Etude rétrospective de l'influence des polymorphismes génétiques de CYP3A4, CYP3A5 et ABCB1 des donneurs et des receveurs sur les effets des immunosuppresseurs en transplantation hépatique

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Thèse dirigée par le Professeur Pierre MARQUET

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A Marie-Bérénice, Marie-Charlotte et Jean-Patrick

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Sommaire

Liste des abréviations

αFP : alpha-foeto-protéine

AMM : autorisation de mise sur le marché

AUC : aire sous la courbe

CHC : carcinome hépato-cellulaire

CNI : inhibiteur de la calcineurine

C0 : predose trough concentration

C0/D : dose adjusted through levels

CsA: cyclosporine

CYP : cytochrome P-450

IL : interleukine

IMDPH : inosine mono phosphate déshydrogénase

KTR : kidney transplantation

LT: liver transplantation

MELD : model end stage of liver disease

MMF : mycophénolate mofétil

m-TOR : mammalian target of rapamycin

PD : pharmacodynamie

PG : pharmacogénétique

P-gp : P-glycoprotéine

PK : pharmacocinétique

pmh : par million d'habitants

STP : suivi thérapeutique pharmacologique

SNP : single nucleotid polymorphism

SU : super urgence

TH : transplantation hépatique

Tac: tacrolimus

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Résumé

La transplantation hépatique est une technique chirurgicale maîtrisée, mais le devenir à long terme du greffon et de l'hôte doit encore être amélioré. L'étude pharmacogénétique des inhibiteurs de la calcineurine (CNI) devrait permettre de comprendre la variabilité de leurs effets thérapeutiques et toxiques. Dans un premier temps, nous avons réalisé une revue de la littérature concernant la pharmacogénétique des CNI en greffe d'organe et surtout hépatique en particulier les trois polymorphismes les plus impliqués dans la pharmacocinétique des CNI (*CYP3A4*22*, *CYP3A5*3* et *ABCB1* exons 12, 21, 26) et leurs éventuelles associations avec le devenir clinique du patient. L'état actuel des connaissances valide l'intérêt du génotype *CYP3A5*3* pour adapter au mieux la posologie précoce de tacrolimus seulement en greffe rénale.

Dans un second temps, nous avons mené une étude de cohorte rétrospective visant à étudier la pertinence et l'intérêt des génotypes du donneur et du receveur d'organe mentionnés précédemment, intervenant dans le métabolisme (*CYP3A4*22*, *CYP3A5*3*) et le transport membranaire (*ABCB1* exons 12, 21 et 26) de la cyclosporine et du tacrolimus en transplantation hépatique. 170 patients avec un suivi de plus de 10 ans en moyenne ont été inclus. Les principaux résultats montrent que : l'allèle *CYP3A5 *1* du receveur était associé significativement à un risque plus élevé de perte de greffon à long terme comparé à l'allèle *CYP3A5 *3* ; l'allèle TT de l'exon 12 d'*ABCB1* du receveur était associé à un risque moins élevé de rejet chronique ; et l'exposition à des doses élevées de CNI, la valeur initiale de la fonction rénale et l'âge du receveur étaient également indépendamment associés au risque d'altération de la fonction rénale. La caractérisation de ces marqueurs pharmacogénétiques en transplantation hépatique pourrait permettre d'adapter les traitements immunosuppresseurs pour chaque patient transplanté. D'autres voies de recherche (pharmacogénétique de la voie calcineurine, biomarqueurs précoces des lésions du greffon, ...) seront nécessaires pour identifier un profil personnalisé pour chaque greffé afin d'adapter au mieux la stratégie thérapeutique à long terme.

Mots-clés : Transplantation hépatique ; *CYP3A4*22* ; *CYP3A5*; *ABCB1* ; Perte du greffon ; Rejet chronique ; Néphrotoxicité.

Abstract

Liver transplantation is now a well mastered surgery with standardized procedures, but the long-term clinical outcomes of the graft and the patient remain uncertain. The pharmacogenetic study of the calcineurin inhibitors (CNI) cyclosporine and tacrolimus should help to understand the variability of their pharmacokinetics and therapeutic or side effects. In the first part of this work, we reviewed the main pharmacogenetic studies of CNI in liver transplantation, focusing on the three polymorphisms mostly involved in CNI pharmacokinetics (*CYP3A4*22*, *CYP3A5*3* et *ABCB1* exons 12, 21, 26) and their possible associations with clinical outcomes. To date, the only pharmacogenetic test consensually recommended in organ transplantation is the *CYP3A5*3* variant for a better selection of the initial tacrolimus dose in kidney transplantation. The second part of this work was a retrospective cohort study in liver transplantation to investigate the influence of the above mentioned donor's and recipient's genotypes, involved in the metabolism (*CYP3A4*22*, *CYP3A5*3*) and the membrane transport (*ABCB1* exons 12, 21 and 26) of cyclosporine and tacrolimus. 170 patients were enrolled in this study with a mean follow-up of more than ten years. Our main results are that: the recipient *CYP3A5*1* allele was associated with a higher risk of graft loss than the *CYP3A5*3* allele; the recipient *ABCB1* exon 12 TT genotype was associated with a lower risk of chronic rejection than the CC genotype; overexposure to CNI, initial renal function and recipient age were associated with a higher risk of post-transplantation renal dysfunction. No genetic factor was associated with patient survival, acute rejection, liver function tests, recurrence of viral or other initial liver disease, or nephrotoxicity. Prospective characterization of both recipient and donor *CYP3A4*, *CYP3A5* and *ABCB1* polymorphisms could help to optimize immunosuppressive therapy for each candidate to liver transplantation. Further studies (pharmacogenetics of calcineurin pathway, early biomarkers of graft dysfunction, ...), should help to define a personalized profile for each transplant patient in order to best adapt the immunosuppressive strategy on the long term.

Keywords: Liver transplantation; *CYP3A4*22*; *CYP3A5*; *ABCB1*; Graft loss; Chronic rejection; Nephrotoxicity.

I. Introduction

La transplantation hépatique (TH) est le seul traitement curatif des hépatopathies sévères aiguës et chroniques. La maîtrise des techniques chirurgicales et la meilleure efficacité des traitements immunosuppresseurs ont permis une survie globale de 93 % à 1 mois, 84,1 % à 1 an, 72,5 % à 5 ans et 62,3 % à 10 ans pour les patients greffés entre 1993 et 2011.

En France, selon le rapport de l'Agence de la Biomédecine, l'activité cumulée entre 1998 et 2012 était de 20 916 greffes hépatiques, dont 446 à partir de donneurs vivants. 29 % des greffons hépatiques provenaient de donneurs âgés de plus de 65 ans en 2012, avec une augmentation de 17 % entre 2011 et 2012. Le nombre estimé de malades porteurs d'un greffon fonctionnel était de 10 739 au 31 décembre 2012.

En 2013, 1241 greffes hépatiques ont été réalisées en France (contre 1092 en 2010), réparties entre 22 équipes (dont 3 exclusivement pédiatriques), portant le taux de greffe à 18,9 pmh. Treize de ces greffes ont été réalisées à partir d'un don vivant et 59 dans le cadre de greffes combinées. La survie est significativement corrélée à l'indication de la greffe, l'âge du receveur, l'âge du donneur et le degré d'urgence ($p < 0,001$).

Les besoins en transplantation hépatique sont en constante augmentation. Le taux national d'inscription en liste d'attente de greffe hépatique en 2013 était de 26,3 pmh. Les besoins restent supérieurs aux possibilités de greffe et sur les 2924 candidats à la greffe on constatait une hausse du nombre de nouveaux inscrits de 12,3 % en 1 an. Seuls environ 40 % des patients inscrits sur liste d'attente accèdent à la greffe chaque année. Au 1er janvier 2013, 1104 malades étaient inscrits soit une augmentation de 17,3 % par rapport à 2012 sur l'ensemble de la cohorte et de 27,3 % si l'on ne prend en compte que les malades actifs sur la liste. La moyenne d'âge des nouveaux inscrits était de 50,5 ans en 2012 contre 47,3 ans en 2002. Le nombre de sujets inscrits âgés de 55 ans à 65 ans a progressé de 138 % en 10 ans et représente 40 % des nouveaux inscrits en 2012. La durée médiane d'attente en France est de 6,9 mois, sans tenir compte des malades greffés en super urgence pour hépatites fulminantes et des donneurs vivants. 163 demandes de Super Urgence (SU) pour receveurs adultes et pédiatriques ont été reçues en 2012, dont 120 ont pu être satisfaites. En effet, le score « Foie » mis en place en France depuis mars 2007 permet d'accélérer l'accès à la greffe des malades les plus graves. Ce score est calculé en fonction du score de MELD qui prend en compte la créatinine, les taux d'INR et de bilirubine. Un score de MELD inférieur à 17 est l'indicateur

d'une absence d'indication à la transplantation hépatique pour une cirrhose, mais le score de MELD à la greffe est un facteur pronostique de la survie du greffon.

Depuis début 2011 le score Foie est modifié, permettant une cinétique différente d'accès à la greffe pour les patients inscrits pour carcinome hépato-cellulaire (CHC) en fonction de la durée d'attente, leur MELD étant souvent bas. L'ajout d'une composante « experts » dans le score Foie, accordée après avis du collège d'experts depuis juillet 2007, peut permettre à des patients ayant un MELD bas d'être greffés (cirrhose isolée avec MELD < 15 soit 24,4 % des greffes en 2012). Cette composante permet d'attribuer des points supplémentaires pour des malades ayant des particularités cliniques, mais dont le score Foie ne leur permet pas d'accéder à la greffe dans le temps imposé par la gravité de leur maladie. Le nombre maximum de points accordés est de 650 pour l'exception « ascite réfractaire » depuis mai 2011 et pour l'exception « encéphalopathie chronique » depuis septembre 2012. Depuis le 22 février 2011, la priorité locale est modifiée au profit d'un modèle gravitaire (l'attractivité d'un malade sur un greffon décroît en fonction de la distance, mais moins vite si la « nécessité d'être greffé rapidement », mesurée par le score « Foie » hors distance est importante) et « isochrone » (distances horaires à la place du modèle linéaire kilométrique).

En 2012, 292 candidats sont sortis de liste pour aggravation ou décédés (182 décès). L'Agence de la Biomédecine analyse ces données pour adapter au mieux les politiques d'inscription.

Le CHC et la cirrhose alcoolique représentent respectivement 26,3 % et 28,2 % des indications principales de greffe hépatique en 2012. L'augmentation des greffes pour cirrhose alcoolique (+ 53 % en 5 ans) est liée à l'élargissement des indications pour les malades ayant une hépatite alcoolique aiguë grave. Les cirrhoses post-hépatite C et les retransplantations hépatiques représentent respectivement 9,5 % et 5,8 % des inscriptions en 2012, les autres indications n'excédant pas 5 %.

Depuis peu, le score « alpha-foeto » (αFP) est un modèle de prédiction de récidive du CHC sur le greffon. Les patients ayant un score αFP > 2 ont un taux de récidive du CHC sur le greffon de 50 %. Le calcul du score αFP est effectif depuis janvier 2013 et est réévalué tous les 3 mois pour tous les CHC TNM2. Toutefois, ce modèle ne prend pas en compte le caractère évolutif du CHC.

L'analyse récente faite par l'Agence de la Biomédecine de l'impact du score Foie sur les résultats des greffes entre 2007 et 2011 a montré que la valeur du MELD a une mauvaise

valeur prédictive sur la survie post-greffe. Le vieillissement des receveurs est à prendre en compte, d'autant plus qu'il n'y a pas de possibilité d'adéquation à l'âge pour l'allocation des greffons. En 2012, les inscriptions de malades graves avec MELD > 30 avaient augmenté de 26 % par rapport à l'année précédente.
De nombreuses pistes pour améliorer la gestion des greffons hépatiques sont en cours d'évaluation par l'Agence de la Biomédecine. L'allocation des greffons se doit d'être égalitaire tout en priorisant les malades les plus graves. Le greffon est devenu un bien précieux dont il faut assurer l'allocation au meilleur candidat, tout en optimisant sa survie à long terme.

Les médicaments immunosuppresseurs permettent d'éviter le rejet aigu et chronique des greffons hépatiques. En France, l'immunosuppression initiale (phase d'induction) est standardisée et est souvent une triple thérapie associant corticoïdes à fortes doses, un anticalcineurine (ciclosporine ou tacrolimus) avec des concentrations sanguines cibles élevées, et un anti-métabolique (azathioprine ou mycophénolate mofétil). Les corticoïdes sont stoppés dans les 6 mois suivant la TH pour prévenir les complications métaboliques et diminuer le risque cardio-vasculaire. En cas de maladie auto-immune avec un risque élevé de récidive de la maladie initiale sur le greffon hépatique, de petites doses de corticoïdes sont maintenues au long cours. Mais le plus souvent, l'immunosuppression en phase d'entretien est une mono ou une bithérapie avec un anticalcineurine à doses réduites parfois associé au mycophénolate mofétil (Cellcept®) pour minimiser encore la dose d'anticalcineurine, en particulier en cas d'altération de la fonction rénale ou de cancers cutanés. Un inhibiteur de m-TOR, l'évérolimus, a eu l'AMM en TH en 2013. Celui-ci peut être introduit 3 mois après la TH, voire à distance afin d'épargner la fonction rénale et de diminuer au maximum les doses d'anticalcineurine.
Le traitement d'éventuels rejets aigus ou chroniques consiste à administrer des bolus intraveineux de méthylprednisolone (Solu-Médrol®) et à augmenter les doses, et donc les concentrations sanguines résiduelles, d'anticalcineurine et/ou de remplacer la ciclosporine par le tacrolimus, plus efficace.
L'objectif à long terme de l'immunosuppression est de maintenir un greffon hépatique fonctionnel et d'éviter les complications des immunosuppresseurs, en particulier la néphrotoxicité, l'insulinorésistance et les cancers.
Nous nous intéresserons dans ce travail aux immunosuppresseurs les plus utilisés en transplantation hépatique : la ciclosporine et le tacrolimus (Figures A, B, C, D).

La posologie de l'anticalcineurine est adaptée selon un critère composite : concentration dans le sang (la plus basse possible à distance de la greffe, dans les limites de la « zone thérapeutique »), date de la TH, indication de la TH, fonction rénale, survenue d'un rejet aigu et/ou chronique, existence d'une récidive virale C, survenue de cancers solides (le plus souvent cutanés) ou de lymphomes.

La ciclosporine, extraite du champignon *Tylopocladium Inflatum* a été longtemps le traitement immunosuppresseur de référence en transplantation d'organes (1978). Quand une cellule T reconnaît grâce à son récepteur spécifique un antigène étranger présenté par les cellules de présentation de l'antigène (ou *antigen presenting cells*, APC), une cascade d'évènements intra-cellulaires se produit avec une augmentation des niveaux de calcium et une activation de la calmoduline. La calmoduline interagit avec la cyclophiline A pour réguler l'activité de la calcineurine, une protéine de la superfamille des phosphatases sérine/thréonine. La calcineurine catalyse la déphosphorylation du NFAT (*nuclear factor of activated T-cells*) ce qui lui permet de se transloquer dans le noyau et d'activer l'expression des gènes de cytokines, comme l'IL2 et l'IL4, responsables d'une réaction immunologique. Kronke et al. ont montré que la ciclosporine bloque l'expression du gène de l'IL2 dans les lymphocytes T activés. La ciclosporine (ex. Néoral®) exerce son action immunosuppressive en se liant à la cyclophiline A pour former un complexe inhibiteur qui bloque l'activité phosphatase de la calcineurine.

Le macrolide immunosuppresseur tacrolimus (ex. Prograf®, Advagraf®) isolé du champignon *Streptomyces tsukubaensi*s est un puissant inhibiteur sélectif de la calcineurine découvert en 1984. Le tacrolimus a obtenu l'AMM en France en prévention du rejet de greffe hépatique en 1995, puis en prévention du rejet de greffe rénale en 1998. Le tacrolimus exerce son action immunosuppressive en se liant à une protéine intra-cellulaire, la FK506-binding protein ou FKBP-12, pour former un complexe inhibiteur qui bloque l'activité phosphatase de la calcineurine. Le blocage en chaîne de la voie de la calcineurine entraîne une inhibition de la translocation du facteur NFAT, empêchant la transcription des gènes produisant les cytokines et inhibant l'activation et la prolifération des lymphocytes T.

Le mycophénolate mofétil (MMF) a l'AMM depuis 1996 en France. Il est transformé par des carboxylestérases en acide mycophénolique, qui inhibe l'inosine monophosphate déshydrogénase de type 2, une enzyme clé de la synthèse des purines. L'inhibition secondaire de la synthèse de guanosine-monophosphate (GMP) limite la synthèse des purines dans les lymphocytes B et T.

L'évérolimus a l'AMM en France pour la prévention de rejet de greffe hépatique depuis 2013. Il s'agit d'un inhibiteur de m-TOR, comme le sirolimus qui est utilisé en greffe rénale depuis 2001. L'évérolimus forme un complexe avec la protéine FKBP12 (immunophiline intra-cellulaire) et ce complexe va se lier avec un domaine de la *mammalian target of rapamycin* (m-TOR) qui va interférer avec la transmission du signal de la m-TOR à ses effecteurs. La m-TOR est une protéine kinase qui contrôle la phosphorylation des protéines régulant la traduction d'ARNm importants pour la progression du cycle cellulaire. Le blocage de la signalisation m-TOR par le complexe évérolimus-FKBP12 ne sera pas étudié dans ce travail.

L'optimisation des traitements immunosuppresseurs en greffe hépatique est un objectif complémentaire à la meilleure gestion des greffons. Traiter les patients pour éviter les rejets et ne pas les exposer aux effets secondaires de ces médicaments peut permettre d'améliorer leur devenir à court et long terme et d'allonger la survie des greffons.

Les médicaments immunosuppresseurs sont caractérisés par une grande variabilité interindividuelle en termes d'efficacité et de toxicité. Ces variabilités phénotypiques dépendent de variabilités pharmacocinétiques et pharmacodynamiques. La biodisponibilité des anticalcineurines dépend de l'activité métabolique des cytochromes 450 (CYP) 3A4 et 3A5 et, à un moindre titre, de l'activité de transport de la P-glycoprotéine (P-gp).
Le suivi thérapeutique pharmacologique (STP) a pour but de donner à chaque patient la posologie optimale de chaque médicament immunosuppresseur (étude de la relation dose-concentration). Il est indispensable de connaître l'absorption, la distribution, le métabolisme et l'élimination des molécules immunosuppressives pour individualiser la dose nécessaire à chaque patient. Le STP consiste à maintenir l'exposition au médicament dans un intervalle de concentrations prédéfinies (cibles thérapeutiques), déduites des relations concentrations – effets dans la population des patients traités (en fait le plus souvent, dans la population des essais cliniques de la molécule). L'exposition est mesurée dans le sang, à défaut de pouvoir être mesurée au niveau du greffon ou du système immunitaire (au moins en routine). Les immunosuppresseurs faisant l'objet d'un STP (ciclosporine, tacrolimus, évérolimus, MMF) répondent aux critères généraux suivants :

- mauvaise relation dose/effet mais meilleure relation entre les concentrations sanguines et les effets pharmacologiques (thérapeutiques ou toxiques),
- faible index thérapeutique, c'est-à-dire rapport faible entre les concentrations sanguines minimale toxique et minimale efficace,

- variabilité pharmacocinétique inter-individuelle très importante,
- pas de mesure directe de l'effet permettant d'adapter le traitement,
- existence de méthodes analytiques permettant un dosage dans les milieux biologiques.

La pharmacodynamie (PD) évalue les relations entre la dose (ou la concentration sanguine) et les effets pharmacologiques du médicament : effets thérapeutiques (efficacité) et effets indésirables (toxicité). Il s'agit de relier les effets à la quantité de principe actif présent dans l'organisme en étudiant les relations entre les effets et :

- la dose administrée,
- la concentration sanguine ou la concentration au site d'action,
- l'aire sous la courbe des concentrations sanguines en fonction du temps (AUC).

La pharmacogénétique étudie l'influence des variations ponctuelles de la séquence d'ADN génomique sur la réponse à une molécule thérapeutique chez un individu (« réponse » recouvrant ici la pharmacocinétique, l'efficacité et la toxicité). Les variabilités génétiques sont à l'origine d'une partie des variabilités inter-individuelles physiologiques, pharmacocinétiques et pharmacodynamiques. Lors de l'administration d'un médicament, il est absorbé, distribué à son site d'action, il interagit avec des récepteurs ou des enzymes, puis il est métabolisé et excrété. Des variations génétiques peuvent survenir à chaque étape et expliquer les réponses variables des receveurs de greffes d'organes aux immunosuppresseurs. Les polymorphismes génétiques des enzymes du métabolisme des immunosuppresseurs expliquent la variation de la biodisponibilité et des effets thérapeutiques. L'objectif de la pharmacogénétique est de comprendre cette variabilité génétique et d'identifier les patients susceptibles d'avoir des effets thérapeutiques insuffisants ou des effets indésirables et ainsi de mieux cibler les posologies optimales pour chacun, avec un traitement immunosuppresseur « à la carte ».

Dans la première partie de ce travail, essentiellement consacrée à l'étude pharmacogénétique des immunosuppresseurs en transplantation hépatique, nous nous intéresserons aux principales protéines impliquées dans la pharmacocinétique des immunosuppresseurs et nous détaillerons leurs propriétés, leurs rôles et leur variabilité d'origine pharmacogénétique. Nous exposerons l'état de l'art actuel sur les associations pharmacogénétiques/exposition et pharmacogénétique/effets des anticalcineurines en transplantation hépatique.

Nous présenterons ensuite notre travail personnel sur l'influence des polymorphismes génétiques des protéines du métabolisme et du transport membranaire des anticalcineurines sur les effets de ces immunosuppresseurs en transplantation hépatique, en prenant en compte le génome du donneur et celui du receveur.

Enfin, nous présenterons dans une discussion globale l'apport de nos résultats aux connaissances actuelles et les perspectives de développement dans ce domaine.

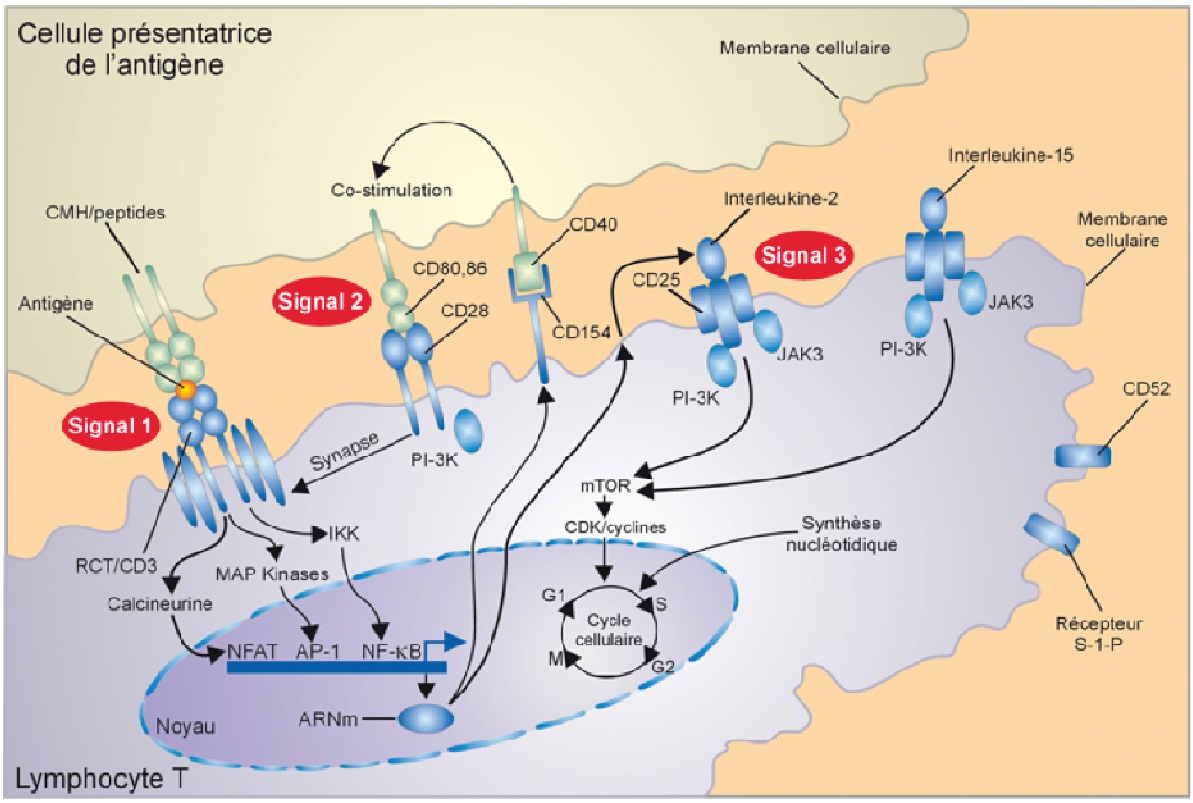

Figure A : les signaux de la réponse cellulaire T. (D'après Halloran, NEJM 2004 ipubli.inserm.fr). AP-1 : activating protein-1 ; CDK : cyclin-dependent kinase ; CMH : complexe majeur d'histocompatibilité ; IKK : IκB kinase ; JAK3 : Janus kinase 3 ; mTOR : mammalian-target-of-rapamycin ; NFAT : nuclear factor of activated T cells ; NF-κB : nuclear factor-κB ; PI-3K : phosphoinositide-3-kinase ; RCT : récepteur de la cellule T ; S-1-P : sphingosine-1-phosphate

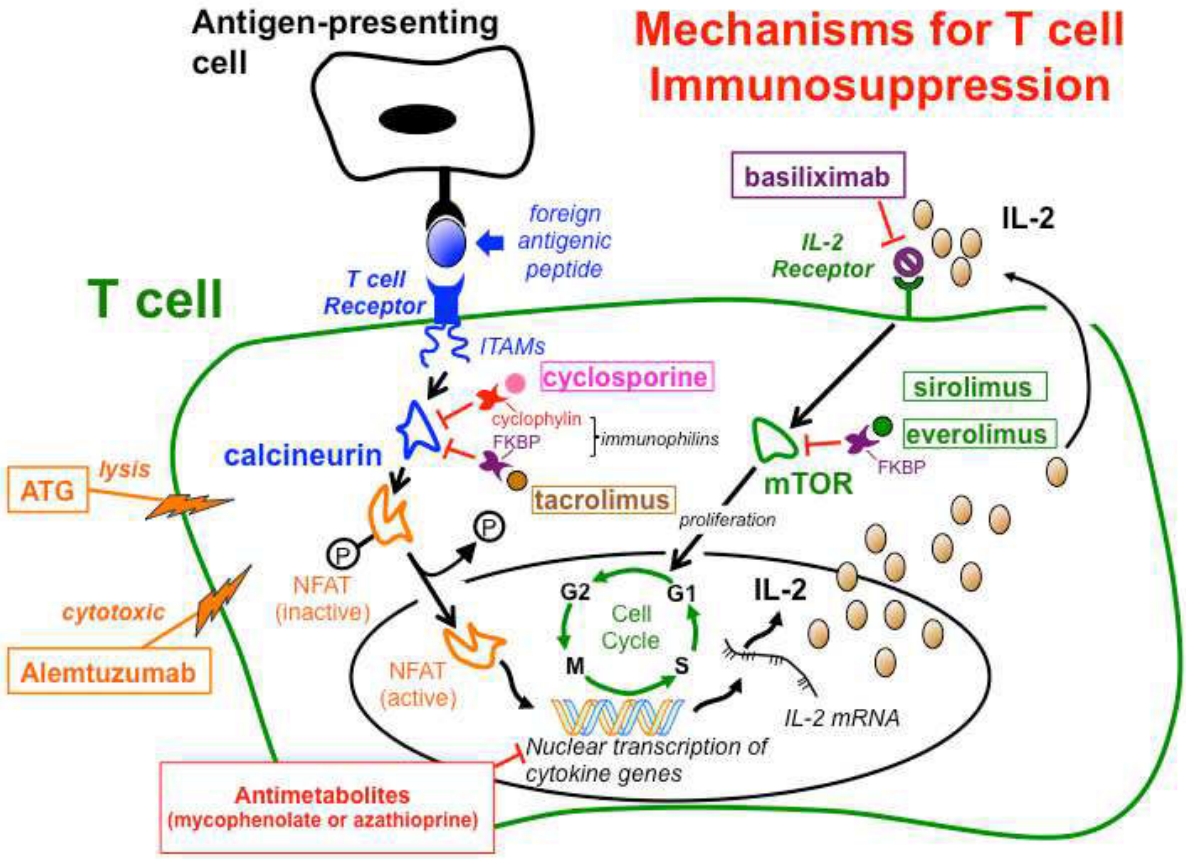

Figure B : La réponse immune : activation des lymphocytes T en réponse à un antigène étranger présenté par une cellule présentatrice d'antigène (cellules dentritiques, macrophages et lymphocytes). From Wood & Goto, Transplantation 2012.

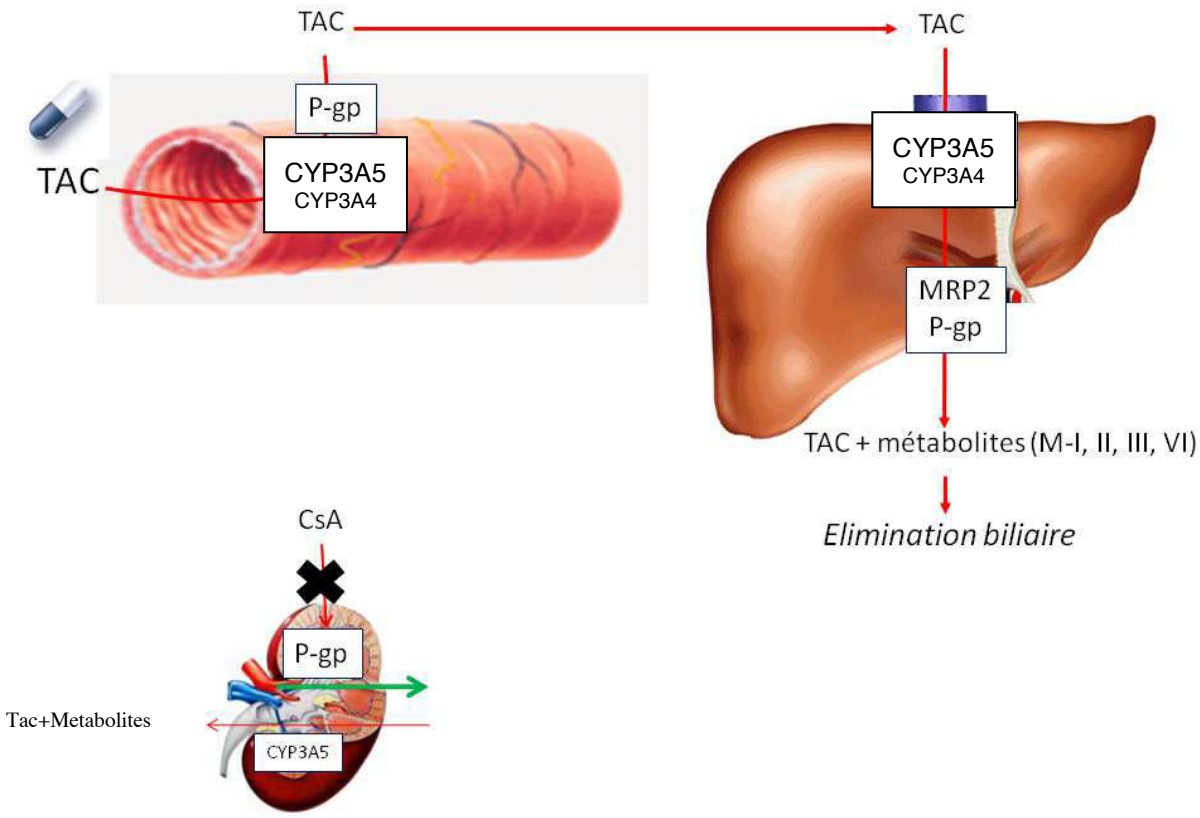

Figure C : Transport et métabolisme du tacrolimus, d'après Woillard JB.

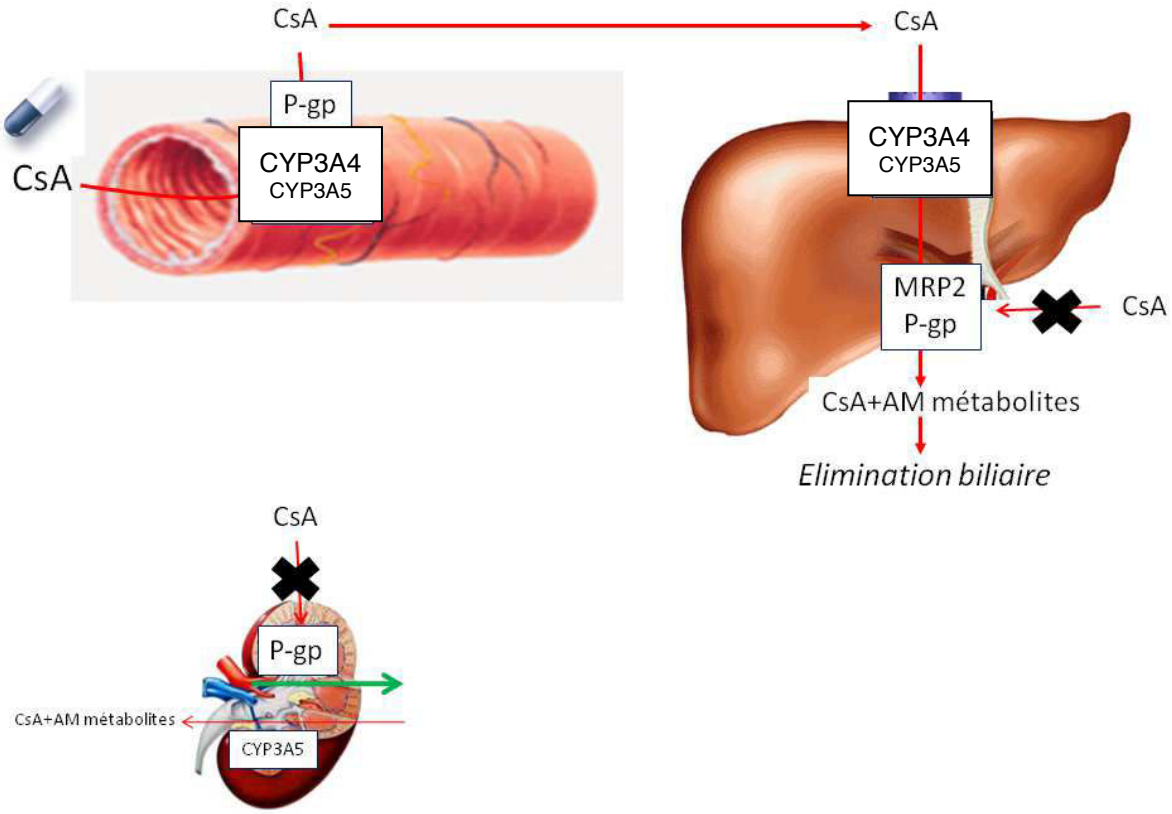

Figure D : Transport et métabolisme de la cyclosporine, d'après Woillard JB.

II. Article 1 : Revue de la Littérature

Influence of *CYP3A4*, *CYP3A5* and *ABCB1* genotypes on clinical outcomes in liver transplantation: Myth or reality?

Importance of the field: Immunosuppressive drugs (calcineurin inhibitors) have high interindividual pharmacokinetic variability and narrow therapeutic ranges.

Therapeutic monitoring of these drugs, through the assessment of cyclosporine or tacrolimus blood concentrations, reduces rejection rates and side effects (mainly nephrotoxicity). A pharmacogenomic approach could help avoiding adverse reactions and refining the calcineurin inhibitor (CNI) doses in liver transplantation.

Areas covered in this review: Single Nucleotide polymorphisms (SNPs), the most abundant genetic variation, can affect RNA expression, processing and its traduction in proteins. Numerous SNPs were described in the genes encoding CNI metabolizing enzymes, membrane transporters or receptors. This review concerns the CYP3A4 and CYP3A5 (CNI metabolizing enzymes) and ABCB1 (a CNI efflux transporter abundantly expressed in gut, liver and kidneys). This review will explore the impact of their SNPs on clinical outcomes in liver transplantation.

What the reader will gain: A better understanding of the impact of *CYP3A4*, *CYP3A5* and *ABCB1* polymorphisms on cyclosporine and tacrolimus on the short and long-term clinical outcome (rejection, survival and nephrotoxicity) in liver transplantation.

Take home message:

The *CYP3A5 *3* variant is the only polymorphism recognized to be useful in clinical practice for CNI monitoring but only in kidney transplantation, because of lack of prospective studies in liver transplantation and the ethical and technical concerns associated with DNA genotyping of the donors. The *CYP3A5 *1 /*3* genotype identifies patients being intermediate metabolizers and those with the *CYP3A5 *1 / *1* genotype as extensive metabolizers requiring 1.5 to 2 fold the standard starting Tac doses, while the *CYP3A5 *3 /* 3* carriers were referred to as poor metabolizers. Furthers prospective studies are required to elucidate the real impact of *CYP3A4*, *CYP3A5* and *ABCB1* donor and recipient genotypes on clinical outcomes, onset of cancer or infectious complications in liver transplantation.

Introduction

Multiple polymorphisms in genes related to the disposition or to the pharmacodynamics of immunosuppressive drugs are thought to explain the interindividual variations in treatment responses and adverse effects in patients undergoing liver or kidney transplantation. Pre-transplant screening could potentially help predicting patient metabolic profile, adjusting dose requirement accordingly and avoiding related adverse effects, such as nephrotoxicity for the calcineurin inhibitors (cyclosporine and tacrolimus).

To date, the only polymorphism that seems to be clinically useful is in the *CYP3A5* enzyme (i.e. the *CYP3A5*3* variant) and only in kidney transplantation so far (1). In France, approx. 1500 *CYP3A5* genotyping requests were recorded for about 3500 living kidney transplant recipients in 2013 (Annual report of the French Biomedicine Agency). *CYP3A5* genotyping is primarily useful in the early period post-transplantation. This biomarker helps selecting the initial dose of tacrolimus to reach adequate blood concentration targets. However, the only comparative, randomized study published so far showed no clinical improvement (regarding renal failure, graft survival or rejection) in the group of kidney graft recipients with *CYP3A5* genotyping (1).

The high interindividual variability in the bioavailability and disposition of the calcineurin inhibitors (CNI) is likely accounted for by the genetic variability of the cytochrome *P450(CYP)3A* enzymes and that of the multidrug resistance protein 1 (*MDR1*) also known as the P-glycoprotein (P-gp), an efflux transporter belonging to the ATP-Binding Cassette (*ABC*) transporter superfamily (*ABCB1*) (2) (Figures E, F).

Genotyping is an attractive option for optimizing CNI dosing because these drugs have narrow therapeutic ranges, but most of the published studies failed to find any pharmacogenetic profile significantly linked with the odds of renal failure, graft rejection or any other long-term clinical outcome. Specifically regarding liver transplantation (LT), *CYP3A4*, *CYP3A5* and *ABCB1* polymorphisms have not been consistently linked with any clinical impact.

This review assesses the impact of single nucleotide polymorphisms (SNPs) in these genes on the pharmacokinetic of cyclosporine (CsA) and tacrolimus (Tac), as well as on renal

dysfunction and acute rejection in LT. It will make a parallel with the results published in kidney transplantation (KTR) (3).

The pharmacogenes of interest

CYP3A4

CYP3A4 is a major drug-metabolizing enzyme expressed in the liver, jejunum, colon, and pancreas with at least 42 SNPs identified (Home page of the human cytochrome p450, http://hapmap.ncbi.nlm.nih.gov/) giving rise to 46 different alleles. Among the variant alleles described, none can individually or even commonly explain the 10- to 100-fold differences in *CYP3A4* activity reported in the population (4-6). The most studied alleles are the *CYP3A4*1B* (characterized by the c-392 A>G SNP) and the *CYP3A4*22* (which contains the c.522-191C>T SNP: rs35599367) alleles (7-9). The *CYP3A4*1B* is present in 2%-10% of Caucasians, 4.2%-11% of Hispanics, 35%-67% of Africans-Americans; it is absent in Asians (10). This allele has an unclear functional impact. It is located in the proximal promoter of *CYP3A4* and the results of the studies regarding its role on *CYP3A4* expression are contradictory (11-13). The *CYP3A4*1B* allele is in high linkage disequilibrium with the *CYP3A5*1* functional allele associated with high *CYP3A5* expression in different tissues such as the liver (12,13). Some data suggest an increased transcriptional activity of the *CYP3A4**1B variant allele in vitro (14) but in vivo studies failed to find an association between this allele and the metabolism of various drugs (7,9,15-19). Zhou et al. published the results of a meta-analysis showing that the *CYP3A4*1B* GG genotype was associated with an increased risk of cancer in particular prostate cancer (18).

The *CYP3A4*22* allele was first described in 2011 by Wang D. et al. It is located in the *CYP3A4* intron 6 and the T-variant allele was associated with decreased hepatic *CYP3A4* mRNA expression and with decreased *CYP3A4* enzymatic activity (i.e. 6β-testosterone hydroxylation in human liver microsomes) (20,21). This allele has a frequency of 3-4% in Caucasians. A study showed that it was associated with the dose requirement of statins (21). This promising biomarker might contribute to the interindividual variability of *CYP3A4* activity (20,22).

The others *CYP3A4* alleles (numbered from *2 to *21) are rare or apparently without functional effect on CNI pharmacokinetics (5). The *CYP3A4**2 allele (characterized by the c.664T>C SNP) was associated with a defective CYP3A4 activity but its allelic frequency is very rare in Caucasians (23-26).

Another polymorphism *CYP3A7* has a low frequency (3%) in Caucasians and Africans-Americans and has an unknown impact in the liver and intestine. This SNP is located in *CYP3A4* intron 7 (*CYP3A4* (rs4646437C>T)) and may have an impact with *CYP3A4* expression and enzymatic activity in vitro (27). CYP3A7*1 / *1 C genotype results in high expression CYP3A7.

The *CYP3A4*18B* is only found in Asians. It would increased CNI metabolic capacity (+25%-30%) in vivo and patients with CYP3A4*18B alleles would required higher doses of CsA or Tac to reach target concentrations (28-30). The following *CYP3A4* alleles: *CYP3A4*3, CYP3A4*4, CYP3A*5, CYP3A4*6, CYP3A4* 7-13* and *CYP3A4*20* have very low allele frequencies or no (or at least unclear) impact on CYP3A4 activity in Caucasians (31-34). *CYP3A4* 14-*19* and *CYP3A4*21* are not present in Caucasians.

CYP3A5

CYP3A5 is located in the liver, small intestine, stomach and kidney. At least 11 different alleles have been described for this gene. A single (A>G) nucleotide substitution in CYP3A5 intron 3 (rs776746) results in the *CYP3A5**1 and *CYP3A5**3 alleles. *CYP3A5**3 causes alternative RNA splicing and protein truncation of the enzyme (35). *CYP3A5**3 is thus a defective allele. The *CYP3A5**1 allele is necessary for the expression of a functional *CYP3A5* protein. CYP3A activity in liver and small intestine seems to be correlated with the *CYP3A5* genotype (36,37). This wild-type allele (CYP3A5 expressers) is present in only 5-30% of Caucasians, as opposed to 50-80% of Africans-American and Chinese people.

ABCB1 (MDR-1, P-gp)

The *ABCB1* gene encodes P-gp, an ATP-dependent efflux pump wich is largely expressed in the liver, kidney as well as at the blood-brain and blood-testis barriers, the maternal side of

the placenta, in adrenal glands and in the small intestine. At least 60 SNPs have been described in *ABCB1*, but three have been more studied: the c.*1236C>T* SNP in exon 12 (rs1128503), the c.*2677G>A/T* SNP in exon 21 (rs2032582) and the c.*3435C>T* SNP in exon 26 (rs1048642). This haplotype occurs in 32% of Caucasians, 35% of Mexicans, 27% of Asians and 5% of African-Americans (38). The gastro-intestinal absorption of P-gp substrates is inversely correlated to the gut expression of P-gp level. The frequency of the variant T in the case of c.2677G>T/A in exon 21 is approx. 40-50% in Caucasians and 0.9%-13% in Africans or Africans-Americans (39). The frequency of the T variant for c.3435C>T in exon 26 is about 33-65% among Caucasians (40-42). The patients homozygous for the exon 26, 3435T variant, may have lower P-gp function (two-fold reduction in intestinal P-gp expression) (43-46). The 3435C>T variant allele may indeed reduce *ABCB1* mRNA stability in liver or affect the insertion and folding of the P-gp into the membrane (47).

The *ABCB1* exon 26, exon 21 and exon 12 polymorphisms exhibit a linkage disequilibrium between them (48) and the *ABCB1* haplotype comprising these three variant alleles might result in diminished P-gp expression in vivo.

Pharmacogenetics effects on cyclosporine (CsA) pharmacokinetics (PK) and clinical outcomes

1- Impact on CsA PK

CsA is metabolized primarily by *CYP3A4* and *CYP3A5* in the small intestine and the liver. Three CsA metabolites, AM1 (hydroxylation at amino acid 1), AM9 (hydroxylation at amino acid 9) and AM4n (*N*-demethylation at amino acid 4) are produced by *CYP3A4*; only AM9 is produced by *CYP3A5*. *CYP3A4* is thus the major contributor to the oxydative metabolism of CsA (49,50). The total metabolic clearance of CsA is not substantially affected by CYP3A5 expression.

Several studies found no impact of the *CYP3A4**1B allele on CsA PK (51,52). In contrast, in 14 healthy volunteers the mean oral clearance of CsA was: CL/F (L/hr) = 49.4 +/- 13.9 (A/A, wild-type, n=4), 83.5 +/- 16.0 (G/G, homozygous variant, n=4), and 52.5 +/- 5.6 (A/G, heterozygous, n=6), P = 0.0024 (53). Although patient numbers are really low, this suggests increased enzymatic activity in vivo in patients with at least one mutant *CYP3A4*1B* allele. In

100 renal transplant patients studied at an average of 7.3 years post-transplantation, Zochowska et al. found lower CsA dose-adjusted trough blood concentrations in *CYP3A4**1/*1B than in *CYP3A4**1/*1 carriers (54), but the allelic frequency of the *CYP3A4*1B* allele was only 2.5%. These significant results are presumably the results of the functional effect of *CYP3A5*1*, which is in linkage disequilibrium with *CYP3A4*1B* and the proper role of *CYP3A4*1B* in CsA metabolism is thus not convincing.

The study of Crettol et al. found that the CYP3A7 *1C carriers required a 1.4 fold to 1.6 fold higher CsA dose during the first year post transplantation ($P<0.05$) in 64 renal and 9 lung transplant (27). Sharaki et al. confirmed that CYP3A4 rs4646437C>T influenced significantly cyclosporine kinetics, the T carriers requiring higher cyclosporine dose in KTR (28). None study was reported in LT. Chinese homozygous wild-type *CYP3A4*18B* carriers (GG genotype) had a higher risk of CsA-related liver injury in renal transplantation over the first three months post-transplantation (30-32). As mentioned before, the *CYP3A4*18B* polymorphism may be helpful for Asian renal transplants treated by CsA or Tac. In a cohort of renal transplant patients, the pre-dose CsA C(0) at 3 months post-transplantation was higher in CYP3A4*1/*1 (GG alleles) and *CYP3A4*1/*18B* carriers than in *CYP3A4*18B*/*18B carriers ($p<0.05$) (30). Only these studies investigated the *CYP3A4*18B* allele and both concerned Asian patients.

The *CYP3A4*22* allele seems the most promising to study for cohort of solid organ transplantation (55).

Elens et al. found in 50 renal transplant recipients that the *CYP3A4*22* allele was associated with 1.6 fold higher CsA dose-adjusted concentrations (95%CI: 1.1-2.6; $p=0.019$). Homozygous wild-type patients need higher CsA doses to achieve the target levels than carriers of the variant *CYP3A4*22* allele (56,57). A recent study in renal transplant recipients, showed that *CYP3A4*22* carriers had a significantly, 15% lower CsA clearances than non carriers (58). In another study with a longitudinal follow-up over twelve months post KTR (n=172), the *CYP3A4*22* allele was not associated with CsA PK, but it was associated with an increased rate of delayed graft function compared to *CYP3A4*1/*1* carriers (59-61).

CYP3A5 reportedly accounts for up to 50% of total *CYP3A* protein in the small intestine and the liver when at least one copy of the *CYP3A5*1* allele is present (50). Anglicheau et al. showed that the *CYP3A5**3 polymorphism was not associated with the CsA PK in 106 renal recipients (48). They concluded that the *CYP3A5* polymorphism cannot explain the variability

of CsA PK in kidney graft (48). Other studies failed to prove that CYP3A5 expression had an impact on CsA dosing in KTR or on CsA dose-normalized concentrations in Asian renal transplants (57,58,62,63). Recently, Zheng et al demonstrated that, although the mean CsA oral clearance was similar between *CYP3A5* expressers and non-expressers, its urinary clearance was 20.4% lower in *CYP3A5* expressers, which suggests a CYP3A5-dependent intra-renal CsA metabolism (64). In contrast with these studies, in 103 Asian renal transplant patients, CsA dose-adjusted trough levels were 25.5% and 30.7% higher in patients with the CYP3A5*3/*3 genotype than in those with the wild-type genotype, at day 8-15 (p=0.011) and day 16-30 post-transplantation (p=0.015), without influence on CsA 2-h post-dose (C2) levels (29).

A meta-analysis by Tang et al., encompassing 14 studies with 1821 renal transplant patients concluded to a significant difference in mean daily dose between the non-CYP3A5*1 allele carriers and the CYP3A5*1 allele carriers (weighted mean difference -0.19 mg/kg; 95%CI: -0.31 to – 0.07; p=0.002) in Asian patients but not in Caucasian (65). The consequence of *CYP3A5*3* on CsA PK remains thus uncertain with no clear clinical impact (31).

CsA is a substrate and an inhibitor of the P-gp. In the small intestine, the P-gp forms a cooperative barrier with *CYP3A* and pump CsA out of the enterocytes. CsA can thus be exposed longer to *CYP3A* and the process can stabilize the intracellular CNI concentration in the range of enzyme-metabolizing capacity (66).

To the best of our knowledge, only a few studies conducted so far demonstrated that the *ABCB1* 3435C>T, 1236C>T, 2677G>T/A SNPs affect CsA PK or excretion in renal transplants. The *ABCB1* haplotype may be more influent than individuals SNPs regarding CsA PK (49). Turolo et al., in kidney transplants on CsA (n=61) found that *ABCB1* polymorphisms can affect CsA PK during the immediate period (at day 6) post renal transplantation: C1236T and G2677T/A homozygotes required a lower daily CsA dose than CT and GT heterozygotes (18.76±8.42mg/kg *vs*. 25.82±10.48mg/kg; p<0.05; 18.60±8.42mg/kg *vs*. 23.20±10mg/kg; p<0.05) but not at later periods (67). CYP3A5 polymorphism was not associated with CsA PK in this pediatric study.

However, most other studies found no influence of *ABCB1* SNPs on CsA PK, which might be explained by the fact that CsA is both a substrate and a potent inhibitor of *ABCB1* (68).

A recent meta-analysis on the effect of the *ABCB1* C3435T SNP on CsA dose requirement demonstrated a significant difference of CsA dose adjusted through levels (C0/D) and peak concentrations (Cmax/D) between 3435CC and 3435TT genotype carriers (weight mean dose (WMD) of C0/D= 4.18 mg/kg, 95%CI: 1.00-7.37, p=0.01; WMD of Cmax/D=20.85mg/kg,

95%CI: 2.25-39.46, p=0.03) (69). Significant differences in C0/D were found between CC and TT carriers at one week and 1-3 months post-transplantation.
In LT, pharmacogenetic studies of CsA are rare and finally, *CYP3A4*22* appears as the most interesting SNP explaining some of the variability in CsA PK.

2- Impact on clinical outcome in patients on cyclosporine

2-a. Nephrotoxicity

Most studies on CNI nephrotoxicity were conducted in kidney transplant recipients.
Individual variability of the production of CsA metabolites could contribute to the individual risk of renal toxicity in solid organ transplantation. Studies on the relationship between *CYP3A5* genotype and CsA nephrotoxicity are however contradictory (70-73).
The *CYP3A4*22* allele was found to be associated with a higher risk of delayed graft function in KTR (*22 carriers versus non-carriers: HR= 6.34; 95% CI 1.38-29.3, p=0.015). Patients with the *CYP3A4*22* allele had a 1-year overall creatinine clearance 20% lower than patients carrying the *CYP3A4 *1/*1* genotype (95% CI: -33.1 to -7.2%; p=0.002) (74,75).
Bouamar et al. found no significant influence of the recipients *CYP3A4**1B, *CYP3A5*3* and *ABCB1 1236C>T*, *2677G>T/A* and *3435C>T* SNPs on renal function with a follow-up of 12 months (52).
Garcia et al, in 68 kidney transplant patients followed over one year showed that the incidence of nephrotoxicity was higher in carriers of *ABCB1* 3435 TT genotype and in those with four to six variants in the three *ABCB1* loci (HR: 4.2, 95% CI: 1.3-13.9, p=0.02 and HR: 3.6, 95%CI: 1.1-11.8, p=0.05) but other genotypes (CYP3A4*1B and CYP3A5) had no impact (76).
Finally, a long-term retrospective cohort study of 259 renal transplant patients treated with CsA, showed that the *ABCB1* 1236T, 2677T an 3435T variant alleles and their corresponding variant haplotype in kidney donors were correlated to a higher risk of graft loss beyond the 4th year post-transplantation. The donor *ABCB1* TTT haplotype was also predictive of renal function deterioration (73).
In transplantation, the genotype carried by the graft is from the donor and not from the recipient. In KTR, the kidney can thus have a different CYP3A5 expression than intestinal and hepatic cells. In LT, the recipient CYP3A5 or *ABCB1* genotypes are expressed in the intestine and kidneys and influence the amount of CsA in the systemic circulation and kidney

tubular cells, as well as the amount metabolites formed in these cells (64). Renal CYP3A5 expression may thus lead to local accumulation of nephrotoxic metabolites.

The role of *ABCB1* polymorphisms is important. P-gp expressed in tubular epithelial cells transports CsA in urine and reduces intra-cellular concentrations in the tubular epithelium. High P-gp activity may independently influence the intra-renal exposure to CsA metabolites and be associated with risks of CsA nephrotoxicity.

2-b. Impact on serious adverse events and survival

In 2015, Traynor et al. showed in a cohort of 255 white kidney transplant patients treated with CsA that the *CYP3A4*22* allele was protective against the development of cancer (HT=0.20; 95%CI: 0.07-0.57; p=0.003). 84% of cancer cases were non-melanoma skin cancer with a lower incidence if patients were not CYP3A4*22 carriers (16% vs 36% of cumulative incidence at ten years p=0.003). None of the variants studied in CYP3A4 (*22= rs 35599367), CYP3A5 (*3/*3), PPARα (rs4253728 and rs 4823613) or POR (*28= rs 1057868) were correlated with graft survival or with the time to first cancer (77) .

Pharmacogenetics effects on tacrolimus (Tac) pharmacokinetics and clinical outcome

1- Impact on Tac PK

Tac is extensively metabolized by intestinal and hepatic CYP3A enzymes. 15 metabolites have been described, including 13-O-desmethyl-tacrolimus (M-I), 31-O-desmethyl-tacrolimus (M-II), 15-O-desmethyl-tacrolimus (M-III) and 12-hydroxy-tacrolimus (M-VI) (60). Only M-II retains pharmacological activity. In vitro, the metabolic clearance of Tac by CYP3A5 was found to be two-fold higher than by CYP3A4.

One study by Zuo et al, suggested that the *CYP3A4*1G* polymorphisms may be a determinant of Tac clearance in Chinese renal transplant (22). The Tac C0/D was higher in carriers of the GG haplotype of CYP3A4*18B during the first month after renal tranplantation (30). In a recent review (including LT and KTR), Provenzani et al. concluded that the *CYP3A4* genotype had no influence on Tac PK and that a sufficient number of studies had confirmed

that the *CYP3A5* genotype is an important determinant of Tac PK (78). However, Hesselink et al., in a study on 64 kidney recipients, showed that patients with the *CYP3A4**1B allele had lower Tac dose-adjusted through levels as compared to patients with the wild-type *CYP3A4**1/*1 genotype at the third and twelve months post-transplantation (57 (40-163) ng/mL per mg/kg versus 89 (34-398) ng/mL per mg/kg, p=0.003). *CYP3A5*3/*3* carriers had Tac dose-adjusted through levels higher than carriers of either the *CYP3A5*1/*3* or the *CYP3A5*1/*1* genotypes: 94 (34-398) ng/mL per mg/kg versus 61 (37-163) mg/mL per mg/kg; p< 0.001) (51).

Also, Gervasini et al. in a study conducted over a period of 1 year (n=103 kidney graft recipients) found that, among *CYP3A5*1* carriers, those carrying the *CYP3A4*1B* variant allele had significantly lower Tac dose-corrected exposure than *CYP3A4*1/CYP3A5*1* carriers at one year post-transplantation (57.01±17.34 vs. 100.09±24.78; P=0.016) (79).

*CYP3A4*22* allele was tested in a study by Elens et al., in a cohort of 185 kidney transplants with a follow-up of one year (80). The mean Tac dose requirement to reach the same Tac pre-dose concentration was 33% lower in *CYP3A4*22* carriers than in non-carriers (95%CI, -46% to -20%; P = 0.018) and the result was independent of the *CYP3A5*3* allelic status. The increase of Tac dose-adjusted blood concentration was +179% in patients carrying the CYP3A4*22 allele in combination with a CYP3A5*3/*3 genotype (p<0.001) as compared to patients with no CYP3A4*22 allele with functional CYP3A5*1 allele.

Pallet et al, recently published post-hoc results of a French prospective randomized multicenter study conducted in 186 kidney transplant recipients, where 9.3% patients (n=18) were heterozygous and none homozygous for the *CYP3A4 *22* genotype (allele frequency of 4.8%) (81). These patients required approx. 30% less Tac daily dose than non-carriers. Pallet showed that ten days post-transplantation (3 days after the introduction of Tac), 11% of the *CYP3A4 *22* carriers were in the target range of Tac C0 (10-15ng/mL) versus 40% for *CYP3A4 *1/ *1* carriers (HR=0.19 [0.03; 0.69]; p=0.02). 90% of the *CYP3A4*22* carriers were *CYP3A5* no- expressers. They suggested that *CYP3A4*22* is the most important variant of *CYP3A4* with a clinical impact because patients with the *CYP3A4*22* variant allele may reach Tac supra-therapeutic concentrations.

In stable renal graft recipients at 3 months (n= 59) and 1-5 years post-transplantation (n=80), De Jonge reported that in CYP3A5 non-expressers, the presence of one *CYP3A4*22* T allele was associated with a reduction of CYP3A4 activity. At one year post-transplantation, Tac clearance was 36.8% lower compared with homozygous CYP3A4*22CC wild-type patients, with a 50% lower dose requirement (82).

Recently the Clinical Pharmacogenetics Implementation Consortium provided guidelines related to *CYP3A5* genotyping and tacrolimus dosing. Birdwell et al referenced all the articles published in organ transplantation and ranked them based on level of evidence. Patients with the *CYP3A5 *1 /*3* genotype were defined as intermediate metabolizers and those with the *CYP3A5 *1 / *1* genotype as extensive metabolizers. The latter require 1.5 to 2 fold the standard starting Tac doses (without exceeding 0.3 mg/kg/day). The *CYP3A5 *3 / *3* carriers were referred to as poor metabolizers and require standard Tac dosing (83).

Many studies in KTR or LT have indeed found a strong influence of the CYP3A5 polymorphism on Tac PK. Patients homozygous for the CYP3A5*3 allele have lower Tac dose requirement and higher trough blood concentrations because of increased bioavailability and decrease oral clearance (25%-45% lower as compared to patients with the CYP3A5*1 allele) (83).

The majority of the studies in kidney transplantation showed that patients with at least one CYP3A5*1 functional allele (expressers: carrying the A nucleotide) require an average two-fold higher Tac dose to reach the target concentrations and that they have lower C0/dose than CYP3A5*3/*3 patients (non expressers, homozygous for the G nucleotide) at least during the first 6 months post-transplantation (84,85).

Finally, one major study with a randomized design showed that the recipient *CYP3A5* status can help to select the initial Tac dose to achieve adequate blood levels in the first weeks post kidney transplantation. This early period is thought to be very important to avoid rejection and future graft loss (86).

Thervet et al. conducted a prospective multicenter clinical trial in 280 kidney transplant recipients, evaluating initial Tac dose adjustment based on the CYP3A5 genotype and showed that it was more efficient to reach predefined target blood concentrations than standard practice. Indeed, after six Tac doses the proportion of patients of the adapted arm who had reached the target range was significantly higher than that of patients of the control arm (43.2 *vs*. 29.1%; p=0.030). Tac initial dose based on CYP3A5 genotype was 0.25 mg/kg/day for CYP3A5*1/*1 carriers, 0.20 mg/kg/day for CYP3A5*1/*3 allele carriers and 0.15mg/kg/day for CYP3A5*3/*3 carriers). However, pharmacogenetic dose adjustment did not result in less acute rejection episodes within the first months post-transplantation (86).

Table A summarizes major studies published in LT regarding the impact of *CYP3A5* on Tac PK. Almost all the studies showed that patients with at least one *CYP3A5*1* allele genotype had a higher Tac dose requirement to reach therapeutic concentrations and/or a lower dose-

adjusted exposure than CYP3A5 *3/*3 carriers (87). The donor CYP3A5 genotype carried by the liver graft may be another determinant of Tac PK variability in LT (88).

A meta-analysis about the impact of the donor and the recipient CYP3A5 genotypes on Tac PK in LT analyzed six studies (n=254 patients) of the donor CYP3A5 and four (n= 180 patients) of the recipient's (89). The authors concluded that Tac trough blood concentration normalized by the daily dose per kg (C/D ratios) was higher in recipients of a CYP3A5*3/*3 donor, at all time-points from one week to one year post-transplantation. Tac C/D ratio was also higher in carriers of the recipient CYP3A5 *3/ *3 genotype at all time-points but it was only significant at week 2 (mean difference of the C/D ratio=49.3 ng/ml/mg/kg/day; 95%CI: 16.1-82.4).

In 2014, another meta analysis of eight studies confirmed that dose-adjusted Tac trough concentrations were lower in patients with the CYP3A5*1 allele or transplanted with a donor carrying this variant, as compared to non-carriers (donor or recipient) at 7 days, months 2, 3, 6 and 12 post-LT. The recipient CYP3A5*1 allele appears as a major determinant in the early period post-transplantation, while the effect of the donor genotype for CYP3A5 on Tac PK may increase with time post LT (90).

A physiologically based pharmacokinetic (PBPK) model of Tac was recently proposed in LT, in which C0 Tac was influenced by CYP3A5 polymorphism of the liver donor, as well as by CYP3A4 inhibitory drug-drug interactions, plasma unbound fraction of Tac, typical intrinsic clearance, bioavailability, body weight, hematocrit, proportion of body fat, and hematocrit. They proposed an initial Tac dosing regimen, as in kidney transplantation, to reach a Tac C0 of 10 ng/ml at day 5, however without taking into account drug interactions (88).

Regarding ABCB1, most studies did not find any significant association between *ABCB1* genotypes and Tac PK (daily dose requirement or trough levels) in LT (91-96). Tac is a weak inhibitor of P-gp and a substrate of this efflux pump. The P-gp can influence Tac hepatic clearance and intestinal absorption.

However, a study showed that the 3435TT variant genotype was associated with higher Tac concentration/dose ratio and lower dose requirement as compared to the 3435CC wild-type genotype while others found that intestinal ABCB1 mRNA level was inversely correlated with Tac concentration/dose ratio in the early period post LT (97). The most convincing effect of the P-gp genotype would be on Tac clearance and would concern the recipient genome in LT. P-gp is indeed mainly implicated in Tac efflux from enterocytes in the native intestine (44,98-99).

Hawwa et al reported a significant association of the recipient ABCB1 exon 21 and exon 26 SNPs, as well as TTT haplotype and higher Tac dose adjusted trough concentrations in LT pediatric patients with a long-term follow-up (100). In Caucasian patients significantly higher C/D Tac ratios were found at 3, 4 and 5 years post LT for patients carrying the exon 12 TT (5 years: mean=69 vs. 46 ng/ml/kg, p=0.036) or the exon 26 TT (5 years: mean 68 vs. 47 ng/ml/kg, p=0.046) variant genotypes. This study confirmed a long-term effect of *ABCB1* genotypes on Tac PK. The same author published a review on the influence of ABCB1 polymorphisms on outcomes in liver transplanted children (101). They concluded that further studies were required to explore the real impact of *ABCB1* donor genotype and ABCB1 expression level in the intestine and leukocytes. Indeed, the role of ABCB1 may be complex: (i) *ABCB1* polymorphism is correlated with the intestinal expression of CYP3A4; (ii) recipient intestinal *ABCB1* and *CYP3A5* genotypes needs to be considered together with the CYP3A5 liver donor genotype; (iii) the role of *ABCB1* polymorphisms in exon 12 and 26 in the liver possibly correlated with higher intra-hepatic Tac concentrations (i.e. reduced biliary excretion as a consequence of reduced expression of the P-gp) (102).

A recent analysis of a cohort of 298 de novo KTR showed that fast metabolizers (*CYP3A5*1/POR*28T* carriers) had two- to three-fold higher tacrolimus dose requirements as compared to slow metabolizers (*CYP3A5*3/*3/CYP3A4*22* carriers). They also required significantly more time to achieve the target C0 of tacrolimus (i.e.>10 ng/ml (3.3±1.7 *vs*. 1.34±0.75 days; p<0.0001) (103).

In the study of Gomez-Bravo et al., on day 7, patients with one native *CYP3A5 *1* allele had lower Tac trough C0 (p=0.03) and C0/D (p=0.02) than *CYP3A5 *3/ *3* homozygous patients. At three months, patients with a liver carrying the *CYP3A5 *1* allele (donor genotype) had lower C0/D (p=0.03) and took higher doses of Tac (p=0.03) than those grafted from a *CYP3A5 *3 / *3* donor. *ABCB1* genotype did not have impact on Tac PK (104).

In a Japanese study on living donor transplantation, the C/D ratio of Tac was higher in recipients with the *CYP3A5 *3 / *3* genotype than in recipients with the *CYP3A5 *1 / *1* allele at all periods during 5 weeks post LT (post operative 1-7 days p<0.001; post operative 8-35 days, p<001) (105).

2- Impact on clinical outcome

2-a. Rejection

The study of the cohort of KTR mentioned above showed that fast metabolizers did not have a higher incidence of acute rejection or diabetes mellitus during the first year post-transplantation (103).

Few studies investigated the incidence of rejection in relation to genetic polymorphisms, in particular in Caucasians patients.

In 164 Japanese living-donor LT, during the very early period post LT (10 days), high levels of intestinal *ABCB1* mRNA were associated with a higher incidence of acute rejection before 10 days post LT (HR: 2.306; 95%CI: 1.058-5.028). Of note, acute rejection rate was also significantly associated with Tac trough blood concentrations on days 2 – 4 post-transplantation: 45.1% for <7 ng/mL *vs*. 22.9% for >7ng/mL (p= 0.040) (106).

A study in 98 LT in the first 3 months post-transplantation found no relation between CYP3A5 or ABCB1 genotypes of either the recipient or the donor and BPAR occurrence (overall incidence of 10.2% with a median time of 37 days) (104).

A study in 410 Japanese living donor LT with intestinal biopsies and 412 donors (graft biopsies) showed that patients grafted with a liver carrying the *CYP3A5 *1* allele had a higher risk of acute cellular rejection between days 14 and 23 post LT (14.5% *vs*. 5.7%, p=0.0134) than those with a liver of the *CYP3A5 *3 / *3* genotype (105). The authors hypothetized that local hepatic concentration of Tac may be lower in the former than the latter patients. The measurement of Tac concentrations in the liver will be necessary in further studies to clearly assess the impact of local exposure on the risk of rejection.

Tang et al, in 2011, published a meta-analysis encompassing 18 studies, 1443 renal transplant patients and 5 studies, 336 liver transplants and concluded that, in LT, higher Tac daily doses were required not only in CYP3A5 expresser of the organ donors than non expressers (by 0.024mg/kg; 95%CI: 0.019-0.028) but also in CYP3A5 expresser of the organ recipients than non expresser (by 0.012 mg/kg; 95%CI: 0.005-0.018) at week 2, and month 1, 3, 6 and 12 post LT (36). They also concluded that in LT, the rate of acute liver rejection is three-fold higher in recipients CYP3A5 expressers than in non expressers only at one month (HR: 3.27; 95%CI: 1.57-6.81; p=0.002). Recipients CYP3A5 expressers compared to non expressers required higher daily Tac dose by 0.017 (95%; CI (0.000-0.028) at week 2 and 0.000 (95%CI -0.013-0.013) at one year. There was no difference at one year regarding LT graft survival

between CYP3A5 expressers and non expressers. In KTR, this meta-analysis demonstrated that CYP3A5 expressers required higher Tac daily doses than non expressers.

2-b. Nephrotoxicity

A prospective cohort study in 252 KTR showed that higher donor age and combined donor-recipient homozygosis for the c.3435 C>T variant (TT genotype) in *ABCB1* is associated with increased susceptibility to chronic tubulointerstitial allograft damage within the first 3 years post-transplantation, but not with graft survival (107). The authors suggested that the effect resulted from a change in renal P-glycoprotein function. They did find any influence of *ABCB1* genotype on Tac PK or on the incidence of acute rejection and systemic Tac exposure was not predictive of graft histology. In this study, 88.8% of kidney biopsies were performed during the first 3 months post-transplantation.

A study in 219 KTR with a 2-year follow-up failed to show any association between donor and recipient *CYP3A5*3* and *ABCB1* 3435C>T genotypes on the one hand, and renal function or histological evaluation of renal biopsies on the other hand (84).

A study conducted in 216 LT with a mean follow-up of 52 months (108) showed that recipients carrying the *CYP3A5 *1 / *1* genotype had lower urine transferrine concentrations than those with **1/ *3* and **3 / *3* genotypes ($p<0.001$), while *ABCB1* polymorphisms were not related to early nephrotoxicity (estimated through the urine levels of transferrine, α1microglobulin, microalbumin and immunoglobulines).

The review of Gijsen et al. on the pharmacogenetics of Tac induced nephrotoxicity identified 4 articles in adult LT and seven in renal transplantation with a possible association between recipients CYP3A5 and ABCB1 polymorphisms and Tac nephrotoxicity (109). One study in 51 pediatric LT revealed a higher incidence of renal dysfunction (≥ 30% decrease in estimated glomerular filtration rate) for carriers of the *ABCB1* TTT haplotype at 6 months post-transplantation, and for *ABCB1* TT genotype at exon 26 at 12 months (101).

Conversely, a study in LT with a median follow-up of 5.7 years found no association between the polymorphisms in either donor or recipient CYP3A5 or ABCB1 3435C>T genes (n= 125 adult patients treated by Tac) and chronic kidney disease (110).

2-c. Impact on serious adverse effects or survival

The influence of CNI pharmacogenetics on patient outcome has been widely explored in kidney transplantation. Less is known in LT and the impact of SNPs in *CYP3A* or the P-gp. Most of the studies investigated short term outcomes or dose requirement in the early period post LT and not long-term clinical outcomes.

In living-donor LT, a significant higher risk of CMV and bacterial infections was found in carriers of the recipient *CYP3A5**1 allele, as compared to non-carriers (p=0.0216, p=0.0332 respectively) (111).

This was confirmed by a study of 64 pediatric LT, where donor or recipient expressers had a higher rate of infectious complications (112).

In LT, the hepatic content in CYP3A5 contributes to the metabolism of Tac, in addition to the recipient CYP3A5 in the small intestine that contributes to the first-pass metabolism of Tac. Ethnicity, drug-drug interactions and age may be important contributors and need to be more detailed in further pharmacogenetic association studies.

The major drawbacks of the published studies concern their retrospective nature, their heterogeneity regarding sample size, methodological and statistical approaches, the patient ethnicity, methods used for CNI determination and outcomes considered, and were most often limited to the early post-transplantation period.

The impact of both recipient and donor *CYP3A4*, *CYP3A5* and *ABCB1* genotypes on clinical outcomes such as nephrotoxicity or new onset diabetes in liver transplantation need to be evaluated. Further prospectives studies are needed to elucidate the real impact of pharmacogenetic interventions on patient management post LT as has already been the case in KTR. Individual factors such as genetic factors and intra-patient pharmacokinetic variability, as well as environmental factors, such as food, diarrhea, non adherence to CNI, drug-drug interactions need to be taken into account to optimize LT results (113).

In summary, the *CYP3A5*3* polymorphism is the most promising marker for tailoring Tac immunosuppression in transplantation. *CYP3A4*22*, *CYP3A5* and the *ABCB1* genotypes seems to have the most clinical relevance for long-term outcome and renal dysfunction after liver transplantation. The study of the combined effects of multiple polymorphisms rather than that of individual SNPs, combined with non-genetic factors, may provide even better tools for treatment personalization in liver transplantation. To improve CNI drug monitoring in LT and confirm its interest, we suggest the development of pharmacogenetic approach with

study of individual profiles of CNI pharmacokinetics. *CYP3A4*22*, *CYP3A5* and the *ABCB1* genotypes seems to have the most clinical relevance for long-term outcome and renal dysfunction after liver transplantation.

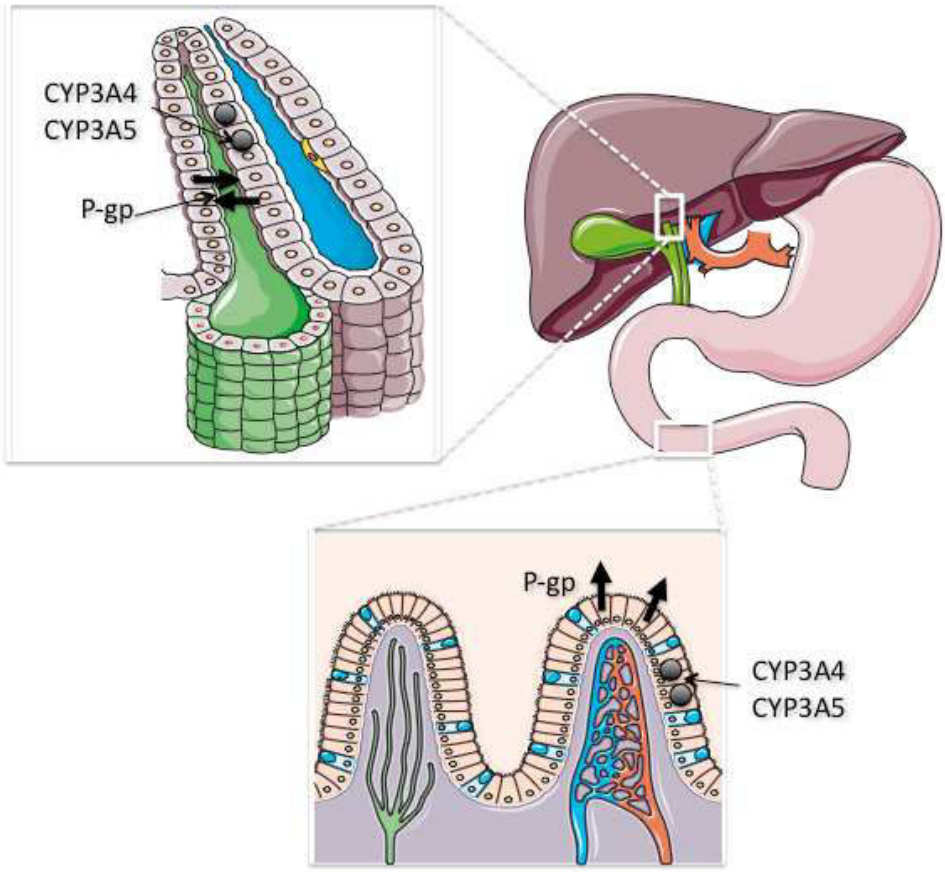

Figure E: Metabolism of calcineurin inhibitors. Influence of cytochromes P450 and P-glycoprotein (P-gp; ABCB1) in the biovailability of calcineurin inhibitors. Calcineurin inhibitors are metabolized by the cytochrome P-450 (CYP) 3A4 and 3A5 in the gut lumen before they reach the portal vein. P-glycoprotein (PGP) prevents drug absorption from the gut by promoting efflux into the lumen of the intestine. PGP also has a role in systemic clearance of drugs by promoting efflux into the bile for excretion. The drugs are subject to first-pass metabolism and systemic metabolism by CYP3A4 and CYP3A5 in the liver. When CYP3A5 is expressed, it accounts for 50% of the total hepatic CYP3A content. A change in the level of expression of CYP3A4, CYP3A5, or PGP would theoretically affect both the bioavailability and metabolism of calcineurin inhibitors.

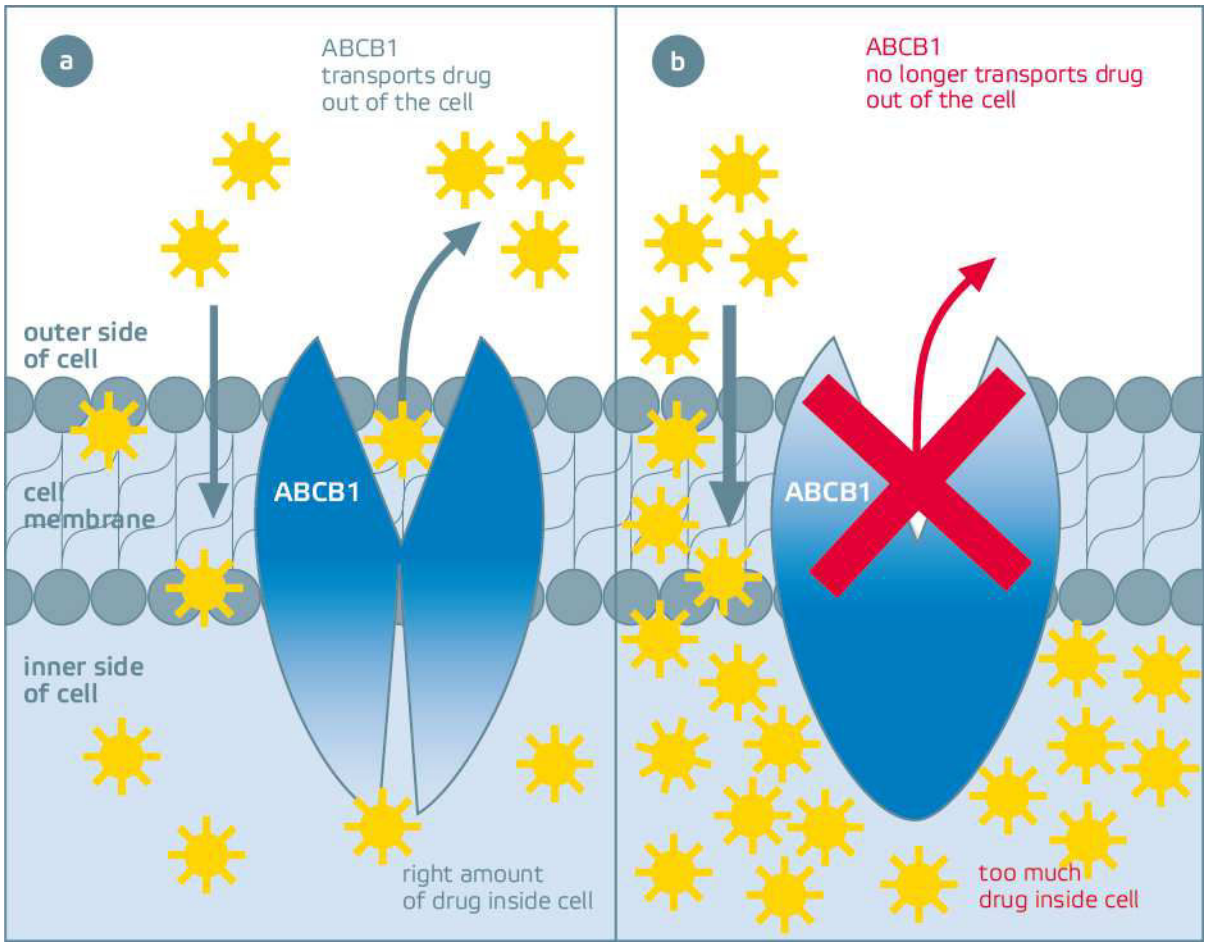

Figure F: Mechanism of action of the protein ABCB1. From Biologis.com.

Table A. Impact of the *CYP3A5*3* single nucleotide polymorphism on tacrolimus pharmacokinetic in liver transplantation

Reference	**Study population Patients (n)**	**DNA origin, donor and/or recipient**	**Significant pharmacogenetic associations**	**Follow-up**
Goto et al., 2004 (114)	181 Japanese living donor LT (143 treated with Tac) and 114 donors Prospective study	Recipients and donors	Donor *CYP3A5* *3/ *3 genotype with lower hepatic mRNA level of CYP3A5	5 weeks
Wei-Lin et al., 2006 (115)	50 Chinese LT Prospective study	Recipients and donors	Donor *CYP3A5 *3/ *3* with higher Tac C/D ratios than the others. Recipient *ABCB1* 3435CC with lower Tac C/D ratios.	One month
Yu et al., 2006 (116)	53 Chinese LT Prospective study	Recipients and donors	Donor *CYP3A5* *1/* 1 or *1/ *3 with lower Tac C/D ratios at 2 weeks (p=0 .036) and one month (p=0.021), but not at one week post LT. (Not significant with recipient CYP3A5*1 / *1 and *3/*3).	One month
Elens et al., 2007 (102)	150 Belgian LT Retrospective study	Recipients	At least one recipient *CYP3A5* * 1 allele with higher Tac dose requirement. Donor *ABCB1* 1199G>A and 2677G>T/A with greater Tac hepatic concentrations.	7 days
Barrera-Pulido et al., 2008 (94)	53 Caucasian patients Prospective study	Recipients and donors	Combination of recipient *CYP3A5*1/*3* and donor *CYP3A5*1/*3* with lower Tac levels during the 1st month post TH. Combination of recipient *CYP3A5*3/ *3* recipients and donor *CYP3A5*1/*3* required higher dose Tac at 1 and 2 months. GG recipients (*CYP3A5)* from C/T donors (*MDR1*): lower frequency of renal dysfunction	3 months

Provenzani et al., 2009 (117)	32 Caucasian LT Prospective study	Recipients and donors	Recipient *CYP3A5*1* allele with higher dose requirement at 3 and 6 months post LT. *ABCB1* (exons 21 and 26): no influence.	6 months
Provenzani et al., 2011 (118)	Caucasian patients 51 LT 50 KTR Prospective study	Recipients and donors for liver, recipients for kidney	Donor *CYP3A5**1 allele with increased Tac C0/D ratios at 1, 3 and 6 months. Recipient *CYP3A5* and *ABCB1* (exon 21, exon 26): no effect in liver.	6 months
Shi et al., 2013 (108)	216 Chinese LT Prospective study	Recipients	Recipient *CYP3A5**1/ 1 genotype with higher Tac dose than *CYP3A5* *3/ *3. Recipient *CYP3A5* *3 allele with increased risk for early renal glomerular injury. *MDR1* (exon 26 and 12): no effect.	52 months
Rojas et al., 2013 (89)	Meta analysis on LT 6 studies 254 patients (donor genotypes) 4 studies : 180 patients (recipient genotypes)	Recipients and donors Asians and whites. Not combined.	Donor *CYP3A5* *3 / *3 liver donor with higher Tac C/D ratio at all time points over the first month post LT.	One week to 12 months
Durand et al., 2013 (119)	179 pediatric LT Retrospective study	Donors (graft)	Donor *CYP3A5* *1 allele with higher mean stable Tac daily dose requirement.	First month
Gomez-Bravo et al., 2013 (104)	98 LT Retrospective study	Recipients and donors	Recipient *CYP3A5*1* allele with lower Tac C0 and higher Tac doses on day 7 post LT. *.ABCB1* exons 12, 21 and 26: no significant association. Donor *CYP3A5* or *ABCB1* polymorphisms: no influence on incidence of BPAR	3 months
Guy-Viterbo et al., 2014 (120)	114 pediatric TH Retrospective study	Recipients and Donors	Donor *CYP3A5*1* with a reduced C0/DBW12h on days 2 and 30. Donor *CYP3A4*22 T* allele with 29% decrease in Tac clearance.	3 months

Jalil et al., 2014 (121)	43 pediatric LT Retrospective study	Recipients	Equation between time post LT, Tac clearance and recipient *CYP3A5**1 allele Mean difference between observed and predicted Tac concentrations: 35.4%.	1st year
Chen D et al., 2014 (122)	96 Chinese LT Prospective study	Recipients and donors	Donor *CYP3A5* polymorphism accounts for 14.3% of total variation in Tac PK.	One month
Chen YK et al., 2014 (123)	90 pediatric LT Retrospective study	Recipients and donors	Recipient *CYP3A5*1* with lower Tac C/D ratio (from day 3 to day 14), lower Tac dose on day 30. Recipient and donor CYP3A5*3/*3 with higher Tac C/D ratio at 1, 2 3 and 12 months. *ABCB1* polymorphisms: no association with Tac PK. No association of CYP3A5 and ABCB1 with infections and acute cellular rejection.	One year
Gerard c et al., 2014 (88)	66 adult LT Prospective study	Recipients and donors	Tac clearance significantly related with the donor CYP3A5 genotype. Proposed initial Tac doses for a standard patient whithout drug interaction, to reach Tac C0 of 10 ng/ml at day 5.	Day 1 to 25

C/D: concentration /dose

C0: predose trough concentration

$C0/D_{BW12h}$: drug concentration weighted-adjusted Tac 12h: µg/l per mg/kg/12h

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III. Article 2

Influence of donor and recipient *CYP3A4, CYP3A5* and *ABCB1* genotypes on clinical outcomes and nephrotoxicity in a cohort of liver transplant recipients on anticalcineurin therapy.

Abstract

The goal of this study was to investigate the influence of polymorphisms in the *CYP3A* enzymes and *ABCB1* membrane transporter from both organ transplant donor and recipient on clinical outcomes and renal function in liver transplant patients on cyclosporine or tacrolimus, which are both substrates of these proteins.

Methods – Data from 170 adult liver transplant recipients receiving cyclosporine (CsA) or tacrolimus (Tac) collected over 10 years post-transplantation were retrospectively investigated. The recipient and donor *CYP3A4*22*, *CYP3A5* and *ABCB1* exons 26, 12 and 21 polymorphisms were genotyped. Multivariate time-dependent Cox proportional hazard and generalized estimating equation (gee) multiple linear regression were used for statistical analysis.

Results – Multivariate analyses showed that recipients expressing the *CYP3A5* enzyme (HR=2.53; 95%CI (1.17-5.46); p=0.01870), recurrence of the initial liver disease (HR=2.29; 95%CI (1.19-4.43); p=0.01315) and percent time spent in the high quantile of exposure to calcineurin inhibitors (CNI) (HR=8.36; 95%CI (2.54-27.50); p=0.00047) were significantly and independently associated with a higher risk of graft loss. Only the recipient *ABCB1* exon 12 CC genotype (exon 12 CC *vs*. TT, HR=3.12; 95%CI (1.35-7.24); p=0.0078) adjusted on the CNI (Tac *vs*. CsA HR=3.22; 95%CI (1.57-6.60); p=0.0015) was associated with a higher risk of chronic rejection. CNI exposure expressed as high (3), middle (2) or low exposure (1) calculated using exposure quantiles at each visit ($\beta \pm SD = -2.41 \pm 0.59$; $p<0.0001$), recipient age ($\beta \pm SD = -0.37 \pm 0.14$; $p=0.0084$), baseline MDRD ($\beta = 0.51 \pm 0.05$; $p<0.0001$) and duration of patient follow-up (per visit, $\beta = -0.98 \pm 0.22$; $p<0.0001$) were significantly associated with post-transplantation renal function. No genetic factor was associated with patient survival, acute rejection, liver function tests, viral or other initial liver disease recurrence, or nephrotoxicity.

Conclusion: Prospective exploration of recipient *CYP3A5* and *ABCB1* polymorphisms before liver transplantation could help to evaluate the risk of graft loss and chronic rejection, together with the CNI type and exposure and recurrence of the initial disease.

Key-words: Liver transplantation; *CYP3A4*22*; *CYP3A5*; *ABCB1*; Graft loss; Chronic rejection; Renal function.

Introduction

Liver transplantation is a life-saving technique for patients with end-stage liver disease. Cyclosporin A (CsA) and Tacrolimus (Tac) are calcineurin inhibitors (CNI) widely used in all types of solid organ transplantation, but their bioavailability varies greatly among individuals. Graft loss in the long term is a risk of all transplantations and is favored by underexposure, while nephrotoxicity, one the most frequent side effects of CNIs, is favored by overexposure. Monitoring CNI blood concentration, especially in the early phase, is thus necessary to prevent acute cellular and chronic graft rejection as well as renal failure, resulting in better graft and patient survival.

A part of CsA and Tac inter-individual pharmacokinetic variability is accounted for by polymorphisms in the genes encoding the metabolizing enzymes cytochrome *P450 (CYP) 3A4* and *3A5* and P-glycoprotein (Pgp; *ABCB1* gene) (1,2). The most frequent polymorphisms of *ABCB1* are located in exons 12 (1236 C>T), 21 (2677 G>T) and 26 (3435 C>T). In kidney transplant recipients receiving CsA: (i) the donor *ABCB1* variant TTT haplotype (combining these 3 SNPs) was significantly associated with a steeper decrease in renal function and an increased frequency of graft loss (3,4); carriers of the *CYP3A4*22* allele had a higher risk of delayed graft function (5,6), an overall 15-20% lower creatinine clearance (7) and a significantly higher dose-standardized exposure (8) than *CYP3A4*1/*1* homozygous carriers; and (iii) a meta-analysis showed that carriers of two *CYP3A5*3* alleles required a lower dose of CsA to reach target levels compared with carriers of at least one *CYP3A5*1* allele (9). In patients on Tac: (i) those carrying at least one *CYP3A5*1* allele, i.e. expressing a functional protein, had an approx. 50% higher dose requirement than non–expressers (10) and the target concentration of Tac was reached later after transplantation (11); (ii) carriers of the *CYP3A4*22* T-variant allele had a lower Tac dose requirement, independently of their *CYP3A5* genotype; (iii) the *POR*28* T variant allele was correlated with a higher Tac dose requirement than *POR*28* CC, however only in *CYP3A5* expressers (12,13). Regarding the *CYP3A4**1G genotype, Uesugi et al. recently showed that it was significantly related to mRNA expression of *CYP3A5* (rather than of *CYP3A4*) in the liver graft and in the intestine, and acute cellular rejection tended to be lower at 14 and 26 post-operative days in liver grafts carrying the donor *CYP3A4*1/1* than the *CYP3A4**1G allele (14). In LT followed-up for 52 months, the daily dose of Tac was higher with the recipient *CYP3A5*1/*1* (AA) than *CYP3A5*3/*3* (GG) genotype, and recipients carrying the *CYP3A5 *3* allele had an increased risk of early renal glomerular injury compared to carriers of the *CYP3A5 *1* allele (15).

ABCB1 polymorphisms (exon 26 and 12) were not significantly associated with Tac pharmacokinetics or renal toxicity. No study has investigated at the same time the impact of *CYP3A4*, *CYP3A5* and *ABCB1* polymorphisms of the donor and the recipient on long term graft outcomes, nor on CsA or Tac chronic nephrotoxicity in liver transplantation.

The objective of this study of a large retrospective cohort of liver transplant recipients with a long follow-up was to investigate whether the *CYP3A4*, *CYP3A5* and *ABCB1* genotypes of the donor and the recipient were associated with graft and patient survival at 5 and 10 years, acute and chronic graft rejection, ductopenia, liver function and calcineurin inhibitor nephrotoxicity.

Materials and Methods

Patients

We included 257 patients who underwent liver transplantation between January 1996 and December 1999 at the Hepatobiliary center (Villejuif, France). To be included, patients had to fulfill the following criteria: aged more than 18 years at the time of transplantation; received CsA or Tac from the first day post-transplantation; first transplantation during the study period; alive at one year post-transplantation; liver graft tissue obtained on the day of transplantation (reperfusion biopsy) available; tissue obtained from the native, explanted liver available; clinical and biological patient follow-up of at least 1 year. The exclusion criteria were: recipient age < 18 years; graft survival < 1 year; no recipient or donor liver tissue available for the pharmacogenetic study, or not all genotyping data available; no routine CNI concentrations available; no liver function test results available; patient lost to follow–up.

This study complied with the Declaration of Helsinski and a written informed consent was obtained from each patient enrolled. Data were collected from the medical file of the intensive care and hepatology units and from the pathology department. For all patients, the following data were collected: date of birth, sex of the donor and recipient, age of the donor, indication for liver transplantation, creatinine clearance (ClCr) just before transplantation; and over the follow-up period: bodyweight, immunosuppressive regimen, CNI daily dose, CNI concentrations, laboratory test results (serum creatinine, ClCr, liver function tests), graft loss, histological examination of the graft and patient survival at 5 and 10 years. All the acute rejection (AR), chronic rejection (CR) or ductopenia episodes were proven by histological examination of biopsies. Over the first year post-transplantation, biological data were collected during hospitalization and at every visit until stable CNI concentrations within the

therapeutic range were reached (at least 4 visits over the first year). Beyond the first year, data were collected annually, except that all modifications in CNI therapy were recorded.

Outcomes

The outcomes considered were: patient survival at 5 and 10 years, and global survival; graft loss as defined by the loss of hepatic function (thrombosis, chronic rejection...); acute rejection, ductopenia and chronic rejection defined as any histologically confirmed episode for which a mild, moderate or severe Banff score was recorded on any of the biopsy histological examinations available (16-18); viral recurrence (hepatitis C and B); recurrence of the initial liver disease; and renal function was evaluated by estimated glomerular filtration rate calculated at each visit using the MDRD formula (Modification in the Diet of the Renal Disease, Levvey 2000) based on patient serum creatinine (µmol/l), age and sex (19) and adjusted on the initial value and time for each patient.

Exposure to immunosuppressants

Exposure to the CNI was recoded, using the quantile of exposure at one visit with respect to all values at the same visit, as low = 1 (0-25th percentiles), medium = 2 (25th-75th percentiles) or high exposure = 3 (75th-100th percentiles). In a second step, the time-weighted average quantile of a given patient was calculated as the mean of the quantiles since the visit considered divided by the time spent from transplantation to this visit (mean quantile/time to the visit). In addition, the time spent in the highest and in the lowest exposure quantiles, divided by the time of patient follow-up was considered as a covariate for graft loss and death.

Donor and recipient DNA extraction.

Each donor's and recipient's DNA was extracted from archival formalin-fixed paraffin-embedded liver biopsies (obtained from the Pathology Department of the Hepatobiliary Center). The blocks of native liver and graft biopsy after reperfusion were processed as follows: each paraffin block was cut and about 10 mg collected in a plastic microtube. Paraffin was removed by adding 1.2 mL xylene for 15 min, followed by washing twice with 1.2mL ethanol for 10 min. After ethanol drying, 180µL lysis buffer (ATL buffer, 20µL proteinase K, QIAmp DNA minikit, ref 51036) were added and incubated at 56°C overnight until all tissue fragments were dissolved completely. DNA extraction and purification were

performed using QIAmp DNA minikits (Qiagen, France). An average of about 20 µg DNA was obtained from each donor and recipient liver biopsy. DNA quantification was carried out using a Nanodrop apparatus.

Genotyping

Donor and recipient genotypes for *CYP3A4* rs35599367 C>T (*CYP3A4*22*), *CYP3A5* 6986 A>G (*CY3A5*3* allele, rs776746 A>G) and *ABCB1* 3435C>T (exon 26, rs1045642), 1236 C>T (exon12, rs1128503), 2677 G>T (exon 21,rs2032582) SNPs were determined using TaqMan allelic discrimination assays on an ABI PRO ISM 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). PCR was performed using standard methods. To 4.75 µl of sample diluted to a target DNA concentration of 2ng/µl were added 5 µl of Master Mix and 0.25 µl of the SNP probe of interest. The PCR protocol was carried out with an initial 10-min denaturation step at 95°C coupled to a repeating cycle of 1 min at 92°C (denaturation) and 30 sec at 60°C (annealing and extension) for 45 cycles. A few genotyping data were missing, due to limited amount of DNA in either the graft or the native liver biopsy. 25 samples among the samples were randomly chosen for validity control and reanalyzed to check the reliability of the results.

Statistical analyses

Statistical analyses were performed using R software version 3.1.1 (R foundation for statistical computing, http://www.r-project.org). Conformity of genotyping data with Hardy–Weinberg equilibrium was assessed using the Fisher exact test in the "SNPassoc" package. The most probable *ABCB1* haplotype in each DNA sample was inferred using the haplo.stat R package. The effects of genetic polymorphisms (SNPs or haplotypes) and covariates on death and graft loss were investigated using the Cox proportional hazard model. The determinants of acute rejection, ductopenia, chronic rejection, viral C and B recurrence and initial liver disease recurrence were investigated using the time-dependent Cox proportional hazard model, including an autoregressive correlation matrix to take into account the correlation between visits for a given subject. The potential sources of renal function evaluation (MDRD) were investigated using generalized estimating equation (gee) multiple linear regression with correlations between within patients, and adjusted on the MDRD baseline value and the time after transplantation (visit). In a first step, univariate analyses were performed and the covariates with $p<0.05$ were included in an intermediate model. The final model was selected

using a backward stepwise process based on the likelihood ratio test. The influence of covariates on time-to-event data was estimated using Kaplan-Meier analysis and groups were compared using the logrank test (for death or graft loss) or cumulative incidence curves for competing risks (for acute rejection, ductopenia, chronic rejection, viral recurrence, initial liver disease recurrence) as implemented in the cmprsk R package.

Analyses were adjusted on the immunosuppressive drug (CsA or Tac) for *CYP3A5*3* and *CYP3A4*22* analyses, except for graft loss for which a global and subgroup analyses for CsA and Tac were performed. The proportionality of risk for the final Cox models was assessed based on Schoenfeld residues.

Results

A total of 170 patients were included in the present study, after exclusion of 54 patients who died during the first year post-transplantation. A flow-chart of patient selection is presented in Figure 1.

1/ Clinical and genotyping data

Clinical and demographic characteristics of the 170 recipients are presented in Table 1. The patients were followed up for a median of 11.85 (5.82-13) years. The grafts were sourced from 164 cadaveric donors and 6 living donors with amylosis neuropathy, aged 40.5 years on average, with 109 males and 61 females. The recipients were 48 years on average, with 112 males and 58 females.

Table 2 lists the frequencies of the variant alleles and genotypes for *CYP3A4*, *CYP3A5* and *ABCB1* in the 170 donors and recipients. The Hardy-Weinberg equilibrium was respected for all genotypes (p-value > 0.05).

2/ Factors linked with patient survival at 5 and 10 years

The patient survival curve is presented in figure 2. In this cohort, 25/170 (14.6%) and 40/170 (23%) deaths occurred in the first 5 and 10 years, respectively. No significant influence on either 5-year or 10-year patient patient survival of the different recipient or donor SNPs, or non-genetic factors was found (data not shown).

3/ Factors of graft loss

Only 161 patients were available for this analysis. A total of 40/161 graft losses (25% of the recipients) were observed within the period considered (Table 1). Univariate analysis as well

as the final multivariate Cox model adjusted on the CNI drug taken (Table 3) revealed significant associations between graft loss and: (i) the recipient *CYP3A5*1* genotype (expresser *vs*. non expresser HR= 2.53; 95% CI (1.17-5.46); p=0.01870); (ii) the percent time spent in the high exposure quantile (HR= 8.36; 95% CI (2.54-27.50); p=0.00047); and (iii) recurrence of the initial liver disease (HR=2.29; 95% CI (1.17-5.46); p=0.01315).

Because CsA and Tac are differentially metabolized by *CYP3A4* and *CYP3A5*, subgroup analysis was performed to evaluate the robustness of these results. Out of the 49 patients treated with CsA (30% of the recipients), 11 graft losses occurred (22.4%). Univariate and multivariate analysis did not confirm the influence of *CYP3A5*, CsA exposure and recurrence of the initial disease in this subgroup, but revealed a strong association between graft loss and the recipient *CYP3A4*22* CT versus CC (HR=6.94; 95%CI (1.97-24.35); p=0.00255).

In the group of 109 patients under Tac (68% of total), 28 graft losses occurred (26% of this subgroup). Univariate and multivariate analysis confirmed that the percent time spent in the high exposure quantile (HR= 7.63; 95%CI (2.12-27.42); p=0.0018) and the recipient *CYP3A5*1* expresser genotype (expresser vs. non expressers, HR=3.39; 95%CI (1.52-7.58); p=0.0028) were significantly and independently associated with graft loss, while the recurrence of the initial disease was not. No other significant association was found with graft loss in this subgroup.

4/ Factors linked with graft histological lesions

On the 791 biopsies available in this cohort of 161 patients, 123 acute rejection episodes were diagnosed and most (84%) occurred in the first year post-transplantation. None of the genetic or non-genetic variables studied was significantly associated with acute rejection.

75 cases of biopsy-proven chronic rejection were noted, with a median [25-75th] time of occurrence of 5 [2.19-7.34] years post-transplantation. Univariate analysis showed that recipients carrying the *ABCB1* homozygous wild-type exon 12 CC and exon 21 GG genotypes had a significantly higher risk of chronic rejection (exon 12 CC *vs*. TT, HR = 3.22; 95% CI (1.37-7.41); p=0.0072, and exon 21 GG *vs*. TT, HR = 2.78; 95% CI (1.18-6.67); p=0.02). Consistently, recipients carrying the *ABCB1* TTT/TTT triple haplotype versus other/other had a significantly lower risk of chronic liver graft rejection (other/other *vs*. TTT/TTT, HR= 2.34; 95% CI (1-5.43); p=0.05). Finally, treatment with Tac was associated with a higher risk of chronic rejection than with CsA (HR= 3.25; 95% CI (1.57-6.75); p=0.0016).

The results of multivariate analysis (Cox model) taking into account either the three *ABCB1* genotypes separately or the corresponding haplotype showed that the risk of chronic rejection was associated significantly with the recipient *ABCB1* exon 12 *SNP* CC vs. TT (HR = 3.12; 95%CI (1.35-7.24; p=0.0078) and the CNI drug used (Tac vs. CsA, HR = 3.22; 95%CI (1.57-6.60); p=0.0015). The recipient *ABCB1* haplotype other/other vs. TTT/TTT tended to be protective against chronic rejection (HR= 2.27, 95%CI (0.99-5.21); p=0.0537), but this did not reach statistical significance (Table 4 and figure 4a, 4b and 4c, 4d).

Subgroup analysis was conducted in patients on Tac, while it was not possible in patients on CsA owing to the low number of events (n = 7). In the Tac group (68/571 observations), only the recipient exon 12 CC genotype was significantly associated with chronic rejection (CC *vs*. TT, HR=2.83[1.21-6.62], p=0.016).

There were 49 cases of ductopenia without chronic rejection reported during patient follow-up (median occurrence time of 5.21 years [2.65-7.93]). Univariate analysis showed no significant association with either genetic or non-genetic variables.

5/ Factors of HCV and HBV recurrence or recurrence of initial liver disease

Only recipient age was strongly associated with a higher risk of viral recurrence (per year increase, HR = 1.06; 95%CI (1.03-1.09); p=<0.0001). The same result was observed for recurrence of the initial liver disease (per year increase, HR = 1.06; 95%CI (1.03-1.09); p=0.00013).

6/ Factors associated with liver function

None of the genetic or non-genetic factors was significantly associated with the evolution of the liver functions test (total bilirubin, ASAT, ALAT, ϒGT, alkalin phosphatases).

7/ Factors of renal function

We excluded the patients with combined liver and kidney transplantation (n=19) from this analysis. Univariate analysis and the final multivariate model adjusted on the CNI revealed a significant and independent association between renal function and: (i) baseline MDRD (β=0.51 ± 0.05; p<0.0001); (ii) duration of patient follow-up (per visit, β=-0.98 ± 0.22; p<0.0001); (iii) CNI exposure (per quantile increase, β=-2.42 ± 0.59; p<0.0001); and (iv) recipient age (per year increase, β=-0.37 ± 0.14; p=0.0084). None of the genetic factors was associated with the renal function.

Discussion

To our knowledge, this is the first study to evaluate the clinical impact of pharmacogenetic factors from both the donor (expressed in the liver graft) and the recipient (expressed in the rest of the body, in particular the small intestine and the kidney) in a large group of CNI-treated LT with a long-term (retrospective) follow-up. Indeed, we were able to extract DNA from liver biopsies, even many years after inclusion in paraffin.

On the basis of data collected retrospectively in 170 LT treated with CsA or Tac, we found that carriers of the recipient *CYP3A5*1* allele, i.e. expressers of the intestinal CYP3A5 protein, had a 2.5 fold higher risk of graft loss than non-expressers, i.e. homozygotes for the recipient *CYP3A5 *3/*3*. The analysis by CNI subgroup showed that this was true for patients on Tac but not for those on CsA. In patients on Tac, graft loss was also associated with the percentage of time they were overexposed to the drug and the recurrence of the initial liver disease, while in patients on CsA it was associated with the recipient *CYP3A4*22* CT genotype.

The risk of chronic rejection was associated with the recipient *ABCB1* exon 12 *SNP* CC vs. TT and the CNI drug used, while the recipient *ABCB1* haplotype other/other vs. TTT/TTT tended to be protective. Subgroup analysis in patients on Tac confirmed that the recipient exon 12 CC genotype was associated with a higher risk of chronic rejection.

Finally, renal function was strongly associated with high exposure to CNI and recipient age in the overall population, in addition to pre-transplant renal function and duration of patient follow-up post-transplantation.

The influence of the recipient *CYP3A5*1* allele on graft loss in patients on Tac is consistent with the corresponding extensive or intermediate metabolizer phenotype, as well as with previous study reports that, in the early period post-transplantation, recipient CYP3A5 expressers (carriers of the *1/*3 or *1/*1 genotypes) had a lower concentration/dose ratio (20-23) and received higher Tac daily doses (15,29) than CYP3A5 non-expressers. A meta-analysis suggested a major influence on Tac PK of the recipient *CYP3A5* *1 allele at the beginning of LT and the possible impact of the donor *CYP3A5* *1 thereafter (23). Another meta-analysis confirmed that higher Tac daily doses were required during the first year post-LT when either the liver graft or the recipient expressed *CYP3A5* (24). However, all previous studies failed to prove the influence of the recipient or donor *CYP3A5*3* polymorphism on LT clinical outcome (25-28). An explanation to our significant results might be that the carriers of the recipient *CYP3A5*1* allele have a relative (but long-term) under-exposure of their graft to Tac, which would result in a higher risk of rejection and graft loss, while the donor

CYP3A5 enzyme would influence more systemic blood concentrations than graft interstitial concentrations. Indeed, the recipient *CYP3A5*1* genotype reduces Tac bioavailability through increased intestinal clearance, a mechanism that might weaken with time post-transplantation. The intestinal CYP3A5 activity determines Tac concentration in the portal vein, hence in the liver interstitium where T cells can translocate to aggress the graft. Still, rather than being of cause of graft loss (as Tac is not hepatotoxic), increased Tac doses might be a consequence of hepatic function alterations, leading clinicians to prescribe higher doses of CNI in the long term (29). Moreover, Xue et al. recently reported a higher risk of infectious complications and lower immune response in Chinese transplanted patients if they carried either a donor or a recipient *CYP3A5* *1 allele. They failed to demonstrate an impact on outcome, but the study concerned pediatric recipients only followed-up over the first year post-transplantation (30). Further investigations are thus needed to confirm the influence of the recipient *CYP3A5*3* polymorphism on liver graft outcome and understand the mechanisms involved.

In the subgroup of patients receiving CsA, strong association between graft loss and the recipient *CYP3A4*22* T allele was found. Elens et al. showed that this allele was associated with 1.6-fold higher CsA dose-adjusted concentrations in 50 renal transplant recipients (95%CI: 1.1-2.6; p=0.019) and that the influence of the *CYP3A4* *22 genotype may depend upon the *CYP3A5*3* status (5,6). We failed to show an association between the recipient *CYP3A4* status and CsA concentration or dose, because of the small size of the group (49 patients) and probably also because the liver expresses the donor CYP3A4 genotype. However, one cannot exclude that these patients were slightly over-exposed to CsA in the long term, increasing the risk of recurrence of initial disease.

Concerning the influence of the ABCB1 polymorphism, P-gp is an efflux transporter and is expressed in the lymphocytes, kidney and intestine. P-gp prevents the luminal entry of CNI at apical membranes and both CYP3A4 and ABCB1 may play the role of an “absorptive barrier” in the intestine of the recipient. The *ABCB1* T, TT or TTT alleles transport Tac at the apical membrane of kidney tubular epithelial cells less efficiently than the corresponding wild-type alleles (31). Higher intra-individual variability of the concentration/dose ratio of Tac and risk of early acute cellular rejection were associated with high *ABCB1* mRNA expression in living-donor liver transplant recipients (32-40). Homozygotes for the exon 26 TT haplotype had lower intestinal P-gp expression and higher drug bioavailability, with higher dose-normalized blood Tac concentrations (41). In contrast, recipients with the CC genotype of the 3435C>T SNPs were possibly associated with significantly lower dose-adjusted concentration

and needed higher doses of Tac to reach the target blood level in renal transplantation (41). This is consistent with our results, where the exon 12 TT genotype was significantly associated with a lower risk of chronic rejection compared to CC, while the difference was not significant with the heterozygous CT genotype. The hypothesis of a lower CNI dose requirement for the homozygous variant genotype is possible. Moreover, Elens et al. confirmed that the donor ABCB1 SNPs with at least one mutated allele for 1236C>T or 2677G>T/1 in LT showed higher Tac hepatic concentrations than wild-type homozygous donor carriers. The Banff score was also significantly lower among 1236C>T than 1236CC carriers in the first 7 days post LT (42). Wei-lin et al., showed that Chinese carriers of the recipient ABCB1 3435CC genotype required higher Tac doses in LT (43). Gomez-Bravo et al. did not confirm that at three months post LT, recipients of a liver carrying the CYP3A5*1 allele took significantly higher Tac doses than those of a CYP3A5*3/*3 liver, while there was no association of donor or recipient ABCB1 with Tac pharmacokinetic or acute rejection during the first three months post-transplantation (44) . Knowledge of the recipient *ABCB1* exon 12 genotype can help to identify patients with higher risk of chronic rejection in LT. Tac hepatic concentrations might also be a biomarker of interest.

In the present study, renal function at the beginning of LT and CNI overexposure over the follow-up period were predictive of further loss of renal dysfunction. Several studies showed that up to 33% of LT had an already altered renal function at the time of LT (45-47). The etiology of chronic kidney disease after liver transplantation is reportedly mainly attributable to CNI toxicity (48%) and/or hypertensive vascular changes (44%) (48). We did not find a significant pharmacogenetic influence on renal function, similar to a previous study but contrary to Hawwa et al., who showed that the TTT haplotype (C1236T, G2677T, C3435T) was associated with decreased renal function at 6 months post-transplantation in 51 paediatric LT on Tac (49). Most previous studies only investigated the initial post-LT period, while the effect of *ABCB1* SNPs or haplotypes may be stronger in the longer term (50,51). Such discrepancies can be due to a lack of statistical power in the negative studies, a false positive result in the positive one, or different definitions of decreased renal function.

CNI adversely alter renal function by mechanisms of acute and chronic toxicity. Both CsA and Tac were associated with alterations in the tubular epithelium, mesangium, afferent glomerular arterioles and interstitium (52-54). Some authors hypothesized that decreased P-gp expression and activity can favor intra-renal accumulation of CNI, leading to alteration of the renal function.

Association studies between renal dysfunction and the donor and recipient *ABCB1* genotypes in kidney transplantation reported contradictory results (3,55-56). Cattaneo et al. described an increased risk of CsA related adverse renal events in carriers of T allelic variants in either *ABCB1* exons 21 or 26 recipients (57). Naesens et al. showed that the combined donor-recipient homozygosity for the *ABCB1* exon 26 3435T variant was associated with chronic tubulointerstial allograft damage under CNI over the first 3 years post-transplantation (56). Our unit confirmed in a cohort of kidney transplant recipients on CsA that the donor *ABCB1* TTT variant haplotype was associated with altered renal function and showed for the first time that it was a determinant of graft survival over the first 10 years (3). At odds with these three studies, one study reported that kidney transplant recipients homozygous for the *ABCB1* 2677T allele (exon 21) would have a reduced risk of nephrotoxicity (but they did not investigate the donor genotype) (54), and another found an increased risk of graft failure for patients carrying the donor exon 26 3435CC genotype, as compared to other genotypes (58).
Therefore, as in liver transplantation, the impact of ABCB1 on renal function in renal transplantation on CNI is still a matter of debate that a unified definition of renal function decrease, as well as maybe a meta-analysis of the available studies may help settle.
In any case, Tac blood concentrations are probably poor indicators of the presumably local, intra-tissue drug accumulation that might be involved in such effects.
They are some limitations in this single-center, retrospective study. First, patients were treated with either tacrolimus or cyclosporine. Adjustment on CNI therapy helped us assessing risk factors of adverse outcomes in the whole cohort of patients, including those who were switched from one CNI to the other during the follow-up. We tested the internal validity of the significant results in the two subgroups of CNI treatment, in search of differential effects, but the small number of patients on cyclosporine resulted in poor statistical power in this group. Secondly, the incidence of chronic rejection may have been underestimated, as some patients may develop chronic lesions before their liver tests are altered. Chronic rejection is characterized by loss of bile ducts (inflammatory and degenerative alterations) and an obliterative arteriopathy on large and medium-sized arteries, and possible centrilobular inflammation and fibrosis. Hübscher published a review on the long-term outcome of liver allograft that emphasizes that the terminology used to describe late graft lesions needs to be clarified (59). Late rejection, de novo autoimmune hepatitis and “idiopathic” post-transplant hepatitis may belong to the same family of immune-mediated diseases. In our study, we only considered biopsy-confirmed chronic rejection, but many “chronic hepatitis” cases may belong to the same overlapping spectrum. Indeed, physicians can modify the

immunosuppressive regimen on the basis of the liver biopsy results, hence reversing histological lesions. Another point is the prevalence of hepatitis C in this cohort. The distinction between hepatitis C and acute or chronic rejection can be a problem for pathologists. Patients with hepatitis C recurrence were under antiviral treatment and often received minimized immunosuppressive regimen to avoid high viral loads. However, this study showed a low frequency of acute and chronic rejection and good survival at 10 years.

In summary, genetic factors may be useful to identify liver transplant patients with a high risk of graft loss or chronic rejection and to personalize their immunosuppressive regimen. The recipient *CYP3A5*3* polymorphism could become a predictive marker of Tac dose requirement in LT. Prospective, randomized studies will be necessary to determine the usefulness of *CYP3A5* genotyping in LT. The base renal function of the candidates to liver transplantation may help to select an immunosuppressive regimen sparing renal function, which in itself may be a determinant of poor outcomes in liver transplantation.

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Nonstandard abbreviations
LT Liver transplantation
CsA Cyclosporine
Tac Tacrolimus
MMF mycofenolat mofetil
CYP3A4 cytochrome P450 3A4
CYP3A5 cytochrome P450 3A5
P-gp P-glycoprotein
SNP single-nucleotide polymorphism
CNI calcineurin inhibitor
C0 residual blood concentration

Table 1. Patient demographics and clinical data

Variable	**Categories**	**Values**
Recipient Sex (male/female) Donor Sex (male/female)		112/58 [66%/44%] 109/61 [64%/46%]
Recipient age (years) Donor age (years)		48 [37.25-55] 40.5 [28-51]
Follow-up duration (years)		11.85 [5.82-13]
Indication for liver transplantation	Cirrhosis Carcinoma Cholestatic or metabolic liver diseases or others Fulminant hepatitis Chronic rejection	86 [50.6%] 36 [21.2%] 33 [19.4%] 15 [8.8%] 0
Graft type	Cadaveric Domino	164 [96.5%] 6 [3.5%]
Type of graft	Liver Combined liver kidney	151 [88.8%] 19 [11.2%]
Serum creatinine (µmol/L)		106 [88-130]
Estimated glomerular filtration rate using the MDRD formula (ml/min/1.73m^2)		62 [48-79]
Total bilirubin (µmol/L)		13.7 [10-18]
ASAT (UI/L)		25 [19-38]
ALAT (UI/L)		28 [19-51]
ϒGT (UI/L)		54 [25-121]
Alkaline phosphatases (UI/L)		86 [63-132]
CNI therapy	CsA (patient number) Dose CsA (mg/day) C0 CsA (ng/mL) Tac (patient number) Dose Tac (mg/day) C0 Tac (ng/mL) NA	55 [32.3%] 150 [100-200] 108 [70-167] 112 [65.9%] 3 [2-5] 6.5 [4.6-8.9] 3 [1.8%]

MMF	Patient number	19 [11.2%]
Quantile of CNI exposure	1 = low exposure quantile (<25th percentile) 2 = average exposure quantile (25th to 75th percentiles) 3 = high exposure (> 75th percentile)	2 [1-2]

Continuous values are expressed as median [25-75th quartiles], categorical data are expressed as n (%)

Table 2. Frequency and distribution of the studied polymorphisms in donors and recipients

Polymorphism	**SNPs**	**n (%)**
Recipient *CYP3A4* *22	CC	148 [87%]
	CT	19 [11.2%]
	TT	0
	NA	3
Recipient *CYP3A5*3*	AA	2 [1.2%]
	GA	21 [12.3%]
	GG	147 [86.5%]
Recipient CYP3A5 phenotype	Expressers	23 [13.5%]
	Non expressers	147 [86.5%]
Recipient *ABCB1* genotypes	3435 C>T (exon 26)	
	CC	54 [31.8%]
	TT	72 [42.3%]
	CT	44 [25.9%]
	G2677T G>T (exon 21)	
	GG	70 [41.2%]
	GT	73 [43%]
	TT	27 [15.8%]
	C1236 C>T (exon 12)	
	CC	64 [37.6%]
	CT	78 [45.9%]
	TT	28 [16.5%]
Recipient *ABCB1* haplotype	Other other	79 [46.5%]
	TTT other	71 [41.8%]
	TTT TTT	20 [11.7%]
Donor *CYP3A4* *22	CC	148 [87%]
	CT	22 [13%]
	TT	0
Donor *CYP3A5*3*	AA	2 [1.1%]
	GA	26 [15.3%]
	GG	141 [82%]
	NA	1 [0.6%]

Donor CYP3A5 phenotype	Expressers	28 [16.4%]
	Non expressers	141 [83%]
	NA	1 [0.6%]
Donor *ABCB1* genotypes	3435 C>T (exon 26)	
	CC	40 [23.5%]
	TT	95 [55.9%]
	CT	34 [20%]
	NA	1 [0.6%]
	G2677T G>T (exon 21)	
	GG	59 [34.7%]
	GT	83 [48.8%]
	TT	27 [15.9%]
	NA	1 [0.6%]
	C1236 C>T (exon 12)	
	CC	56 [33%]
	CT	92 [54.1%]
	TT	21 [12.3%]
	NA	1 [0.6%]
Donor *ABCB1* haplotype	Other other	66 [38.8%]
	TTT other	86 [50.6%]
	TTT TTT	17 [10%]
	NA	1 [0.6%]

Table 3. Multivariate analysis (Cox Model) of the risk of graft loss adjusted on CNI therapy

Variable	**Category**	**Adjusted hazard ratio**	**Adjusted 95% CI**	***P***
Recurrence of initial liver disease	Yes *vs*. no	2.29	1.19-4.43	0.01315
Percent time spent in the high quantile	Per 1% increase	8.36	2.54-27.50	0.00047
Recipient CYP3A5 Phenotype	Expressers *vs*. non expressers	2.53	1.17-5.46	0.01870

Table 4. Multivariate analysis (Cox model) of the risk of chronic rejection (taking into account either the three *ABCB1* genotypes separately or the corresponding haplotype)

Variable	**Category**	**HR**	**95% CI**	***P***
Recipient *ABCB1* exon 12 SNP	CC *vs*. CT	1.27	0.68-2.38	0.4511
	CC *vs*. TT	3.12	1.35-7.24	0.0078
CNI therapy	Tac *vs*. CsA	3.22	1.57-6.60	0.0015
Recipient *ABCB1* haplotype	Other/other *vs*. TTT/other	1.28	0.68-2.41	0.4467
	Other/other *vs*. TTT/TTT	2.27	0.99-5.21	0.0537
CNI therapy	Tac *vs*. CsA	3.14	1.51-6.53	0.0022

Table 5. Multivariate analysis of renal function (MDRD) degradation using generalized estimating equation (gee) multiple linear regression.

Variable	**Category**	**β ± SD**	***P***
Intercept		4.88 ± 8.76	< 0.0001
Duration of patient follow-up	Per visit	-0.98 ± 0.22	<0.0001
Baseline MDRD value	Per unit increase	0.51 ± 0.05	<0.0001
Quantile of CNI exposure	Per quantile	-2.42 ± 0.59	<0.0001
Recipient age	Per year	-0.37 ± 0.14	0.0084

Figure legends:

Figure 1: Enrollment and outcomes in 257 liver transplant recipients over the study period (the 54 patients who died during the first year post-transplantation were excluded)

Figure 2: Kaplan–Meier curves of patient survival after liver transplantation over the 15-years follow-up period

Figure 3: Kaplan–Meier curves of cumulative survival without graft loss as a function of recipient *CYP3A5*3* status and CNI received

Figure 4: Cumulated incidence curve of chronic rejection as a function of: (4a) recipient *ABCB1* exon 12 SNP; (4b) recipient *ABCB1* exon 21 SNP; (4c) *ABCB1* haplotype; (4d) CNI received

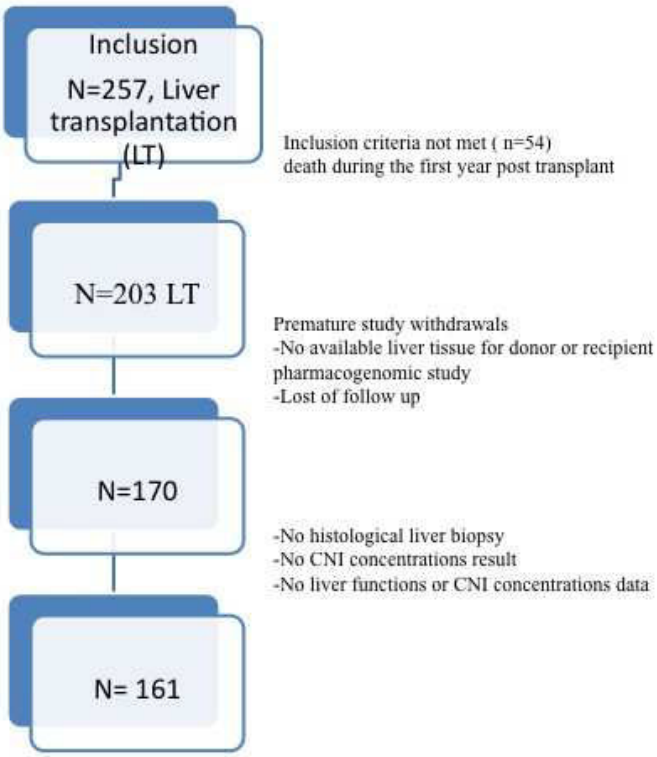

Inclusion
N=257, Liver transplantation (LT)
Inclusion criteria not met (n=54)
death during the first year post transplant
N=203 LT
Premature study withdrawals
-No available liver tissue for donor or recipient pharmacogenomic study
-Lost of follow up
N=170
-No histological liver biopsy
-No CNI concentrations result
-No liver functions or CNI concentrations data
N= 161

Figure 1

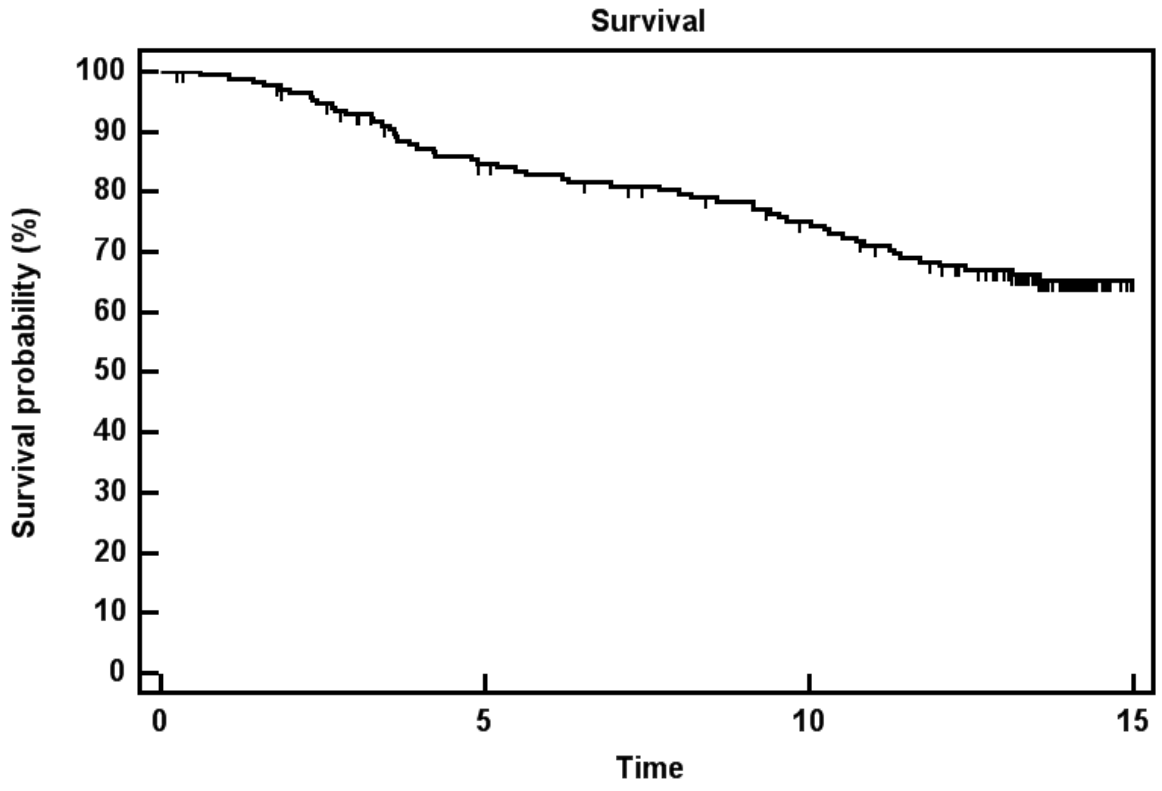
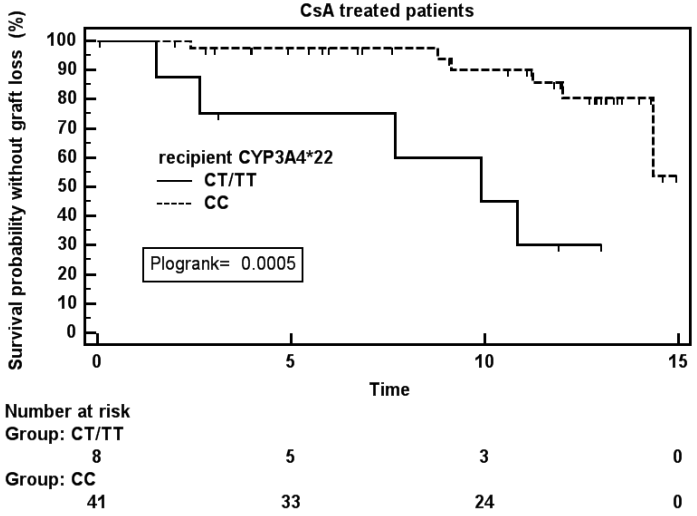
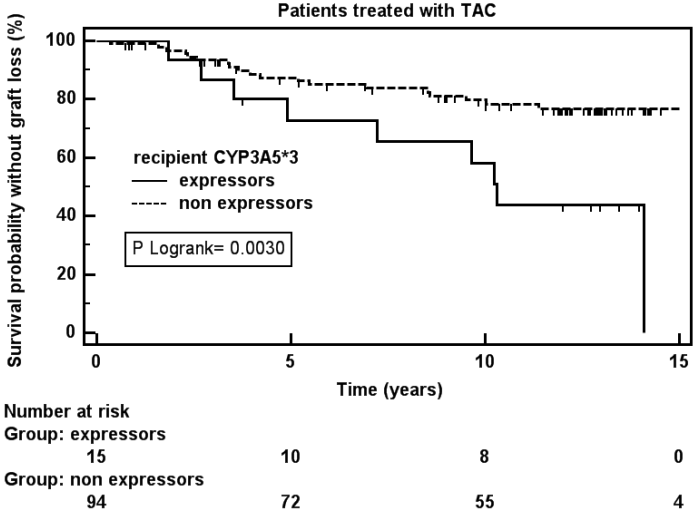

Survival
Survival probability (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
Time

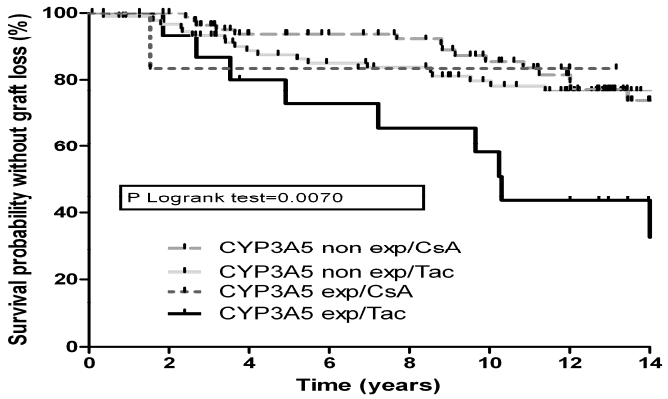
Figure 2



3a



3b



3c

Figure 3

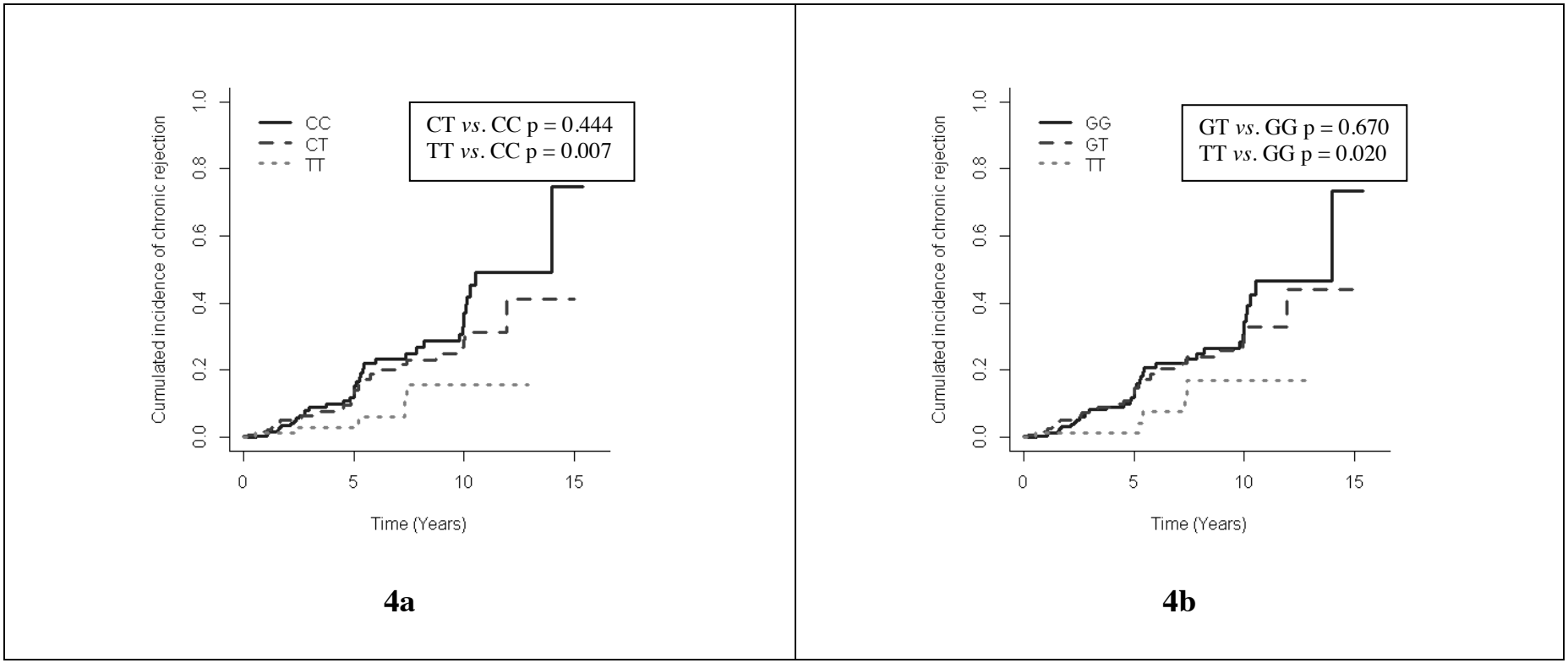
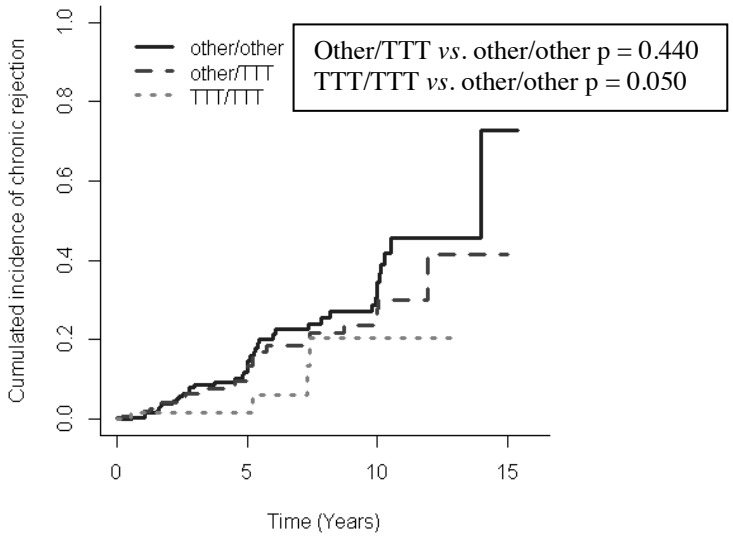
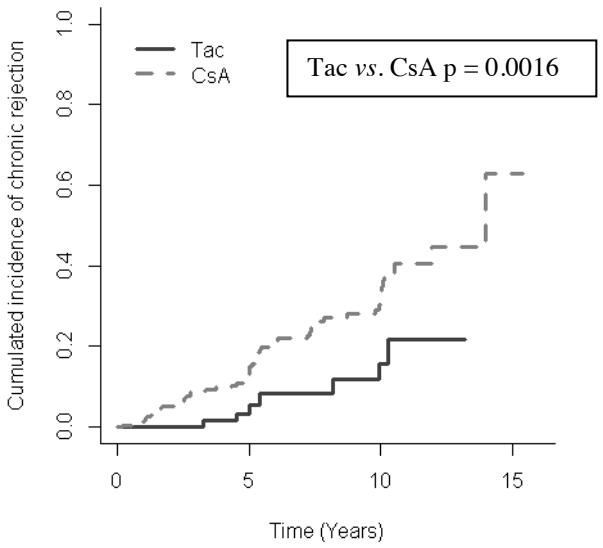

Cumulated incidence of chronic rejection
0.0
0.2
0.4
0.6
0.8
1.0
CC
CT
TT
CT vs. CC p = 0.444
TT vs. CC p = 0.007
0
5
10
15
Time (Years)
4a
Cumulated incidence of chronic rejection
0.0
0.2
0.4
0.6
0.8
1.0
GG
GT
TT
GT vs. GG p = 0.670
TT vs. GG p = 0.020
0
5
10
15
Time (Years)
4b

Figure 4


other/other
other/TTT
TTT/TTT
Other/TTT *vs*. other/other p = 0.440
TTT/TTT *vs*. other/other p = 0.050
Cumulated incidence of chronic rejection
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Time (Years)

4c


Tac
CsA
Tac *vs*. CsA p = 0.0016
Cumulated incidence of chronic rejection
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Time (Years)

4d

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IV. Perspectives et conclusion

La pharmacogénétique largement étudiée en transplantation rénale demeure encore confidentielle dans le domaine de la greffe hépatique, à la fois dans la preuve de son intérêt et dans sa réelle influence sur le devenir clinique. Notre travail de revue nous a montré la disparité des études cliniques et leurs différences méthodologiques très variées rendant souvent difficile les comparaisons. De nombreuses publications valident l'impact des polymorphismes CYP3A4*22 et CYP3A5 sur la pharmacodynamie des CNI, en particulier le tacrolimus devenu le premier immunosuppresseur en greffe d'organes. La pharmacogénétique est appliquée en virologie, en cancérologie et a un intérêt médico-économique potentiel. La prescription de la dose adaptée pour un patient donné peut permettre d'éviter des effets secondaires graves voire une hospitalisation (sepsis, rejet, insuffisance rénale…). Les CNI ont une grande variabilité inter-individuelle pharmacocinétique et une marge thérapeutique étroite avec des risques d'effets secondaires graves en cas de sur ou sous dosage. Le suivi thérapeutique pharmacologique est essentiel et permet aux cliniciens d'adapter au mieux les posologies des CNI. Le STP permet de compenser rapidement les variabilités intra-individuelles de concentrations thérapeutiques pour les cliniciens mais peut-être reste-t-il insuffisant pour limiter les risques sur le devenir à long terme. En transplantation rénale, le génotypage CYP3A5 permet de déterminer les doses précoces de tacrolimus afin d'éviter les modifications de doses et les variations trop importantes de posologie depuis l'étude de Thervet et al. qui reste la référence. En greffe rénale, cardiaque ou pulmonaire, les patients porteurs des génotypes *CYP3A5*1/*1* ou *CYP3A5 *1/*3* ont besoin de 1.5 à 2 fois la dose pour atteindre une concentration sanguine identique à ceux porteurs du génotype *CYP3A5*3/*3*. Birdwell et al. ont publié des recommandations thérapeutiques pour l'initiation du tacrolimus selon le phénotype du receveur (« poor, extensive ou intermediate metabolizer »). Il ne semble pas exister jusqu'à présent d'influence du dosage de tacrolimus selon le génotypage sur le devenir clinique à long terme. La période initiale est souvent la plus exposée aux rejets aigus et peut avoir un impact sur le devenir du greffon et à l'inverse, le surdosage en CNI peut s'accompagner d'insuffisance rénale ou de risques infectieux voire entraîner un risque accru de mortalité dans la première année (10 % dans la première année en greffe hépatique surtout par cause infectieuse).

En transplantation hépatique, quand on étudie le génotype du donneur, on s'intéresse aux polymorphismes des enzymes de métabolisme et des transporteurs hépatiques alors que quand

on étudie le génotype du receveur, on s'intéresse aux polymorphismes des enzymes de metabolisme et transporteurs intestinaux et rénaux.

Le fait de n'avoir pas d'impact du génotype du donneur sur le devenir des greffons est conforme aux résultats de nombreuses études en transplantation rénale qui n'ont jamais mis en évidence d'influence des polymorphismes du receveur (incluant les polymorphismes hépatiques et intestinaux) sur le rejet ou la survie du greffon rénal alors que les polymorphismes des donneurs (ceux du greffon rénal) avaient une influence.

Le fait d'avoir un effet des polymorphismes du receveur uniquement serait en faveur d'un effet du métabolisme ou du transport membranaire intestinal ou rénal. Il est connu que l'activité intestinale de la P-gp (du receveur) prédomine (par rapport à celle du greffon) en post-greffe hépatique immédiate. Il est probable qu'il en soit de même pour l'activité des cytochromes P450. Ceci pourrait expliquer le rôle prédominant des SNPs du receveur en greffe hépatique en post-opératoire. On pourrait également faire l'hypothèse que lors du 1er passage hépatique, les CNI soient en concentration trop importante pour qu'un effet éventuel des polymorphismes des enzymes hépatiques (donc du donneur) ne se manifeste. Un effet significatif de la P-gp et des CYP3A rénaux (donc du receveur) sur les concentrations de CNI peut constituer une hypothèse de régulation de la quantité de CNI arrivant secondairement au niveau hépatique. Ceci pourrait être particulièrement favorisé chez un expresseur du CYP3A5 au niveau rénal qui présenterait des concentrations plus faibles au niveau hépatique et donc un risque de rejet chronique plus important.

En greffe hépatique, les résultats concernant le génotypage CY3A5 sont discordants. Le génotype CYP3A5 peut différer entre le receveur et le greffon. Les recommandations de Birdwell et al. concernent seulement les greffés hépatiques ayant un génotypage *CYP3A5* identique entre greffon et receveur du fait d'un nombre d'études bien moindre et moins concluantes qu'en greffe rénale.

Nous avons pu rappeler dans notre revue de littérature que les polymorphismes les plus fréquents, les plus étudiés et impliqués en greffe hépatique étaient néanmoins les *CYP3A4*22*, *CYP3A5* et *ABCB1*, surtout avec le tacrolimus. L'impact sur le devenir clinique en dehors des dosages immédiats reste cependant incertain selon les données actuelles de la littérature. La question « mythe ou réalité » demeure non résolue en transplantation hépatique et a suscité tout l'intérêt de notre deuxième partie de thèse.

Notre travail expérimental était volontairement consacré à une étude à très long terme afin de valider la possible influence des polymorphismes sur le devenir clinique de la greffe hépatique. Notre cohorte était plus faible que prévu initialement du fait de la difficulté à avoir

à la fois des données cliniques, biologiques et pharmacogénétiques pour chacun. Pourtant, l'avantage de centre expert en greffe hépatique est la richesse des données potentielles rétrospectives disponibles pour ce type d'étude. Disposer des données à la fois sur les donneurs et les receveurs est essentiel en matière de greffe d'organe. On ne peut concevoir une étude pharmacogénétique sans disposer des données sur les deux acteurs principaux que sont l'hôte et le greffon. Cette étude a montré l'association significative de CYP3A5 expresser du receveur, de la récidive de la maladie initiale et du temps passé dans le quantile haut d'exposition aux CNI sur un risque plus élevé de perte du greffon. Cette notion de perte de greffon était difficile à évaluer initialement mais nous avons été vigilant pour définir la perte de greffon comme un arrêt de fonctionnement du greffon d'origine strictement hépatique. La récidive de la maladie initiale était incluse dans cette définition de manière logique, les patients récidivant peut-être plus leur maladie virale, néoplasique ou auto-immune selon leur exposition aux CNI. Nous avons pu souligner l'intérêt immense de disposer de programmes de biopsies hépatiques systématiques pour la surveillance à long terme. Les anomalies histologiques sont souvent plus précoces que les anomalies biologiques et permettent seul de modifier la stratégie immunosuppressive à la carte. Les méthodes non invasives d'évaluation de la fibrose ne pourront pas remplacer la richesse des données histologiques, ce d'autant que les lésions décrites sur un greffon « âgé » sont parfois difficiles à interpréter. Seuls des histopathologistes entraînés à la lecture des biopsies hépatiques des greffés peuvent analyser ces lames et ce d'autant qu'il s'agit d'anciens greffés. Nous avions volontairement décidé de prendre une « outcome » comme la ductopénie car elle peut être le signe précurseur d'un début de rejet chronique. Celle-ci peut être signalée dans les compte rendus dès qu'elle atteint au moins 10 % des canaux biliaires. Aucun facteur génétique ou non génétique n'a été significativement associé à la ductopénie mais il semble que cette donnée ne soit pas systématiquement rapportée dans les compte-rendus lorsqu'elle demeure isolée. L'allèle TT de l'exon 12 d'ABCB1 du receveur est associé à un risque plus élevé de rejet chronique comparé à l'allèle CC de l'exon 12 d'ABCB1, peut-être par un mécanisme de besoin accru en CNI pour atteindre les doses thérapeutiques par l'augmentation de la P-gp expression intestinale pour le variant CC. Finalement, le génotype du receveur dans notre étude apparait comme essentiel pour le devenir clinique du fait d'une expression ubiquitaire dans le rein, l'intestin et les leucocytes entre autre. La néphrotoxicité est associée significativement avec la sur-immunossuppression, la fonction rénale initiale ainsi que l'âge du receveur. Ces facteurs sont classiques mais méritent d'être plus pris en compte pour le devenir à long terme des greffés hépatiques. Les stratégies thérapeutiques restent très

standardisées pour l'immunosuppression initiale et sont encore peu personnalisées au profil de chaque greffé. Un greffé hépatique dont les résultats biologiques restent dans des valeurs normales est rarement soumis à des changements thérapeutiques, alors même que les tests biologiques des fonctions hépatiques peuvent ne pas refléter l'exposition in situ du greffon ou du rein aux CNI, par exemple. L'analyse statistique a été très riche et très novatrice. Elle a permis d'étudier, en ajustant les résultats, des patients sous ciclosporine et sous tacrolimus voire même des patients ayant changé d'immunosuppresseurs dans le temps. L'exposition aux CNI a pu être exprimée en terme de quantile d'exposition bas, moyen ou haut selon les dosages résiduels de chaque patient à chaque visite anniversaire et selon les autres patients et leurs dosages moyens respectifs. Ceci a permis ensuite d'évaluer pour chaque greffé une moyenne des quantiles d'exposition aux CNI rapporté au temps d'exposition total depuis la greffe hépatique. Cette variable « temps passé dans le quantile haut ou bas d'exposition aux CNI » a été construite pour prendre en compte au mieux l'exposition réelle dans le temps aux CNI. Pour l'analyse de la perte de greffon et du décès, un modèle de Cox avec un seul événement a été choisi, l'événement ne pouvant être cumulé. L'évaluation de la dégradation de la fonction rénale a pu être étudiée en continu, chaque patient étant son propre témoin et tout baisse étant prise en compte dans l'évaluation finale. L'ajustement de l'estimation de la fonction rénale se fait sur la valeur initiale pour chaque patient. Chaque variable testée a été étudiée en analyse univariée puis reprise en modèle multivarié. Au final, seuls les résultats en analyse multivariée ont été pris en compte.

Notre travail rappelle toute la complexité de la prise en charge immunosuppressive des greffés hépatiques dans le long terme. Le rejet chronique est peut-être sous-estimé prenant parfois des aspects histopathologiques différents. Parallèlement, une sur exposition à des doses élevées d'immunosuppresseurs est associée à des risques plus élevés de perte de greffon et à un risque d'aggravation de la fonction rénale. Les risques de récidive néoplasique et de diabète de novo sont deux risques pour lesquels la pharmacogénétique peut apporter une aide dans le profil personnalisé de chaque greffé. Tous les programmes de recherche actuels visent à optimiser la survie du greffon. Notre étude est la première étude à démontrer l'importance de la pharmacogénétique sur le devenir clinique à long terme des greffés hépatiques sur une cohorte suivie sur 10 ans en moyenne. L'étude des polymorphismes *CYP3A4*22*, *CYP3A5* et *ABCB1* des donneurs et des receveurs en greffe hépatique peut permettre de donner à la pharmacogénétique un intérêt « réel » pour répondre à notre question initiale. Les polymorphismes *CYP3A5* et exon 12 d'*ABCB1* du receveur sont les seuls significativement associés au risque de perte de greffon ou de rejet chronique. Une étude prospective

multicentrique pour valider l'intérêt du génotypage en greffe hépatique pour les polymorphismes *CYP3A5* et *ABCB1* pourrait être proposée. La pharmacogénétique des polymorphismes *CYP3A4, CYP3A5* et *ABCB1* doit s'intégrer à d'autres recherches visant à améliorer le devenir de la greffe. Des biomarqueurs précoces du greffon, la pharmacogénétique de la voie de la calcineurine voire le screening complet du génome de l'hôte doivent compléter l'approche globale du greffé.

En définitif et de façon pragmatique, en l'état actuel des connaissances, aucun génotypage systématique n'est à recommander en transplantation hépatique et le suivi thérapeutique reste basé sur les taux résiduels des CNI. Les AUCs estimées à partir d'un nombre de prélèvements limités pourraient permettre un meilleur monitoring des patients.

V. Bibliographie

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Marilyne DEBETTE-GRATIEN

TITRE : Etude rétrospective de l'influence des polymorphismes génétiques de *CYP3A4*, *CYP3A5* et *ABCB1* des donneurs et des receveurs sur les effets des immunosuppresseurs en transplantation hépatique.

RESUME : La transplantation hépatique est une technique chirurgicale maîtrisée, mais le devenir à long terme du greffon et de l'hôte doit encore être amélioré. L'étude pharmacogénétique des inhibiteurs de la calcineurine (CNI) devrait permettre de comprendre la variabilité de leurs effets thérapeutiques et toxiques. Dans un premier temps, nous avons réalisé une revue de la littérature concernant la pharmacogénétique des CNI en greffe d'organe et surtout hépatique en particulier les trois polymorphismes les plus impliqués dans la pharmacocinétique des CNI (*CYP3A4*22*, *CYP3A5*3* et *ABCB1* exons 12, 21, 26) et leurs éventuelles associations avec le devenir clinique du patient. L'état actuel des connaissances valide l'intérêt du génotype *CYP3A5*3* pour adapter au mieux la posologie précoce de tacrolimus seulement en greffe rénale.
Dans un second temps, nous avons mené une étude de cohorte rétrospective visant à étudier la pertinence et l'intérêt des génotypes du donneur et du receveur d'organe mentionnés précédemment, intervenant dans le métabolisme (*CYP3A4*22*, *CYP3A5*3*) et le transport membranaire (*ABCB1* exons 12, 21 et 26) de la cyclosporine et du tacrolimus en transplantation hépatique. 170 patients avec un suivi de plus de 10 ans en moyenne ont été inclus. Les principaux résultats montrent que : l'allèle *CYP3A5 *1* du receveur était associé significativement à un risque plus élevé de perte de greffon à long terme comparé à l'allèle *CYP3A5 *3* ; l'allèle TT de l'exon 12 d'*ABCB1* du receveur était associé à un risque moins élevé de rejet chronique ; et l'exposition à des doses élevées de CNI, la valeur initiale de la fonction rénale et l'âge du receveur étaient également indépendamment associés au risque d'altération de la fonction rénale. La caractérisation de ces marqueurs pharmacogénétiques en transplantation hépatique pourrait permettre d'adapter les traitements immunosuppresseurs pour chaque patient transplanté. D'autres voies de recherche (pharmacogénétique de la voie calcineurine, biomarqueurs précoces des lésions du greffon, etc.) seront nécessaires pour identifier un profil personnalisé pour chaque greffé afin d'adapter au mieux la stratégie thérapeutique à long terme.

Mots-clés : Transplantation hépatique ; *CYP3A4*22* ; *CYP3A5* ; *ABCB1* ; Perte du greffon ; Rejet chronique ; Néphrotoxicité.

TITLE: Retrospective study of the influence of donor and recipient *CYP3A4, CYP3A5* and *ABCB1* genotypes on the effects of anticalcineurin therapy in liver transplantation.

ABSTRACT: Liver transplantation is now a well mastered surgery with standardized procedures, but the long-term clinical outcomes of the graft and the patient remain uncertain. The pharmacogenetic study of the calcineurin inhibitors (CNI) cyclosporine and tacrolimus should help to understand the variability of their pharmacokinetics and therapeutic or side effects. In the first part of this work, we reviewed the main pharmacogenetic studies of CNI in livert transplantation, focusing on the three polymorphisms mostly involved in CNI pharmacokinetics (*CYP3A4*22*, *CYP3A5*3* et *ABCB1* exons 12, 21, 26) and their possible associations with clinical outcomes. To date, the only pharmacogenetic test consensually recommended in organ transplantation is the *CYP3A5*3* variant for a better selection of the initial tacrolimus dose in kidney transplantation. The second part of this work was a retrospective cohort study in liver transplantation to investigate the influence of the abovementioned donor's and recipient's genotypes, involved in the metabolism (*CYP3A4*22*, *CYP3A5*3*) and the membrane transport (*ABCB1* exons 12, 21 and 26) of cyclosporine and tacrolimus. 170 patients were enrolled in this study with a mean follow-up of more than ten years. Our main results are that: the recipient *CYP3A5*1* allele was associated with a higher risk of graft loss than the *CYP3A5*3* allele; the recipient *ABCB1* exon 12 TT genotype was associated with a lower risk of chronic rejection than the CC genotype; overexposure to CNI, initial renal function and recipient age were associated with a higher risk of post-transplantation renal dysfunction. No genetic factor was associated with patient survival, acute rejection, liver function tests, recurrence of viral or other initial liver disease, or nephrotoxicity. Prospective characterization of both recipient and donor *CYP3A4*, *CYP3A5* and *ABCB1* polymorphisms could help to optimize immunosuppressive therapy for each candidate to liver transplantation. Further studies (pharmacogenetics of calcineurin pathway, early biomarkers of graft dysfunction, etc.), should help to define a personalized profile for each transplant patient in order to best adapt the immunosuppressive strategy on the long term.

Keywords: Liver transplantation; *CYP3A4*22*; *CYP3A5*; *ABCB1*; graft loss; chronic rejection; nephrotoxicity.

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