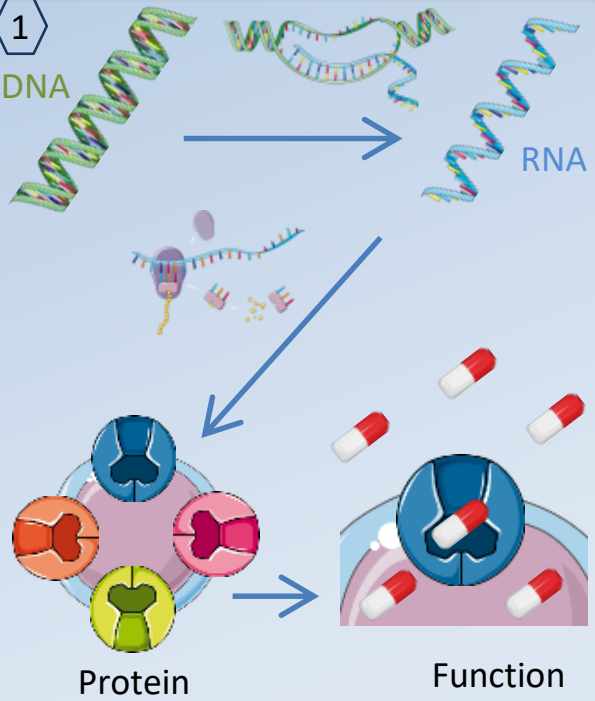
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2

- Tacrolimus (TAC) is the keystone of post-transplantation immunosupression
- Genetic polymorphisms of drug transporters influence its pharmacokinetics
- Drug transporters could influence drug diffusion to T lymphocyte, i.e. the action site

Their impact on whole blood over peripheral blood mononuclear cells TAC concentration ratio ($C_{PBMC/blood}$) was clinically investigate in 60 organ transplant recipients at the genetic, mRNA, and proteic levels, and functionnally *in vitro*

3

$C_{PBMC/min}$ vs :	*ABCB1*/ P-gp	*ABCC2*/ MRP2	*SLC28A3*/ CNT3	*SLC29A1*/ ENT1
Genetic polymorphism	Tendency	Not tested	Tendency	Not tested
mRNA				
Proteic				
Functionnal (in vitro models)	Transport	No transport	Non conclusive	Non conclusive

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- Innovative study
- Multiple approaches
- This work should encourage other in the field of drug transporters of tacrolimus

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Title Page

Title

Drug transporters are implicated in the diffusion of tacrolimus into the T lymphocyte in kidney and liver transplant recipients: genetic, mRNA, protein expression, and functionality

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Abstract

Because of a narrow therapeutic index and a wide inter- and intra-patient variability, therapeutic drug monitoring of the immunosuppressant drug tacrolimus (TAC) based on whole-blood concentrations (C_{blood}) is mandatory in solid organ transplant recipients. Using peripheral blood mononuclear cells concentrations (C_{PBMC}) could improve patient outcomes. The poor correlation between C_{blood} and C_{PBMC} makes hypothesize that drug transporters are implicated in the intracellular accumulation of TAC. The aim of this work was therefore to clinically study: i) the role of genetic variants and ii) the effect of mRNA and protein expression of 4 drug transporters on the TAC $C_{PBMC/blood}$ ratio. In addition, functional *in vitro* experiments were performed to mechanistically validate the clinical observations. Genetic variants of *ABCB1*/P-gp and *SLC28A3*/CNT3 did not influence TAC C_{PBMC} in liver transplant recipients (LTR). *ABCC2*/MRP2 at the mRNA level; *ABCB1*/P-gp, *SLC28A3*/CNT3 and *SLC29A1*/ENT1 at the protein level; correlated with the $C_{PBMC/blood}$ in kidney and LTR. *In vitro* results suing transporter-expressing cells confirmed that TAC is substrate of P-gp but not MRP2, whereas experiments remained inconclusive for CNT3 and ENT1. In conclusion, the genetic-transcription-protein-functional approach presented in this work provides new insights in the understanding of TAC transport at the T lymphocyte plasma membrane.

Keywords

Tacrolimus, solid organ transplantation, therapeutic drug monitoring, pharmacokinetics, drug transporter, personalized medicine.

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Statements and Declarations

Funding

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Ethics approval

The studies were approved by the local ethics committee of Rennes University Hospital (authorization numbers n°14.05 and n°19.17). The studies were performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

Consent to participate

Informed consent (written when needed) was obtained from all individual participants included in the studies, in accordance with French law.

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Drug transporters are implicated in the diffusion of tacrolimus into the T lymphocyte in kidney and liver transplant recipients: genetic, mRNA, protein expression, and functionality

1. Introduction

Tacrolimus (TAC) is the keystone immunosuppressive drug used after solid organ transplantation [1]. Despite unquestionable efficacy in graft rejection prevention when combined with mycophenolate mofetil MMF and corticosteroids [2], TAC treatment presents several limitations. First, TAC has a narrow therapeutic index and a large inter- and intra-individual pharmacokinetic (PK) variability [1]. Then, its use is associated with some adverse drug reactions (ADR), the most concerning being its concentration-dependent nephrotoxicity [3]. These elements justify the iterative measurement of whole-blood TAC trough concentrations (C_{blood}) and subsequent dosage adjustment to maintain concentrations within an appropriate range, this process being named therapeutic drug monitoring (TDM) [1]. TDM based on C_{blood} improves patient's outcomes by decreasing the risk of graft rejection and limiting ADR. But some patients still experience loss of effects (graft rejection) and/or ADR, despite having C_{blood} within the therapeutic range [4]. Thus, whole blood TDM appears as a required but imperfect tool to optimize TAC therapy.

TAC exerts its immunomodulatory effects by inhibiting the serine/threonine phosphatase calcineurin, located inside the T lymphocyte cytosol, thereby inhibiting interleukine-2 production and lymphocyte activation. Intra-lymphocyte concentration of TAC, in practice approached by peripheral blood mononuclear cell (PBMC) concentrations (C_{PBMC}), has already been suggested to be more directly linked to the immunosuppressive effect of the drug compared to C_{blood}, in renal and hepatic

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transplant recipients [5, 6]. C_{blood} and C_{PBMC} display limited correlation [7-9], highlighting a potential cause for effects discrepancies in patients. Plasma membrane transporters are expected to be involved in TAC distribution into the T lymphocyte. However, the influence of transporters on TAC intracellular concentration is not clearly elucidated. This gap must be fulfilled to understand the determinants of TAC exposure in its target cells and to better understand interpatient variability in the drug response. Additionally, a better knowledge of TAC transport at the T lymphocyte membrane could help providing an indirect monitoring of TAC C_{PBMC}, based on C_{blood}.

A recent systematic review by our team underlined the importance of several polymorphisms of genes coding for drug transporters, in the PK of TAC [10]. Among these transporters, the efflux pumps P-glycoprotein (P-gp) coded by the ATP binding cassette B1 (*ABCB1*) gene, the multidrug resistance-associated protein 2 (MRP2) coded by *ABCC2*, the concentrative nucleoside transporter 3 (CNT3) coded by the solute carrier 28A3 (*SLC28A3*) gene (a secondary active influx transporter), and the equilibrative nucleoside transporter 1 (ENT1) coded by *SLC29A1* (a passive bi-directionnal transporter), are expressed by the T lymphocyte. Taken together, these elements support the hypothesis of a possible involvement of these drug transporters in the diffusion of TAC into PBMC.

Nevertheless, a majority of the medical literature available regarding clinical cohorts reported an influence of genetic polymorphisms in transporters on the concentration of TAC in whole blood, not at the intracellular level. One can hypothesize that these genetic polymorphisms may be relevant regarding TAC concentration in cells as well. Additionally, genotype does not systematically reflect protein expression and functionality. Messenger ribonucleic acid (mRNA) quantitation and protein level expression could also be more relevant to understand TAC disposition in blood and

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cells than genetic polymorphisms. Moreover, there is no modern and definitive *in vitro* evidence that TAC is actually substrate of the four transporters mentioned above. This point must be explored to understand the mechanistic bases of clinical observations. Therefore, the aim of the current study was to use a reverse translational approach to explore: i) the relationship between single nucleotide polymorphisms (SNP) of genes coding for drug transporters, and intra-PBMC diffusion of TAC in a cohort of liver transplant recipients (LTR); ii) the impact of *ABCB1*/P-gp, *ABCC2*/MRP2, *SLC28A3*/CNT3 and *SLC29A1*/ENT1 mRNA and protein levels on the intra-PBMC diffusion of TAC, in stable kidney transplant recipients (KTR) and LTR; iii) and the *in vitro* interaction between P-gp, MRP2, CNT3 and ENT1 and TAC, in cellular models.

2. Materials and methods

Detailed experimental processes can be found in the supplementary methods section of the supplementary material.

2.1 Relationship between *ABCB1* and *SLC28A3* SNPs and intracellular concentrations of TAC in LTR

2.1.1 Study design and objectives

This part was conducted on the data collected during the OPTILTH study (NCT02877628; registration on August the 24th, 2016), which was a prospective, observational, single-center, single-arm, PK-pharmacodynamic (PD) study performed by our team. The original objective of this study was to explore the added value of C_{PBMC} and enzymatic calcineurin activity measurement on TAC TDM in LTR patients. Another objective was to explore the impact of genetic polymorphisms possibly implicated in TAC PK on these parameters. Patients gave their written consent for

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genetic exploration of genes coding for metabolic enzymes and drug transporters, and the study, in accordance with the Declaration of Helsinki, has been authorized by the local ethic committee (authorization number n°14.05). The objective of the present work on this cohort was to explore the influence of genetic polymorphisms on C_{blood} and C_{PBMC} over C_{blood} ratio ($C_{PBMC/blood}$) at day-7 post-liver transplantation.

2.1.2 Inclusion and non-inclusion criteria

All adult orally TAC-treated LTR patients followed at the Rennes University Hospital who provided informed consent and were not under liberty deprivation were eligible. Inclusions spread between September 2015 and October 2017. The number of subjects included was calculated according to the primary outcome of the initial OPTILTH study (NCT02877628).

2.1.3 Patient participation

Patients were included prior to the procedure. During their hospital stay, patients underwent usual laboratory tests, including C_{blood} determination. Using the blood remaining from this analysis, PBMCs were isolated and C_{PBMC} was determined, at various time points including day-7 post-transplantation. Eighty-nine of those TAC-treated LTR patients were genotyped for *ABCB1* rs2229109 (or 1199 G>A, located in exon 11) and *SLC28A3* rs10868152 variants, with TaqMan SNP genotyping assay (Thermofisher scientific, Waltham, MA, USA).

2.1.4 Endpoint

The endpoint was the $C_{PBMC/blood}$ at day-7 according to the genetic status.

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2.2 Impact of *ABCB1*/P-gp, *ABCC2*/MRP2, *SLC28A3*/CNT3 and *SLC29A1*/ENT1 mRNA and protein levels on the intracellular diffusion of TAC in stable KTR and LTR

2.2.1 Study design and objectives

The TRANS-TAC study (NCT03910868) was a prospective, observational, single-center, single-arm, mechanistic and PK study. This work has been conducted in accordance with the Declaration of Helsinki, and the ethic committee of the Rennes University Hospital approved the study protocol (authorization number n°19.17). The objective was to elucidate the impact of the expression of four drug transporters, that may be implicated in the T lymphocyte diffusion of TAC, on the $C_{PBMC/blood}$. The two expression methods applied in this study were mRNA quantification by reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) and protein quantification by flow cytometry.

2.2.2 Inclusion and non-inclusion criterion

All consecutive adult orally TAC-treated KTR or LTR patients followed at the Rennes University Hospital, between 2 months and 2 years after the procedure, were eligible for this study. Inclusions spread between July and December 2020. Patients had to be on TAC therapy regardless of other medications, except for drug transporters inhibitors (verapamil, voriconazole, posaconazole, itraconazole, isavuconazole, macrolide antibiotics, ritonavir, amiodarone) or inducers (rifampicin/rifabutin, bosentan, efavirenz, nevirapine, carbamazepine, phenobarbital). Patients had also to be on stable prescription (no change in dosage) for all medications for the last 2 weeks prior the inclusion. Re-transplantations were authorized, multiple transplantations were not. The main non-inclusion criteria were liberty deprivation

(prisoners and patients under guardianship or curatorship), and pregnancy for women. Groups were designed to include as many KTR and LTR. As no data exists on the variability and size effect of the studied parameter, an inclusion objective of 60 patients (30 for each transplanted organ) was estimated enough to answer the question.

2.2.3 Patient participation

Study participation for patients lasted only one day. After information and non-opposition in accordance with French law on biomedical research, participants normally underwent the transplantation follow-up visit. The latter comprised clinical and biological evaluation with blood count and serum electrolytes, as well as determination of C_{blood}, all being part of the routine protocol. Using leftover blood, PBMC were isolated, allowing mRNA and protein expression quantification, with RT-qPCR and flow cytometry, respectively, as well as C_{PBMC} determination. Primer details can be found in Table S1. The investigators were blind of study-specific parameters.

2.2.4 Endpoints

Primary endpoint was the correlation of mRNA or protein levels of the four studied transporters with $C_{PBMC/blood}$. Secondary endpoints included the correlation between mRNA and protein levels.

2.3 Interaction between P-gp, MRP2, CNT3 or ENT1, and TAC in *in vitro* overexpressing cellular models

2.3.1 Reagents

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Cellular culture medium, phosphate-buffered saline and fetal bovine serum were from Thermo Fisher (Waltham, MA, USA). Rhodamine 123 (Rho), cyclosporin A (CsA), probenecid (PBN), nitrobenzylthioinosine (NBMPR) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). TAC was from LGC standards (Flosheim, France). Carboxy-2',7'-dichlorofluorescein (CF) was from Life Technologies (Saint Aubin, France). Tritiated uridine (URI) with a specific activity of 20,4 Ci/mmol was from PerkinElmer (Courtaboeuf, France). All other reagents were commercial products of the highest purity available.

2.3.2 Objectives and procedures

The objective of the *in vitro* experiments was to validate the mechanisms of the *in vivo* (clinical) findings. For P-gp, parental breast adenocarcinoma MCF7S (Sensible, non-P-gp expressing) and MCR7R (Resistant, P-gp expressing after doxorubicin selection) cells (RRID: CVCL_0031) were used [11]. For MRP2, human hepatoma HuH-7 cells (RRID: CVCL_0336) with MRP-like expression were used [12]. For CNT3, fresh primary human monocyte-derived macrophages (MDM) with strong constitutive CNT3 expression were used [13]. For ENT1, placental BeWo cells (RRID: CVCL_0044) were used [14]. Detailed information about the cells origins and culture can be found in the referenced papers and supplementary material. All cellular models were firstly validated with reference substrates and inhibitors, and TAC transport was thereafter tested, all procedures according to previously published studies by our team [11, 12, 15]. For the P-gp, CNT3 and ENT1, accumulation experiments were performed (incubation in a substrate-containing medium with or without an inhibitor). For MRP2, it was a retention experiment (incubation in a substrate-containing medium without an inhibitor, and then in a substrate-free medium with or without inhibitor). Detail about the experimental design and reference

substrates and inhibitors can be found in Table S2 and Table S3. The endpoint was the intracellular concentration according to the used inhibitors.

2.4 Statistical analysis

Qualitative results are presented as class and percentages, quantitative results are presented as means ± standard deviation. Qualitative/quantitative experiments with two conditions were analyzed using a Mann and Whitney test. When more than two conditions were present, a Kruskal-Wallis test was used with a Dunn's post-test. Quantitative/quantitative experiments were analyzed with non-parametric Spearman's correlation. Non-parametric tests were used because of non-normal distribution of all the data. Prism 5.0 (Graphpad, CA, USA) was used to plot all the graphs. A p-value (p) inferior to 0.05 was considered significant.

3. Results

3.1 *ABCB1* and *SLC28A3* SNP may impact intra-T lymphocyte distribution of TAC in LTR

For the entire cohort, $C_{PBMC/blood}$ was 4.97 ± 2.55, and C_{PBMC} was 25.42 ± 16.67 pg/10^6 cells. rs2229109 variant (*ABCB1*) failed to influence $C_{PBMC/blood}$ and C_{PBMC} (figure 1A and 1B). rs10868152 (*SLC28A3*) had no significant effect on $C_{PBMC/blood}$ and C_{PBMC} (figure 1C and 1D), even if a trend was visible for C_{PBMC} ($p = 0.0768$).

3.2 Impact of *ABCB1*/P-gp, *ABCC2*/MRP2, *SLC28A3*/CNT3 and *SLC29A1*/ENT1 mRNA and protein levels on the intra-T lymphocyte diffusion of TAC in stable KTR and LTR

3.2.1 Patients characteristics and PK parameters

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Baseline patients' characteristics are presented in Table 1. For KTR, C_{blood} was 8.9 ± 2.5 ng/mL, and C_{PBMC} was 23.1 ± 13.1 pg/10^6 cells. For LTR, C_{blood} was 5.6 ± 2.3 ng/mL, and C_{PBMC} was 14.7 ± 10.2 pg/10^6 cells. For all patients, C_{blood} and C_{PBMC} showed a moderate correlation (figure 2A) ($r^2 = 0.4945$; $p < 0.0001$). Although TAC blood levels differed between KTR and LTR, the $C_{PBMC/blood}$ was not different (figure 2B) ($p = 0.5895$); patients' results were consequently pooled for the analysis of correlation between transporters expression and $C_{PBMC/blood}$.

3.2.2 *ABCC2* mRNA expression correlates with $C_{PBMC/blood}$

The figure 3 displays the relationships between *ABCB1*, *ABCC2*, *SLC28A3* and *SLC29A1* mRNA levels in PBMCs, and $C_{PBMC/blood}$. Only *ABCC2* mRNA levels showed a statistical association with the $C_{PBMC/blood}$ (figure 3B) ($r^2 = 0.1427$; $p = 0.0045$), with $C_{PBMC/blood}$ being negatively correlated with *ABCC2* mRNA expression.

3.2.3 P-gp, CNT3 and ENT1 protein expression correlates with $C_{PBMC/blood}$

$C_{PBMC/blood}$ showed a positive significant association with P-gp ($r^2 = 0.1069$; $p = 0.0108$), CNT3 ($r^2 = 0.1292$; $p = 0.0048$) and ENT1 ($r^2 = 0.2360$; $p < 0.0001$) protein expression (figure 4A and 4C–D). MRP2 protein expression did not correlate with $C_{PBMC/blood}$ (figure 4B). In subgroup analysis by transplanted organ, ENT1 retained a significant association with $C_{PBMC/blood}$ in both KTR ($r^2 = 0.3772$; $p = 0.0003$) and LTR ($r^2 = 0.1659$; $p = 0.0255$). Interestingly, KTR were the drivers of the association for P-gp association with $C_{PBMC/blood}$ ($r^2 = 0.2825$; $p = 0.0025$) while there was no association for LTR ($r^2 = 0.0284$; $p = 0.3737$) (figure S1). and LTR were the drivers for CNT3 association with $C_{PBMC/blood}$ ($r^2 = 0.2049$; $p = 0.0120$; while for KTR: $r^2 = 0.0603$; $p = 0.1830$) (figure S2).

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3.2.4 mRNA and protein are poorly correlated, but transporters are co-expressed

mRNA and protein expression of the 4 studied transporters are showed in figure 5. Only *ABCB1* and *SLC28A3* showed a modest and negative statistic association between mRNA and protein expression ($r^2 = 0.0921$; $p = 0.0243$ and $r^2 = 0.1208$; $p = 0.0475$; respectively). Interestingly, the four studied transporters showed statistically significant co-expressions at both the mRNA and protein levels (except CNT3 and MRP2 at the protein level). All p-values are presented in Table S4 and S5. The example of *ABCB1*/P-gp and *SLC29A1*/ENT1 is presented in figure S3.

3.3 Interaction between P-gp, MRP2, CNT3 and ENT1, and TAC, in overexpressing cellular models

3.3.1 TAC is a P-gp substrate in MFC7 cells

P-gp activity in MCF7R cells, as well as absence of the aforementioned activity on parental MCF7S cells, were verified using the reference substrate Rho (figure S4A). TAC accumulation was significantly lower in MCF7R cells when compared to MCF7S cells (figure 6A) (3.4 ± 1.4 vs. 8.3 ± 2.3 ng/mL). Moreover, in MCF7S cells, the P-gp inhibitor CsA did not significantly impair TAC intracellular concentration (8.3 ± 2.3 vs 9.7 ± 1.5 ng/mL), whereas, by contrast, in MCF7R cells, the intracellular concentration of TAC was significantly increased in the presence of CsA (3.4 ± 1.4 vs. 9.6 ± 1.5 ng/mL) ($p = 0.0327$ with Kruskal-Wallis test), to reach levels exhibited by parental MCF7S cells.

3.3.2 TAC is not a MRP2 substrate in HuH-7 cells

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The retention of the reference substrate for MRP2, CF, in HuH-7 cells, was enhanced by PBN, although not reaching significance (p = 0.0614), validating the model as exhibiting MRP2-like activity (figure S4B). TAC retention in HuH-7 cells corresponded approximately to 20.5 ± 18.5 % of initial loading, indicating that a notable efflux occurs, PBN however failed to have any effect on TAC retention by HuH-7 cells, being 19.9 ± 18.2 % with PBN (p = NS versus TAC + PBN -) (figure 6B). p-value with Kruskal-Wallis test was 0.0358.

3.3.3 TAC does not seem to be transported by CNT3 in MDM

In MDM, Na withdrawal resulted in a remarkable decrease in URI uptake, accounting for a strong CNT activity. Furthermore, inosine (INO) and thymidine (THY) both nearly abolished URI uptake, whereas NBMPR did not have any effect, validating MDM as a relevant model for CNT3 activity exploration (figure S4C). TAC uptake was not affected by any of the experimental conditions (figure 6C) (p = NS), supporting the idea that TAC is not a substrate for CNTs, especially for CNT3.

3.3.4 TAC does not seem to be transported by ENT1 in BeWo cells

The uptake of URI on BeWo cell was not affected by Na withdrawal. NBMPR nevertheless significantly reduced it, which was greatly enhanced by both INO and THY. BeWo cell therefore appeared as a cellular ENT1 functional model (figure S4D). Cellular TAC uptake exhibited an increase in the presence of NBMPR (0.12 ± 0.07 vs. 0.37 ± 0.35 ng/mL), but this did not reach significance (figure 6D). Overall p-value with Kruskal-Wallis test was 0.5004.

4. Discussion

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Here we report the results of a translational approach to study the influence of T lymphocyte-expressed drug transporters on TAC distribution in this cellular type, known to host the molecular target of this drug, *i.e.* the calcineurin. This work can be seen as an indirect study of TAC exposure measurement in PBMCs. Starting from genetic data to functional analysis, via mRNA and protein expression in clinical samples, our data, despite displaying some contradictory results, informed the field of TAC PK.

First, we wanted to confirm rs2229109 as influencing TAC distribution into PBMC, to validate our review work [10]. In the present study, this *ABCB1*/P-gp SNP failed to influence TAC intracellular distribution, possibly due to a lack of statistical power. In the OPTHILT study, other SNP of ABCB1 were determined, as presented in figure S5. These variants were rs1128503 (1236 C>T, located in exon 12), rs2032582 (2677 G>T/A, located in exon 21) and rs1045642 (3435 C>T, located in exon 26). They were all far from significance regarding influence on $C_{PBMC/blood}$ and C_{PBMC}, (all $p > 0.25$), except rs1045642 for $C_{PBMC/blood}$ ($p = 0.0237$), with the caveat that the association is mainly driven by two outlier points. As the main objective of the OPTILTH study was not to explore the impact of drug transporters on $C_{PBMC/blood}$, the inclusion criteria did not include the absence of drug transporters inhibitors or inducers on patients' treatment. These possibly co-prescribed drugs could have increased the variability in our cohort. In another one of our previous work, rs2229109 did influence C_{blood} and C_{PBMC} [16]. Despite having included less patients (32 LTR), we had, in this study, full PK profiles, namely area under the concentration-time curve with 10 time-points per day sampling scheme, which are richer PK data than C_{blood}.

Downstream of the genotype lies the gene expression, which we used here as a predictor for TAC distribution. Primers analysis on GTEx Portal (https://gtexportal.org/home/, accessed 21st of February, 2022) revealed that most of all transcripts of the 4 genes were amplified (data not shown). *ABCB1* mRNA expression did not correlate with TAC $C_{PBMC/blood}$. P-gp protein expression however did. The *in vitro* experiments confirmed the efflux of TAC by P-gp. This corroborates studies based on genetics [10, 17], mRNA [18] and *in vitro* cellular models [19]. This corroborates the importance of this drug transporter in TAC PK.

Regarding other mRNA experiments, only *ABCC2*/MRP2 mRNA showed a negative association with $C_{PBMC/blood}$ (the more the cell expresses this efflux pump, the less TAC diffusion into PBMC is important). In contrast, for proteins, MRP2 was not associated with $C_{PBMC/blood}$. Finally, in HuH-7, a robust MRP2-like functional model, this transporter was not involved in TAC transport.

Concerning *SLC28A3*/CNT3, we wanted to confirm also rs10868152 as influencing TAC distribution into PBMC. This SNP however also failed to influence TAC intracellular distribution. A recent genome wide-association study found *SLC28A3* genetic polymorphism to be a major determinant of TAC PK in KTR [20]. Clinical data obtained in our study favor CNT3 implication at the PBMC level, because of the correlation between TAC $C_{PBMC/blood}$ and CNT3 protein expression. Still, mRNA expression of SLC28A3 did not influence TAC $C_{PBMC/blood}$. We provide here elements towards the absence of direct TAC transport by CNT3. To reconciliate these findings, an indirect drug/metabolite interaction may occur in TAC-treated patients, involving an endogenous substrate of CNT3, or a drug-drug interaction with a comedication [21].

Although experiments with *SLC29A1*/ENT1 are not unequivocal, there is a signal towards the contribution of the transporter to the TAC accumulation into cells. *SLC29A1* mRNA expression did not correlate with TAC $C_{PBMC/blood}$, but ENT1 protein expression did. This latter correlation was even the strongest observed of the four studied transporters, with a r^2 of 0.2360 in non-parametric analysis ($p < 0.0001$). On the ENT1-functionnal BeWo model, NBMPR enhanced TAC accumulation, despite not reaching significance, while Na withdrawal had no effect. This does not seem in accordance with clinical findings, where ENT1 participated in the influx of TAC. In the *in vitro* experiments, ENT1 could participate in the efflux of TAC. Nevertheless, ENT1 being a bidirectional transporter, the direction of the transport may change depending on the experimental conditions. Moreover, the BeWo model expresses other transporters such as *ABCG2*/breast cancer resistance protein [22] and as NBMPR inhibits the latter [23], such a mechanism could cause an experimental artefact. Additional experiments with an ENT1-only overexpressing model could bring a more conclusive response. Nevertheless, *in vitro* and clinical data highlighted here the potential value of ENT1 as a novel explicating covariate for TAC PK, in particular regarding its distribution into the effect compartment.

CNT3 (influx transporter) and ENT1 (bidirectional transporter) protein expressions were associated with an increase in $C_{PBMC/blood}$, which is plausible. However, P-gp, which is an efflux pump like MRP2, protein expression also positively correlated with $C_{PBMC/blood}$. Our hypothesis to explain this discrepancy is that intra-T lymphocyte TAC is able to upregulate P-gp expression at the cellular membrane, as already proposed in oncology with anti-cancer drugs [24]. The net effect of ENT1 and/or CNT3 being possibly more efficient to transport TAC, they could reverse the P-gp-mediated transport in stable transplant recipients. We conducted different experiments by

supplementing whole blood samples with TAC and by comparing P-gp expression in clinical samples, but could not confirm this hypothesis (data not shown).

When splitting the cohorts depending on the grafted organ, we found interesting results in terms $C_{PBMC/blood}$ regarding to protein expression. As ENT1 remained significantly associated with $C_{PBMC/blood}$ in both semi-cohorts, the P-gp was only in KTR, and CNT3 only in LTR. The lack of association for P-gp in LTR may be related to a lack of statistical power, as the impact of *ABCB1* polymorphisms on C_{PBMC} has been found in both KTR and LTR [7;10;16]. However, for CNT3, polymorphisms of *SLC28A3* has only been found significant in LTR [20;25]. Thus, *SLC28A3*/CNT3 might be more relevant in LTR than in KTR.

The results regarding the absence of association between mRNA and protein expressions may be related to the post-transcriptional, translational and post-translational regulation steps. A very interesting finding of this study is the strong co-expression between the studied genes, at both the mRNA and protein levels. To our knowledge, there is no description of a common regulation of those genes in non-cancerous tissues. Our results could be used in transplant populations because of the fair replicability in KTR and LTR, however the extrapolation to non-transplanted patients could be hazardous.

Although the TRANS-TAC patients were not genotyped for the studied transporters, which constitutes a major limitation to this study, protein expression seems to add information which is not provided by m-RNA expression or genotype. This is supported by the facts that i) the mechanistic experiments of the *in vitro* part tie in with those of protein expression of the clinical part ii) the transporter function of the genes is directly related to proteins and not mRNA, which “only” serve as a transient

information carrier iii) we found almost no correlation between mRNA and protein expressions. In this study, mRNA levels cannot be seen as quantitative biomarkers of the gene function, in contrast to protein expression. At last, the advantage of the method we used for protein expression determination, flow cytometry, is that it is very tunable and versatile. This procedure can indeed be coupled with PD/immunologic explorations, and has relatively quick pre-analytical and analytical phases [26].

In this study, a definitive evidence is provided for the P-gp and MRP2. The nucleotide transporters CNT3 and ENT1 still required confirmation regarding their potential impact on TAC drug transport, though. However, both of these transporters might be new and major determinants of TAC therapy outcomes.

5. Conclusion

In conclusion, this work explored using various approaches, the mechanisms of TAC diffusion in its effect compartment, the T lymphocyte, in relation with drug transporters. While some results remained to confirmed, our study paves the way towards indirect monitoring of TAC C_{PBMC} using drug transporters expression evaluation. The P-gp/*ABCB1*, CNT3/*SLC28A3* and ENT1/*SLC29A1* appears as significant determinants of TAC diffusion to its compartment of effect.

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Supplementary material

See attached file.

Conflicts of interest/Competing interests

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Author contributions based on CRediT authorship contribution

Gwendal Coste: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. Fabien Robin: Resources. Jonathan Chemouny: Resources, Visualization. Camille Tron: Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. Jérôme Le Priol: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft. Régis Bouvet: Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. Marc Le Vée: Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. Pauline Houssel-Debry: Resources. Michel Rayar: Resources. Marie-Clémence Verdier: Writing - review & editing. Mikael Roussel: Methodology, Supervision. Marie-Dominique Galibert: Supervision. Edouard Bardou-Jacquet: Resources. Olivier Fardel: Formal analysis, Methodology, Supervision, Writing - review & editing. Cécile Vigneau: Resources, Writing - review & editing. Karim Boudjema: Resources. Bruno Laviolle: Funding acquisition, Supervision. Florian Lemaitre: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing - review & editing. All authors have read and agreed to this version of the manuscript.

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Figure captions:

Figure 1 Day 7 post-liver transplantation $C_{PBMC/blood}$ (A and C) or C_{PBMC} (B and D), according to *SLC28A3* rs10868152 (A and B) or *ABCB1* rs2229109 (C and D) genotype (n = 89). *ABC*: ATP binding cassette; C_{PBMC}: intra-peripheral blood mononuclear cells tacrolimus concentration; $C_{PBMC/blood}$: intra-peripheral blood mononuclear cells over whole blood tacrolimus concentrations ratio; *SLC*: solute carrier; TAC: tacrolimus

Figure 2 (A) C_{blood} versus concomitant C_{PBMC}, n = 60, rounds represent KTR and squares represent LTR. (B) $C_{PBMC/blood}$ for KTR (rounds) and LTR (squares), n = 30 each. C_{blood}: blood trough TAC concentration; C_{PBMC}: intra-peripheral blood mononuclear cells tacrolimus concentration; $C_{PBMC/blood}$: intra-peripheral blood mononuclear cells over whole blood tacrolimus concentrations ratio; KTR: kidney transplant recipient; LTR: liver transplant recipient; TAC: tacrolimus

Figure 3 $C_{PBMC/blood}$ according to *ABCB1* (A; n = 55), *ABCC2* (B; n = 55), *SLC28A3* (C; n = 33) and *SLC29A1* (D; n = 55) mRNA expression determined with RT-qPCR. *ABC*: ATP binding cassette; $C_{PBMC/blood}$: intra-peripheral blood mononuclear cells over whole blood tacrolimus concentrations ratio; mRNA: messenger ribonucleic acid; RT-qPCR: reverse transcription quantitative real time polymerase chain reaction; *SLC*: solute carrier

Figure 4 $C_{PBMC/blood}$ according to P-gp (A; n = 60), MRP2 (B; n = 59), CNT3 (C; n = 60) and ENT1 (D; n = 60) protein expression determined with flow cytometry. CNT: concentrative nucleoside transporter; $C_{PBMC/blood}$: intra-peripheral blood mononuclear cells over whole blood tacrolimus concentrations ratio; ENT: equilibrative nucleoside transporter; MRP: multidrug resistance-associated protein; P-gp: P-glycoprotein

Figure 5 P-gp protein according to *ABCB1* mRNA (A), MRP2 according to *ABCC2* (B), CNT3 according to *SLC28A3* (C) and ENT1 according to *SLC29A1* (D) expression. mRNA expression was determined with RT-qPCR, protein expression with flow cytometry. *ABC*: ATP binding cassette; CNT: concentrative nucleoside transporter; ENT: equilibrative nucleoside transporter; mRNA: messenger ribonucleic acid; MRP: multidrug resistance-associated protein; P-gp: P-glycoprotein; RT-qPCR: reverse transcription quantitative real time polymerase chain reaction; *SLC*: solute carrier

Figure 6 TAC accumulation in P-gp-overexpressing MCF7R cells and parental MCF7S cells (A); TAC retention in MRP2-expressing HuH7 cells (B); TAC accumulation in MDM (C) or BeWo cells (D). (A) MCF7S and R cells were incubated with TAC, with or without CsA, n = 4. (B) HuH-7 cells were incubated with TAC (loading phase), and then reincubated in a TAC-free medium with or without PBN (efflux phase); n = 5. (C) and (D) Cells were incubated with TAC and with mentioned inhibitors; n = 4. For all experiments, results are presented as TAC intracellular concentration in ng/ml; Kruskal-Wallis tests were performed except Mann & Whitney for (A); horizontal bars represent significant differences regarding Dunn's post-test (except between TAC + PBN – and TAC + PBN + in (B)). CsA: cyclosporin A; CTRL: negative control; INO: inosine; MDM: monocyte-derived macrophage; MRP: multidrug resistance-associated protein; NBMPR: nitrobenzylthioinosine PBN: probenecid; P-gp: P-glycoprotein; TAC: tacrolimus; THY: thymidine

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Article title: Drug transporters are implicated in the diffusion of tacrolimus into the T lymphocyte in kidney and liver transplant recipients: genetic, mRNA, protein expression, and functionality

Journal name: Drug Metabolism and Pharmacokinetics

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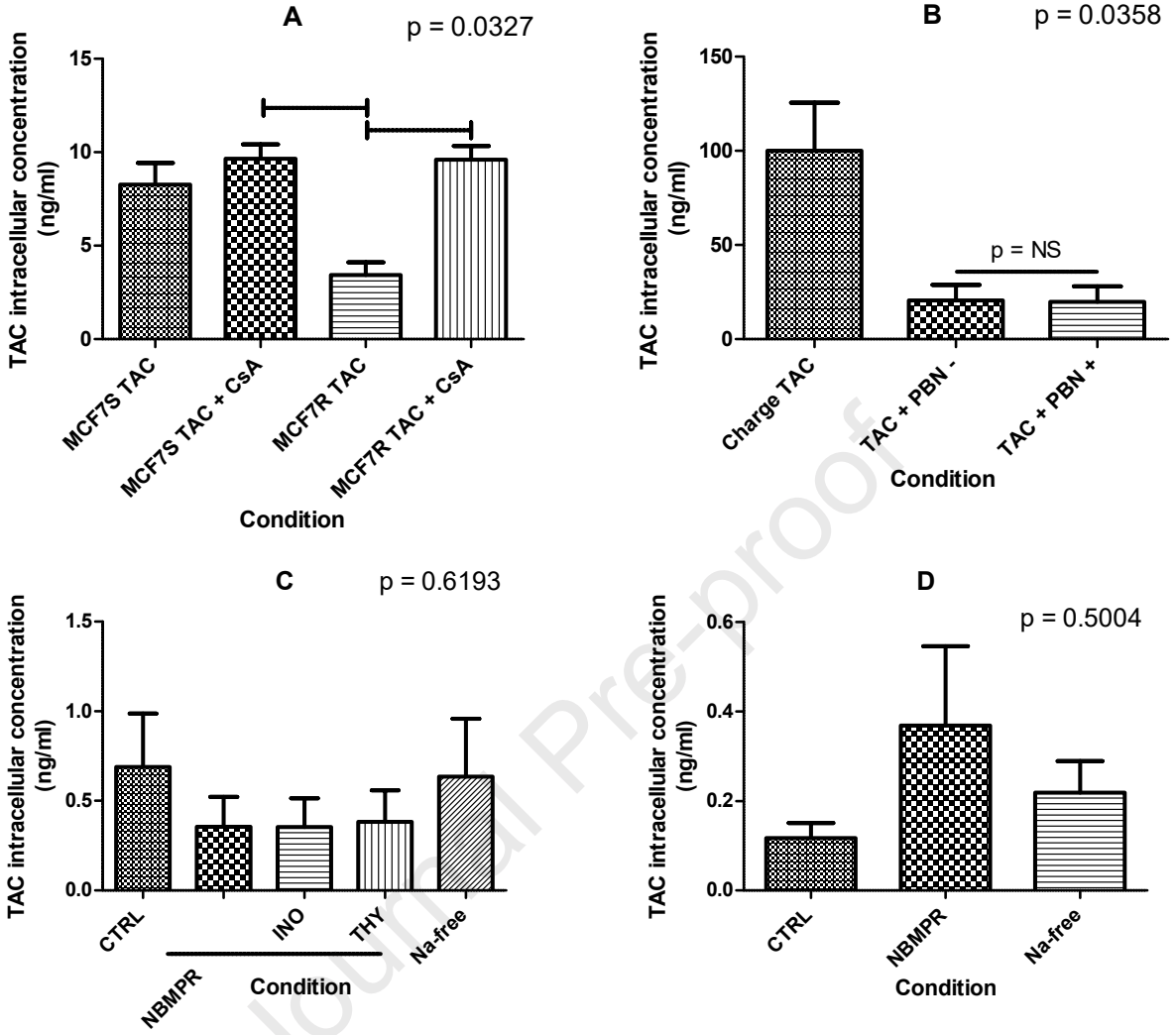
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	Liver transplant recipients	Kidney transplant recipients
Number	30	30
Age (years)	60.4 ± 7.6	52.6 ± 13.2
Weight (kg)	75.9 ± 15.6	77.3 ± 16.0
Male sex (%)	77	63
Post-operative months	2.4 ± 0.2	9.0 ± 5.8
TAC daily dose (mg)	4.6 ± 1.9	7.4 ± 4.3
TAC pharmaceutical form		
IR-TAC (%)	63	67
ER-TAC (%)	30	23
LCP (%)	7	10
TAC trough concentration (ng/ml)	5.6 ± 2.3	8.9 ± 2.5
GFR (ml/min/1.73m²)	77.5 ± 21.7	52 ± 20.3

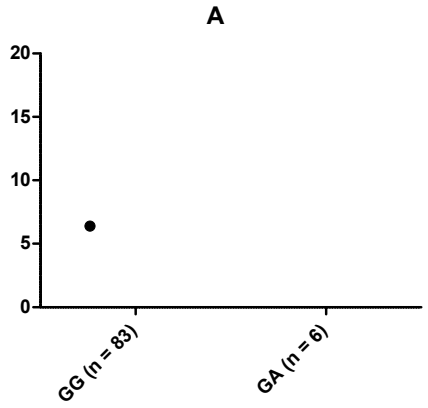
Table 1 Caption: Baseline characteristics of the patients included in the TRANS-TAC study (impact of mRNA and protein expression of four transporters on the TAC distribution in PBMCs)

ER: extended release; GFR: glomerular filtration rate; IR: immediate release; LCP: melt-dose formulation; TAC: tacrolimus

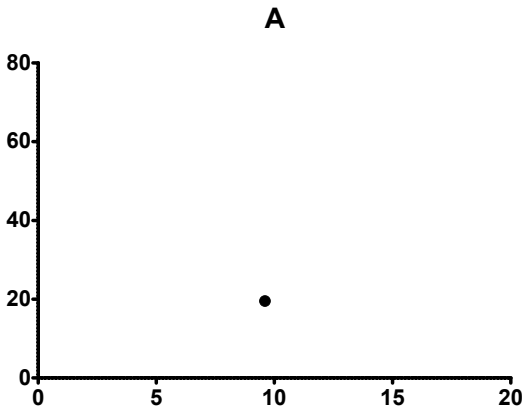
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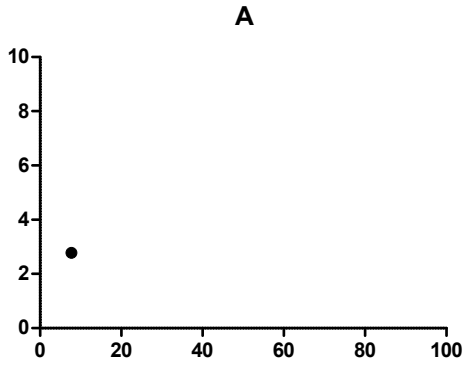
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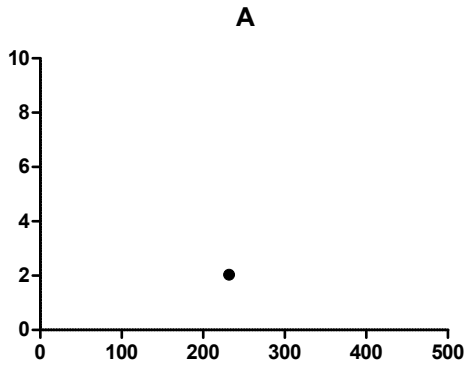
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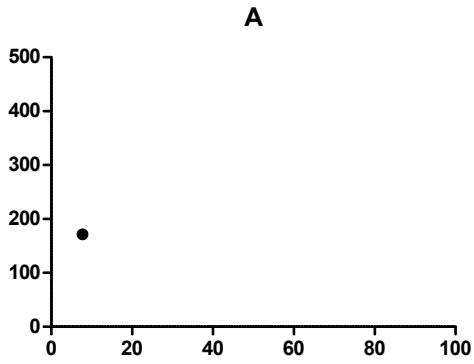
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