralol, chlorzoxazone, midazolam, ebastine, dodecanoic acid or repaglinide) to the incubation mixture (total final volume of 500 μL). Bupropion, chlorzoxazone, ebastine and midazolam were incubated together as a cocktail as previously described whereas the other probe substrates were tested separately. The substrate concentrations used span a range from 38–620 μM for bupropion,

2.5–40 µM for bufuralol, 50–800 µM for chlorzoxazone, 0.125–2 µM for ebastine, 1.25–20 µM for dodecanoic acid, 0.25–4 µM for midazolam, 0.85–14 µM for repaglinide and 25–400 µM for tolbutamide (5 different concentrations were used with liver microsomes while one concentration (>2 km) to ensure saturation was selected to investigate cyp450 activities in extra-hepatic microsomes). After 30 min, the reaction was stopped using 1000 µL of ice-cold internal methanol containing isotope-labeled internal standard probe metabolite(s). Reaction mixtures were put on ice for 10 min, and following a centrifugation at 13,000 rpm for 10 min, and then the supernatant was transferred for analysis. For dodecanoic acid metabolite analysis, the solution was evaporated to dryness at 50 °C under a gentle stream of nitrogen, reconstituted with 200 µL of methanol and transferred to an injection vial for analysis.

2.5. High Performance Chromatography–Mass Spectrometry Analytical Methods

2.5.1. Chromatographic Conditions for the Metabolites of Bupropion, Midazolam and Ebastine

This analysis was performed on a Thermo Scientific Acclaim RSLC Polar Advantage C16 column (75 mm × 3.0 mm, 3 µm) and Phenomenex Security Guard Cartridge (C12, 4 mm × 2 mm) operating at 50 °C. The mobile phase was a gradient elution consisting of (A) 0.1% formic acid in acetonitrile and (B) 10 mM ammonium formate in water adjusted to pH 3; ratio A:B varied from 25:75 to 60:40 (*v/v*), at a flow rate 500 µL/min (a total run time of 10 min). A 10 µL aliquot of the extract was injected into LC-MSMS system.

A Thermo Scientific TSQ Quantiva Triple Quadrupole mass spectrometer (San Jose, CA, USA) was interfaced with a Thermo Scientific Ultimate 3000 XRS UHPLC system (San Jose, CA, USA) using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode using selected reaction monitoring (SRM). Selection of optimal transitions and collision energy and tube lens voltage conditions for the metabolites and their respective internal standard are listed in Table S1.

2.5.2. Chromatographic Conditions for the Metabolites of Chlorzoxazone, Tolbutamide, Dodecanoic Acid, Bufuralol and Repaglinide

These analyses were carried out on a Phenomenex Luna PFP (2) column (150 mm × 3.0 mm I.D., 3 µm) with a Phenomenex PFP security guard cartridge operating at 40 °C. A mobile phase in isocratic mode was composed of acetonitrile and 0.01% formic acid having a fixed ratio of 40:60 (*v/v*) for chlorzoxazone, tolbutamide and dodecanoic acid, while a ratio of 50:50 was used for bufuralol and repaglinide. The flow rate was 0.30 mL/min (total run time 10 min). A 10 µL aliquot of the extract was injected into the LC-MSMS system.

The HPLC system consisted of a Shimadzu Prominence series UFLC pump and auto sampler (Kyoto, Japan). The tandem MS system used was a Thermo TSQ Quantum Ultra (San Jose, CA, USA). The mass spectrometer was interfaced with the HPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in negative ion mode for chlorzoxazone, tolbutamide, and dodecanoic acid metabolites, and in positive ion mode for bufuralol and repaglinide metabolites using selected reaction monitoring (SRM). Selection of optimal transitions and collision energy and tube lens voltage conditions for the metabolites and their respective internal standard are shown in Table S2.

2.6. Statistical Analysis

Calibration curves were calculated from the equation $y = ax + b$, as determined by weighted $1/x$ and $1/x^2$ linear regressions of the calibration lines constructed from the peak-area ratios of metabolites to the internal standard (XCalibur software, Thermo Fisher, San Jose, CA, USA). Relative expression levels of CYP450s mRNAs were analyzed by one-way analysis of variance followed by the Dunnett post-hoc test. A difference with $p < 0.05$ was considered statistically significant. Enzyme kinetic

parameters were determined by non-linear regression analysis using Michaelis–Menten equation and Lineweaver–Burk double reciprocal plot and data points were expressed as the mean ± S.D., K_m and V_{max} values and the 95% confidence interval for the intrinsic clearance. Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and SAS statistical software (Version 9.4 of the SAS System for Windows, Copyright©, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Animal Model

Although no animal model exactly reflects human T2D, some have similar features. Human T2D is a heterogeneous disorder with a complex interplay between genetic, epigenetic and environmental factors. On one hand, diabetic animal models including chemically-induced or surgically-provoked develop hyperglycemia primarily by cytotoxic actions on beta cells rather than through insulin resistance. On the other hand, transgenic/knockout model are more useful to investigate the role of a specific candidate gene unlike heterogeneity as seen in humans. Moreover, the observed diabetes conditions are less stable; chemicals can produce toxic actions and development of digestive problems can be observed which could also affect CYP450 activities. Consequently, a validated nutritionally (high-fat diet; HFD) obese mouse model has been selected to characterize the effects of obesity-induced diabetes on CYP450 activities [51].

Weight, glycemia and insulinemia measured at week 8 were as follow; 29.2 ± 1.1 g, 9.7 ± 0.3 mmol/L and 1.37 ± 0.35 ng/mL in the chow-fed control group (n = 12); 36.4 ± 0.9 g, 9.4 ± 0.5 mmol/L and 2.59 ± 0.44 ng/mL for the LDR group (n = 12) and 43.1 ± 0.6 g, 10.5 ± 0.4 mmol/L and 6.82 ± 1.32 ng/mL, for the HDR group (n = 16), respectively. Weight, glycemia and insulinemia parameters were significantly higher for HFD groups compared to the control group (p < 0.05). Five mice were considered as extreme responders (outliers) since their weight was over 45.0 g and were excluded from our analysis.

3.2. Effects of HFD on cyp450 mRNA Expression Levels

3.2.1. General Pattern of cyp450 Expression

The relative expression of total cyp450 mRNA levels for 15 cyp450 isoforms found in hepatic and extra-hepatic tissues are illustrated for chow-fed control group (ND) and HFD groups in Figure 1 (and Table S3). Major differences were observed in the expression pattern of various cyp450s among tissues. On the other hand, the pattern of cyp450 expression was rather similar among the different diet group: hence, the HFD did not change the pattern of relative cyp450 expression levels in a specific organ. The highest relative levels of mRNAs were cyp2e1 in the liver, cyp2j5/cyp2e1 in kidneys, and cyp2d22 in both heart and lung tissues. The cyp2d, cyp2e and cyp2j subfamilies were expressed in all organs tested (i.e., liver, kidneys, heart and lungs). Moreover, high levels of cyp2b10 (representing approximately 20% of cyp450 mRNA expression) were also observed in the lungs.
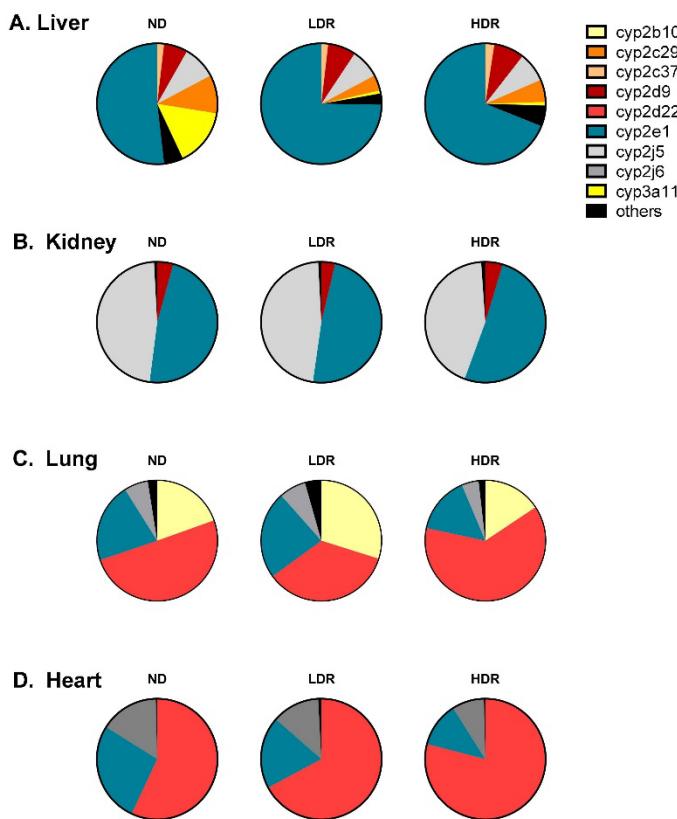
Figure 1. Cyp450 mRNA pie charts. Total mRNA transcripts for each isoenzyme are displayed as expressed in C57BL6 mouse microsomes according to diet group: (**A**) Liver; (**B**) Kidney; (**C**) Lung; and (**D**) Heart. ND, Normal diet; LDR, low-diet responders; and HDR, high-diet responders. Cyp450 mRNA transcript with a relative contribution >1% are illustrated, and "others" have a relative contribution <1%. Others include the following isoforms: Liver, cyp2b10, cyp2b29, cyp2c39, cyp2c40, cyp2d22, cyp2j6, cyp3a13, cyp3a25, and cyp4a10; Kidneys, cyp2b10, cyp2b29, cyp2c29, cyp2c37, cyp2c39, cyp2c40, cyp2d22, cyp2j6, cyp3a11, cyp3a13, cyp3a25, and cyp4a10; and Lungs, cyp2b29, cyp2c29, cyp2c37, cyp2c39, cyp2c40, cyp2d9, cyp2j5, cyp3a11, cyp3a13, cyp3a25, and cyp4a10.

3.2.2. Modulation of cyp450 mRNA Expression by HFD

Table 1 presents the relative mRNA transcripts for each cyp450 isoform found in each individual organ under HFD (LRD and HRD) vs. normal chow-diet (ND). Overall, our results showed that HFD altered the profile of mRNA expression in an isoenzyme-specific manner. Moreover, HFD altered the expression profile of cyp450 mRNAs in a tissue-specific fashion. Our major findings were: (1) mRNA levels of cyp3a11 were significantly decreased (approximately 14-fold) in the liver of both HFD groups (LRD and HRD) compared to ND ($p < 0.001$); (2) in contrast, a two-fold increase of hepatic mRNA levels of cyp2b9 ($p < 0.001$), cyp2c39 ($p < 0.01$) and cyp4a10 ($p < 0.05$) were observed in HDR compared to ND; and (3) cyp2b10 ($p < 0.01$) was also increased by two folds in the lungs.

Table 1. Relative mRNA transcripts for each cyp isoform in C57B/6 mice microsomes according to diet group.

Tissue	Diet	cyp2b9	cyp2b10	cyp2c29	cyp2c37	cyp2c39	cyp2c40	cyp2d9	cyp2d22	cyp2e1	cyp2j5	cyp2j6	cyp3a11	cyp3a13	cyp3a25	cyp4a10
	ND	0.01	0.02	0.49	1.31	0.01	0.44	0.55	0.53	0.56	0.98	1.08	0.58	2.40	0.44	1.25
Liver	LDR	4.89 *	NQ	0.25 *	1.77	0.01	0.20	0.81	0.59	1.02	1.05	0.88	0.04 †	3.07	0.17	1.22
	HDR	8.51 †	0.03	0.30	2.15	0.02 **	0.37	0.71	1.03	0.79	0.90	1.09	0.04 †	2.96	0.21	3.72
	ND	ND	0.54	2.35	ND	ND	8.62	1.65	1.32	1.28	4.89	4.56	1.41	1.37	2.00	1.42
Kidney	LDR	ND	0.46	3.08	ND	ND	3.08	1.35	1.38	1.29	4.68	4.62	0.57	2.12	1.79	1.13
	HDR	ND	0.42	1.59	ND	ND	5.92	1.65	1.50	1.29	4.18	4.03	0.72	2.41	0.95	2.43
	ND	ND	1.21	0.26	ND	ND	ND	0.05	3.24	0.96	0.09	2.19	ND	NQ	ND	1.09
Heart	LDR	ND	2.16	0.49	ND	ND	ND	0.07	3.40	0.62	0.19	1.85	ND	NQ	ND	2.15
	HDR	ND	1.68	0.30	ND	ND	ND	0.07	5.03	0.51 *	0.19	1.67	ND	NQ	NQ	1.47
	ND	ND	0.73	1.11	ND	ND	ND	0.07	2.06	0.21	0.13	1.20	ND	3.26	0.06	3.28
Lung	LDR	ND	1.66 †	2.96	ND	ND	ND	0.08	2.05	0.33 **	0.47	1.88	ND	8.15 **	0.07	9.90 *
	HDR	ND	1.17 **	1.00	ND	ND	ND	ND	5.76	0.32 *	0.83	1.69	ND	5.39	0.11	3.43

Results are expressed as mean N-fold differences in cyp gene relative to the average expression of housekeeping genes and a calibrator ($2^{-\Delta\Delta Ct \, sample}$). ND, Normal diet; LDR, low-diet responders; and HDR, high-diet responders. NQ = Not Quantifiable ($35 < Ct < 38$), ND = Not Detectable ($Ct > 38$). Each experiment was performed three times and in triplicates. One-way ANOVA was performed with Dunnett post-hoc test. LDR or HDR versus ND; * $p < 0.05$, ** $p < 0.01$, † $p < 0.001$.

3.3. Modulation of cyp450 Hepatic Activities by DIO Mouse as a Model of T2D

3.3.1. Hepatic Activities

As shown in Figure 2 (Table 2), HFD induced variations in hepatic cyp450 activities in an isoform-dependent manner. A significant decrease in midazolam metabolism, a marker of cyp3a subfamily, was observed following HFD treatment; the intrinsic clearance of 1-hydroxymidazolam was reduced in LDR and HDR (23 and 40 µL/min/mg prot) groups compared to ND (107 µL/min/mg prot) ($p < 0.001$) (Table S4). This was mostly explained by a decrease in V_{max}; formation of 1-hydroxymidazolam decreased from 0.32 (ND) to 0.06 nmol/mg protein/min (LDR and HDR, $p < 0.001$) (Table 2). HFD treatment was also associated with a diminished hepatic activity of cyp2c subfamilies compared to ND group. The intrinsic clearance of tolbutamide (used as a probe of CYP2C9 in human) was significantly reduced from 0.80 to 0.54 and 0.57 µL/min/mg prot in ND, LDR and HDR groups, respectively ($p < 0.05$) (Table S4). Similarly, HFD affected also the repaglinide hydroxylation (CL_{int} to M1-hydroxyrepaglinide) from 10.6 µL/min/mg prot in ND compared to 1.0–1.4 µL/min/mg prot in HFD groups) (Table S4). Cyp2b activity as measured by bupropion hydroxylation tended to be slightly decreased in HDR group (CL_{int} in HDR vs. ND reduced by 22%). In contrast, no significant effect was observed on the hepatic hydroxylation of bufuralol (cyp2d), dodecanoic acid (cyp4a), chlorzoxazone (cyp2e1) and ebastine (cyp2j) as demonstrated by comparable pharmacokinetics values (Table 2).
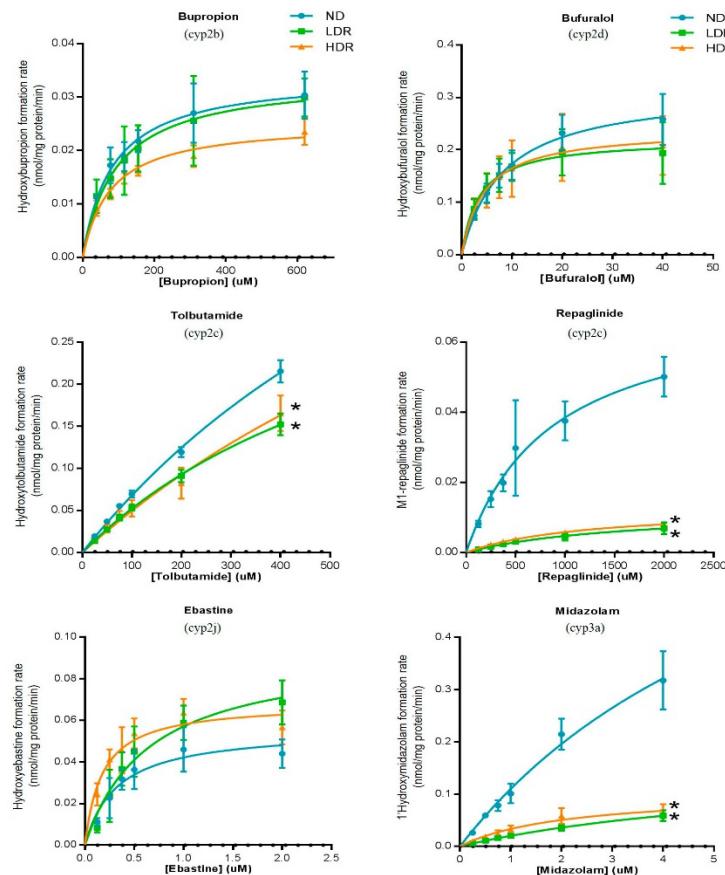

Figure 2. *Cont.*
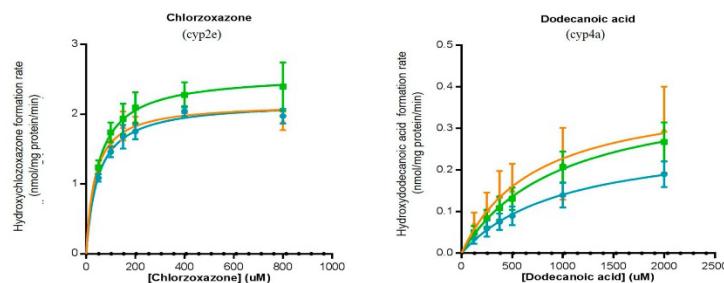
Figure 2. Hepatic cyp450 activities of cyp2b (bupropion), cyp2c (tolbutamide and repaglinide), cyp2d (bufuralol), cyp2e (chlorzoxazone), cyp2j (ebastine), cyp3a (midazolam) and cyp4a (dodecanoic acid) in C57BL6 mice fed a normal diet (ND) or a HFD (LDR, low-diet responders; and HDR, high-diet responders after an eight-week period of treatment). Data were expressed as the mean ± S.D. LDR or HDR vs. ND; * $p < 0.05$. N/F; activity not found, N/A; Not Available (in vitro incubation could not be determined) and dotted line represents the limit of quantification.

Table 2. Hepatic microsome activities for cyp2b (bupropion), cyp2c (tolbutamide and repaglinide), cyp2d (bufuralol), cyp2e (chlorzoxazone), cyp2j (ebastine), cyp3a (midazolam) and cyp4a (dodecanoic acid) in C57BL6 mice fed a normal diet (ND) or a HFD (LDR, low-diet responders; and HDR, high-diet responders after an eight-week period).

Liver	ND	LDR	HDR
	(nmol/mg protein/min)		
Bupropion → Hydroxybupropion	0.030 ± 0.004	0.030 ± 0.004	0.023 ± 0.002 **
Tolbutamide → Hydroxytolbutamide	0.22 ± 0.01	0.15 ± 0.01 ᵗ	0.17 ± 0.02 ᵗ
Repaglinide → M1-repaglinide	0.050 ± 0.006	0.007 ± 0.002 ᵗ	0.0081 ± 0.0003 ᵗ
Repaglinide → Hydroxyrepaglinide	0.0020 ± 0.0001	0.0010 ± 0.0002 ᵗ	0.0013 ± 0.0004 **
Bufuralol → Hydroxybufuralol	0.26 ± 0.05	0.19 ± 0.06	0.21 ± 0.06
Chlorzoxazone → Hydroxychlorzoxazone	2.0 ± 0.1	2.4 ± 0.3 **	1.9 ± 0.1
Ebastine → Hydroxyebastine and carebastine	0.044 ± 0.007	0.07 ± 0.01 ᵗ	0.057 ± 0.008 *
Midazolam → 1′-hydroxymidazolam	0.32 ± 0.06	0.06 ± 0.01 ᵗ	0.06 ± 0.01 ᵗ
Dodecanoic acid → 12-hydroxydecanoic acid	0.19 ± 0.03	0.27 ± 0.05	0.3 ± 0.1 *

Cyp450 activities are reported as the rate of metabolite formation in nmol/mg protein/min ± SD (* $p < 0.05$, ** $p < 0.01$, ᵗ $p < 0.001$ compared to ND).

3.3.2. Extra-Hepatic Activities

Figure 3 (Table 3) illustrates the effects of diabetes induced by HFD on the formation rate of cyp450 probe metabolites measured in extra-hepatic tissues. A tissue-dependent modulation of cyp450 activities by HFD was observed. In the kidneys, HFD treatment produced a significant decrease in cyp3a activity; formation rate of 1-hydroxymidazolam was three- and five-times lower in LDR and HDR groups, respectively, compared to ND group ($p < 0.001$) (Table 3(A)). In contrast, the cyp3a activity measured in the lung was not affected by the HFD indicating a tissue-dependent modulation of cyp450 by HFD. No cyp3a activity was detectable in mouse hearts.

Our data showed that the formation of hydroxybupropion was approximately 20–50-times greater in the lungs compared to the liver and kidneys (regardless of the diet groups, $p < 0.0001$) (Table 3(B)). Renal and lung microsomes displayed a slight decrease in the hydroxylation of bupropion in HDR group vs. control diet group ($p < 0.01$) (Figure 3). Although the magnitude of activity was low, a similar observation was made for the hydroxylation of bupropion in the heart (0.150 vs. 0.037 and 0.025 pmol/mg protein/min in ND vs. LDR and HDR, respectively, $p < 0.001$) (Table 3(C)). In addition, ebastine (cyp2j) and dodecanoic acid (cyp4a) metabolisms were reduced by HFD, particularly in HDR, in renal and lung tissues. Ebastine hydroxylation in heart microsomes was not affected by HFD, whereas cyp4a activity could not be detected. In contrast, our results showed that DIO mouse were

associated with an increase in tolbutamide (~20–50%) and bufuralol (~90–110%) metabolic activities in kidneys, while in lung microsomes, the hydroxylation of tolbutamide and bufuralol tended to decrease ($p < 0.01$ and $p < 0.001$, respectively, in HDR group). Overall, cyp450 activities tended to be reduced in HFD group with greater effects being observed in HDR.
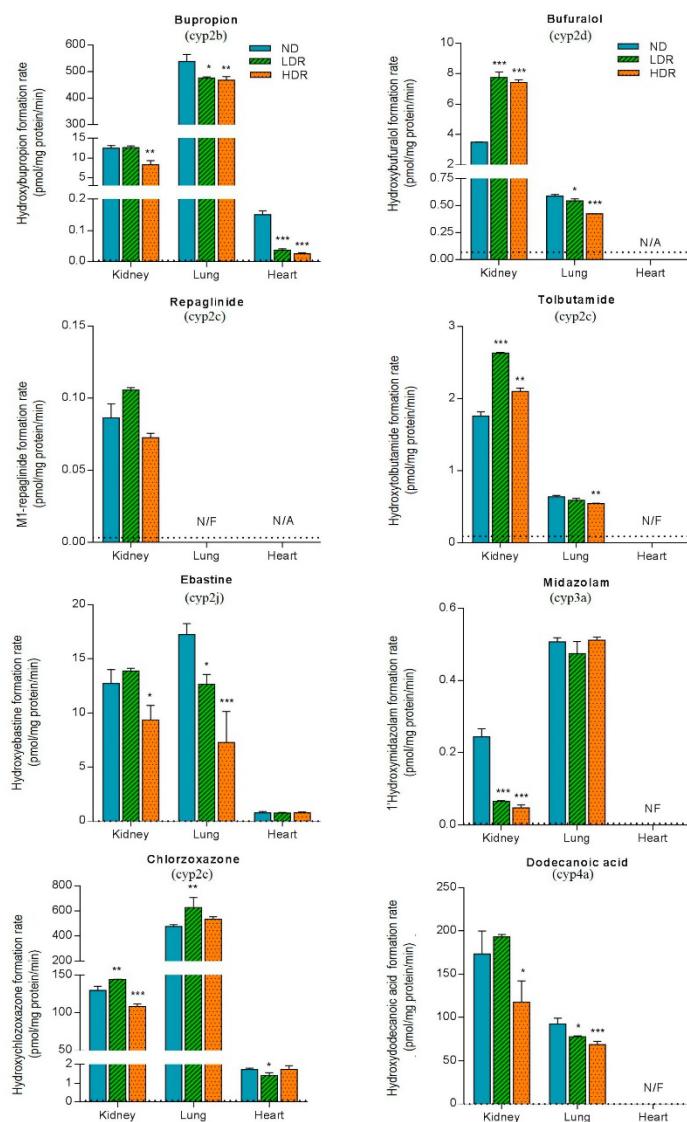

Figure 3. Extrahepatic cyp450 microsomal activities for cyp2b (bupropion), cyp2c (tolbutamide and repaglinide), cyp2d (bufuralol), cyp2e (chlorzoxazone), cyp2j (ebastine), cyp3a (midazolam) and cyp4a (dodecanoic acid) measured in the kidneys, lungs and heart in C57BL6 mice fed a normal diet (ND) or a HFD (LDR, low-diet responders; and HDR, high-diet responders after an eight-week period). Bars and error bars represent the mean ± SD, respectively. LDR or HDR vs. ND; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N/F; activity not found, N/A; Not Available (in vitro incubation could not be determined) and dotted line represents the limit of quantification.

Table 3. Extra-hepatic microsome activities for cyp2b (bupropion), cyp2c (tolbutamide and repaglinide), cyp2d (bufuralol), cyp2e (chlorzoxazone), cyp2j (ebastine), cyp3a (midazolam) and cyp4a (dodecanoic acid) in C57BL6 mice fed a normal diet (ND) or a HFD (LDR, low-diet responders; and HDR, high-diet responders after an eight-week period): (**A**) renal microsomes; (**B**) lung microsomes; and (**C**) heart microsomes.

(A)			
Kidney	**ND**	**LDR**	**HDR**
	(pmol/mg protein/min)		
Bupropion → Hydroxybupropion	12.5 ± 0.6	12.6 ± 0.4	8 ± 1 **
Tolbutamide → Hydroxytolbutamide	1.76 ± 0.06	2.63 ± 0.01 ‡	2.10 ± 0.04 **
Repaglinide → M1-repaglinide	0.09 ± 0.01	0.105 ± 0.002	0.073 ± 0.003
Bufuralol → Hydroxybufuralol	3.50 ± 0.03	7.7 ± 0.4 ‡	7.4 ± 0.2 ‡
Chlorzoxazone → Hydroxychlorzoxazone	130 ± 6	144.6 ± 0.2 **	108 ± 3 **
Ebastine → Hydroxyebastine and carebastine	13 ± 1	13.9 ± 0.3	9 ± 1 *
Midazolam → 1′-hydroxymidazolam	0.24 ± 0.02	0.065 ± 0.002 ‡	0.047 ± 0.008 ‡
Dodecanoic acid → 12-hydroxydecanoic acid	173 ± 27	193 ± 3	118 ± 24 *

(B)			
Lung	**ND**	**LDR**	**HDR**
	(pmol/mg protein/min)		
Bupropion → Hydroxybupropion	538 ± 26	476 ± 4 *	467 ± 14 **
Tolbutamide → Hydroxytolbutamide	0.64 ± 0.02	0.59 ± 0.03	0.54 ± 0.01 **
Repaglinide → M1-repaglinide	N/F	N/F	N/F
Bufuralol → Hydroxybufuralol	0.59 ± 0.02	0.55 ± 0.02 *	0.425 ± 0.001 ‡
Chlorzoxazone → Hydroxychlorzoxazone	477 ± 16	629 ± 79 **	535 ± 19
Ebastine → Hydroxyebastine and carebastine	17 ± 1	12.6 ± 0.9 *	7 ± 3 ‡
Midazolam → 1′-hydroxymidazolam	0.51 ± 0.01	0.47 ± 0.04	0.51 ± 0.01
Dodecanoic acid → 12-hydroxydecanoic acid	93 ± 6	78 ± 1 *	68 ± 4 ‡

(C)			
Heart	**ND**	**LDR**	**HDR**
	(pmol/mg protein/min)		
Bupropion → Hydroxybupropion	0.15 ± 0.01	0.037 ± 0.004 ‡	0.025 ± 0.003 ‡
Tolbutamide → Hydroxytolbutamide	N/F	N/F	N/F
Repaglinide → M1-repaglinide	N/A	N/A	N/A
Bufuralol → Hydroxybufuralol	N/A	N/A	N/A
Chlorzoxazone → Hydroxychlorzoxazone	1.7 ± 0.1	1.2 ± 0.1 *	1.7 ± 0.2
Ebastine → Hydroxyebastine and carebastine	0.8 ± 0.1	0.76 ± 0.06	0.78 ± 0.06
Midazolam → 1′-hydroxymidazolam	N/F	N/F	N/F
Dodecanoic acid → 12-hydroxydecanoic acid	N/F	N/F	N/F

Cyp450 activities determined at 3 km are reported as the rate of metabolite formation in pmol/mg prot/min ± SD (* $p < 0.05$, ** $p < 0.01$ and ‡ $p < 0.001$ compared to ND). N/F, activity not found; N/A, Not Available (in vitro incubation could not be determined).

4. Discussion

This study demonstrated that expression and activities of major cyp450s involved in the metabolism of drugs were modulated in DIO C57BL6 mice used as a model of T2D (Table S5). First, we demonstrated that cyp3a expression and activities were decreased by HFD. Second, cyp2c activities were reduced in all organs tested (except for tolbutamide in the kidneys). Finally, cyp2b activity, largely expressed in the lungs, was also decreased by HFD.

C57BL/6 mice were divided into three groups: ND, LDR and HDR. LDR were less obese and developed intermediate severity of insulin resistance, while HDR were more obese and developed severe insulin resistance. The extent of changes in cyp450 activities by HFD tended to be gradually decreased in LDR compared to HDR and some effects being only observed in HDR group. This observation suggests that modulation of some cyp450 activities happens at early stage

of pre-diabetes and similar pathways of regulation involved in pre-diabetes development can also intervene on cyp450 activities in an isoform-dependent manner.

Obesity and diabetes have been shown to alter the expression and activity of hepatic CYP450s [14,28,29,35–38,40–42,55–57]. Clinical studies and animal experiments report mostly on hepatic CYP3A since it is the most important isoenzyme involved in the metabolism of prescribed drugs [58,59]. In humans, a significant decrease in CYP3A activity has been reported in diabetic human liver microsomes compared to healthy subjects [56], while a significant increase in CYP2E1 activity has been reported in obese T2D human liver microsomes [33]. In our study, we observed a significant decrease in hepatic cyp3a mRNA expression levels in HFD groups compared to normal diet group. Similarly, decreased levels of cyp3a11 mRNA expression have been reported in DIO mice [38,59,60]. Preliminary results from our clinical study using oral CYP450 probe cocktail demonstrate that oral clearance of midazolam was significantly reduced in subjects with T2D compared to subjects without T2D [61]. Our results on RNA transcripts are also consistent with phenotypic findings showing that cyp3a activity determined via hydroxylation of midazolam was significantly reduced in the two HFD groups compared to control group. In agreement with our finding, CYP3A activity has been reported to be also decreased in Zucker diabetic fatty (ZDF) rats using midazolam and testosterone as probes [40].

There are no or very limited data pertaining to the effects of T2D on cyp2c and cyp2b families. We observed that cyp2c and cyp2b subfamilies mRNA expression levels were not changed in the liver by HFD compared to normal group, except for cyp2b9 which was increased in HFD groups. Our results are in agreement with those reported by Yoshinari et al. and Guo et al. who demonstrated unchanged relative mRNA levels of cyp2c and cyp2b subfamilies in the liver [59,60]. In contrast, it has been reported a decrease of cyp2b10 mRNA levels in CD1 mice fed with HFD [38]. However, the relative hepatic levels of cyp2b10 mRNA measured in our study were very low or below the limit of quantification yielding a comparison analysis unreliable. In addition, our results showed that hepatic cyp2c metabolic activities, using repaglinide and tolbutamide as markers, were significantly decreased in HFD groups. Kim et al. reported that CYP2C catalytic activity determined with diclofenac was decreased in chemically induced diabetes in rats [62]. This finding, in agreement with our study, suggest that CYP2C activities are impaired, thereby lower metabolic clearance can be anticipated for drugs metabolized by CYP2Cs, particularly CYP2C8 or CYP2C9, under conditions associated with pre-diabetes or diabetes.

Our results indicate that cyp2e1 mRNA expression levels and activity remained unchanged and comparable among all groups (HFD and ND). In the same way, expression levels of cyp2e1 mRNA were reported to be unchanged in DIO mice [38,59]. In addition, activity of cyp2e1 was also unaffected in DIO and db/db mice [37,38], but increased activity was shown in ZDF [42]. No alteration in hepatic levels of cyp2e1 was found in streptozocin-induced diabetic mice [41]. These differences in cyp2e1 activity modulation could be function of the animal model of diabetes used and the stage of the disease.

Our data demonstrated a tissue-specific modulation of cyp2b activities by HFD. Indeed, no significant difference was observed for the hydroxylation of bupropion in liver microsomes. However, bupropion hydroxylation was significantly decreased in HFD groups, particularly in the lungs, the heart and kidneys (HDR group only).

To our knowledge, no study has been conducted to assess the impact of HFD induced obesity as a model of early diabetes stage in humans on CYP450s expression or activity in extrahepatic tissues. Although the magnitude of metabolic capacity and significance in total body clearance of extrahepatic CYP450s metabolism are much lower in comparison to hepatic CYP450s, extrahepatic CYP450s metabolism may affect the local exposure to xenobiotics and thus, influence their pharmacological and toxicological effects. For instance, renal cyp450 metabolites of arachidonic acid, 20-HETE and EET, play an important role in the control of blood pressure and the development of acute kidney injury [63]. In fact, arachidonic acid is metabolized by CYP4A and CYP4F families to 20-HETE (vasoconstrictor) and by CYP2C and CYP2J families to EETs (vasodilator). Our results showed a significant decrease in renal activity of cyp4a and cyp2j in the HDR group using dodecanoic acid and ebastine hydroxylation

as marker, respectively. This finding indicates that diabetes could influence homeostasis by affecting local biotransformation of endogenous compounds. In high fat diet induced hypertension rats, CYP2C and CYP4A activities were found to be decreased [64]. The discrepancy observed for CYP2C activity can be explained by using different substrate markers (arachidonic acid being not specific for CYP2C but, also a CYP2J substrate).

Little is known about the effects of obesity and diabetes on CYP450 catalytic activities in the lungs, in both humans and animals. Our data showed extensive metabolic activities in lung microsomes for midazolam, bupropion, ebastine and chlorzoxazone corresponding to cyp3a, cyp2b, cyp2j and cyp2e1, respectively. In lung microsomes, HFD was associated with modulation of cyp450 catalytic activity for certain isoforms independently of patterns observed in the liver or in the kidneys. These findings support the concept that CYP450s expressed in the lungs may contribute to drug metabolism for drugs administered intravenously or locally as well as contribute to the first pass metabolism.

In conclusion, the major finding of this study was that HFD affects CYP450 expression and activities in an isoform- and tissue-dependent fashion. Our results clearly indicate that cyp3a and cyp2c metabolic activities were reduced in DIO-T2D mice (Table S5). In humans, these two CYP450 isoenzymes are involved in the metabolism of 80% of medications prescribed in clinical settings. We speculate that modulation of hepatic CYP450s by T2D diabetes may alter drug pharmacokinetics leading to intersubject variability in drug response. In addition, modulation in CYP450s expressed in extra-hepatic organs can cause variations in tissue concentrations of drugs or endogenous compounds leading to impaired pharmacological action of drugs as well as disruption of homeostasis. Therefore, variation in hepatic and extra-hepatic CYP450s makes patients with pre-diabetes and obesity more prone to adverse drug effect, toxicity or inefficacy (e.g., for prodrugs).

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Abbreviations

DIO	diet-induced obesity
DMSO	dimethyl sulfoxide
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
HDR	high-diet responders
HFD	high-fat diet
IFN-γ	interferon-γ
IL-1β	interleukin-1β
IL-6	interleukin-6
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LDR	low-diet responders
NADP	Nicotinamide-Adenine Dinucleotide Phosphate
P450	cytochrome P450
PMSF	phenylmethanesulfonyl

xxxii

RT-qPCR   real time quantitative polymerase chain reaction
T2D   type 2 diabetes
VHDR   very high-diet responders

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