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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

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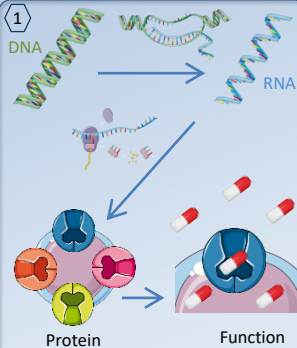
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- 2
- Tacrolimus (TAC) is the keystone of post-transplantation immunosuppression
- Genetic polymorphisms of drug transporters influence its pharmacokinetics
- Drug transporters could influence drug diffusion to T lymphocyte, i.e. the action site
- 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 fonctionnally *in vitro*
- 

3

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

- 4
- 
- Innovative study
  - Multiple approaches
  - This work should encourage other in the field of drug transporters of tacrolimus

1 **Title Page**2 **Title**

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

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**52 Abstract**

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

**71 Keywords**

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

74

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77 This work was partly supported by a research grant by Sandoz and the bourse Ouest  
78 Transplant. The funding source had no involvement in any part of the study design, in  
79 the collection, analysis and interpretation of data, in the writing of the report, and in  
80 the decision to submit the article for publication.

**81 Ethics approval**

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

**85 Consent to participate**

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

88

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

92 **1. Introduction**

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

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



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

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

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

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

## 149 **2. Materials and methods**

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

### 152 **2.1 Relationship between *ABCB1* and *SLC28A3* SNPs and intracellular** 153 **concentrations of TAC in LTR**

#### 154 **2.1.1 Study design and objectives**

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

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

### 167 **2.1.2 Inclusion and non-inclusion criteria**

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

### 173 **2.1.3 Patient participation**

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

### 181 **2.1.4 Endpoint**

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

183 **2.2 Impact of *ABCB1/P-gp*, *ABCC2/MRP2*, *SLC28A3/CNT3* and *SLC29A1/ENT1***  
184 **mRNA and protein levels on the intracellular diffusion of TAC in stable KTR**  
185 **and LTR**

186 **2.2.1 Study design and objectives**

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

196 **2.2.2 Inclusion and non-inclusion criterion**

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

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

### 212 **2.2.3 Patient participation**

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

### 222 **2.2.4 Endpoints**

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

## 226 **2.3 Interaction between P-gp, MRP2, CNT3 or ENT1, and TAC in *in vitro*** 227 **overexpressing cellular models**

### 228 **2.3.1 Reagents**

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

### 237 **2.3.2 Objectives and procedures**

238 The objective of the *in vitro* experiments was to validate the mechanisms of the *in*  
239 *vivo* (clinical) findings. For P-gp, parental breast adenocarcinoma MCF7S (Sensible,  
240 non-P-gp expressing) and MCR7R (Resistant, P-gp expressing after doxorubicin  
241 selection) cells (RRID: CVCL\_0031) were used [11]. For MRP2, human hepatoma  
242 HuH-7 cells (RRID: CVCL\_0336) with MRP-like expression were used [12]. For  
243 CNT3, fresh primary human monocyte-derived macrophages (MDM) with strong  
244 constitutive CNT3 expression were used [13]. For ENT1, placental BeWo cells  
245 (RRID: CVCL\_0044) were used [14]. Detailed information about the cells origins and  
246 culture can be found in the referenced papers and supplementary material. All  
247 cellular models were firstly validated with reference substrates and inhibitors, and  
248 TAC transport was thereafter tested, all procedures according to previously published  
249 studies by our team [11, 12, 15]. For the P-gp, CNT3 and ENT1, accumulation  
250 experiments were performed (incubation in a substrate-containing medium with or  
251 without an inhibitor). For MRP2, it was a retention experiment (incubation in a  
252 substrate-containing medium without an inhibitor, and then in a substrate-free  
253 medium with or without inhibitor). Detail about the experimental design and reference

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

## 256 **2.4 Statistical analysis**

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

## 265 **3. Results**

### 266 **3.1 *ABCB1* and *SLC28A3* SNP may impact intra-T lymphocyte distribution of** 267 **TAC in LTR**

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

### 272 **3.2 Impact of *ABCB1*/P-gp, *ABCC2*/MRP2, *SLC28A3*/CNT3 and *SLC29A1*/ENT1** 273 **mRNA and protein levels on the intra-T lymphocyte diffusion of TAC in stable** 274 **KTR and LTR**

#### 275 **3.2.1 Patients characteristics and PK parameters**

276 Baseline patients' characteristics are presented in Table 1. For KTR,  $C_{\text{blood}}$  was  $8.9 \pm$   
277  $2.5$  ng/mL, and  $C_{\text{PBMC}}$  was  $23.1 \pm 13.1$  pg/ $10^6$  cells. For LTR,  $C_{\text{blood}}$  was  $5.6 \pm 2.3$   
278 ng/mL, and  $C_{\text{PBMC}}$  was  $14.7 \pm 10.2$  pg/ $10^6$  cells. For all patients,  $C_{\text{blood}}$  and  $C_{\text{PBMC}}$   
279 showed a moderate correlation (figure 2A) ( $r^2 = 0.4945$ ;  $p < 0.0001$ ). Although TAC  
280 blood levels differed between KTR and LTR, the  $C_{\text{PBMC}/\text{blood}}$  was not different (figure  
281 2B) ( $p = 0.5895$ ); patients' results were consequently pooled for the analysis of  
282 correlation between transporters expression and  $C_{\text{PBMC}/\text{blood}}$ .

### 283 **3.2.2 ABCC2 mRNA expression correlates with $C_{\text{PBMC}/\text{blood}}$**

284 The figure 3 displays the relationships between *ABCB1*, *ABCC2*, *SLC28A3* and  
285 *SLC29A1* mRNA levels in PBMCs, and  $C_{\text{PBMC}/\text{blood}}$ . Only *ABCC2* mRNA levels  
286 showed a statistical association with the  $C_{\text{PBMC}/\text{blood}}$  (figure 3B) ( $r^2 = 0.1427$ ;  $p =$   
287  $0.0045$ ), with  $C_{\text{PBMC}/\text{blood}}$  being negatively correlated with *ABCC2* mRNA expression.

### 288 **3.2.3 P-gp, CNT3 and ENT1 protein expression correlates with $C_{\text{PBMC}/\text{blood}}$**

289  $C_{\text{PBMC}/\text{blood}}$  showed a positive significant association with P-gp ( $r^2 = 0.1069$ ;  $p =$   
290  $0.0108$ ), CNT3 ( $r^2 = 0.1292$ ;  $p = 0.0048$ ) and ENT1 ( $r^2 = 0.2360$ ;  $p < 0.0001$ ) protein  
291 expression (figure 4A and 4C–D). MRP2 protein expression did not correlate with  
292  $C_{\text{PBMC}/\text{blood}}$  (figure 4B). In subgroup analysis by transplanted organ, ENT1 retained a  
293 significant association with  $C_{\text{PBMC}/\text{blood}}$  in both KTR ( $r^2 = 0.3772$ ;  $p = 0.0003$ ) and LTR  
294 ( $r^2 = 0.1659$ ;  $p = 0.0255$ ). Interestingly, KTR were the drivers of the association for P-  
295 gp association with  $C_{\text{PBMC}/\text{blood}}$  ( $r^2 = 0.2825$ ;  $p = 0.0025$ ) while there was no  
296 association for LTR ( $r^2 = 0.0284$ ;  $p = 0.3737$ ) (figure S1). and LTR were the drivers  
297 for CNT3 association with  $C_{\text{PBMC}/\text{blood}}$  ( $r^2 = 0.2049$ ;  $p = 0.0120$ ; while for KTR:  $r^2 =$   
298  $0.0603$ ;  $p = 0.1830$ ) (figure S2).



299 **3.2.4 mRNA and protein are poorly correlated, but transporters are co-**  
300 **expressed**

301 mRNA and protein expression of the 4 studied transporters are showed in figure 5.  
302 Only *ABCB1* and *SLC28A3* showed a modest and negative statistic association  
303 between mRNA and protein expression ( $r^2 = 0.0921$ ;  $p = 0.0243$  and  $r^2 = 0.1208$ ;  $p =$   
304  $0.0475$ ; respectively). Interestingly, the four studied transporters showed statistically  
305 significant co-expressions at both the mRNA and protein levels (except CNT3 and  
306 MRP2 at the protein level). All p-values are presented in Table S4 and S5. The  
307 example of *ABCB1*/P-gp and *SLC29A1*/ENT1 is presented in figure S3.

308 **3.3 Interaction between P-gp, MRP2, CNT3 and ENT1, and TAC, in**  
309 **overexpressing cellular models**

310 **3.3.1 TAC is a P-gp substrate in MCF7 cells**

311 P-gp activity in MCF7R cells, as well as absence of the aforementioned activity on  
312 parental MCF7S cells, were verified using the reference substrate Rho (figure S4A).  
313 TAC accumulation was significantly lower in MCF7R cells when compared to MCF7S  
314 cells (figure 6A) ( $3.4 \pm 1.4$  vs.  $8.3 \pm 2.3$  ng/mL). Moreover, in MCF7S cells, the P-gp  
315 inhibitor CsA did not significantly impair TAC intracellular concentration ( $8.3 \pm 2.3$  vs  
316  $9.7 \pm 1.5$  ng/mL), whereas, by contrast, in MCF7R cells, the intracellular  
317 concentration of TAC was significantly increased in the presence of CsA ( $3.4 \pm 1.4$   
318 vs.  $9.6 \pm 1.5$  ng/mL) ( $p = 0.0327$  with Kruskal-Wallis test), to reach levels exhibited by  
319 parental MCF7S cells.

320 **3.3.2 TAC is not a MRP2 substrate in HuH-7 cells**

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

### 328 **3.3.3 TAC does not seem to be transported by CNT3 in MDM**

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

### 335 **3.3.4 TAC does not seem to be transported by ENT1 in BeWo cells**

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

## 342 **4. Discussion**

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

350 First, we wanted to confirm rs2229109 as influencing TAC distribution into PBMC, to  
351 validate our review work [10]. In the present study, this *ABCB1*/P-gp SNP failed to  
352 influence TAC intracellular distribution, possibly due to a lack of statistical power. In  
353 the OPTHILT study, other SNP of *ABCB1* were determined, as presented in figure  
354 S5. These variants were rs1128503 (1236 C>T, located in exon 12), rs2032582  
355 (2677 G>T/A, located in exon 21) and rs1045642 (3435 C>T, located in exon 26).  
356 They were all far from significance regarding influence on  $C_{\text{PBMC/blood}}$  and  $C_{\text{PBMC}}$ , (all  $p$   
357  $> 0.25$ ), except rs1045642 for  $C_{\text{PBMC/blood}}$  ( $p = 0.0237$ ), with the caveat that the  
358 association is mainly driven by two outlier points. As the main objective of the  
359 OPTILTH study was not to explore the impact of drug transporters on  $C_{\text{PBMC/blood}}$ , the  
360 inclusion criteria did not include the absence of drug transporters inhibitors or  
361 inducers on patients' treatment. These possibly co-prescribed drugs could have  
362 increased the variability in our cohort. In another one of our previous work,  
363 rs2229109 did influence  $C_{\text{blood}}$  and  $C_{\text{PBMC}}$  [16]. Despite having included less patients  
364 (32 LTR), we had, in this study, full PK profiles, namely area under the concentration-  
365 time curve with 10 time-points per day sampling scheme, which are richer PK data  
366 than  $C_{\text{blood}}$ .

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

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

380 Concerning *SLC28A3*/*CNT3*, we wanted to confirm also rs10868152 as influencing  
381 TAC distribution into PBMC. This SNP however also failed to influence TAC  
382 intracellular distribution. A recent genome wide-association study found *SLC28A3*  
383 genetic polymorphism to be a major determinant of TAC PK in KTR [20]. Clinical data  
384 obtained in our study favor *CNT3* implication at the PBMC level, because of the  
385 correlation between TAC  $C_{\text{PBMC/blood}}$  and *CNT3* protein expression. Still, mRNA  
386 expression of *SLC28A3* did not influence TAC  $C_{\text{PBMC/blood}}$ . We provide here elements  
387 towards the absence of direct TAC transport by *CNT3*. To reconcile these findings,  
388 an indirect drug/metabolite interaction may occur in TAC-treated patients, involving  
389 an endogenous substrate of *CNT3*, or a drug-drug interaction with a comedication  
390 [21].

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

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

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

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

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

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

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

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

## 450 **5. Conclusion**

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

457

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**464 Supplementary material**

465 See attached file.

**466 Conflicts of interest/Competing interests**

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469



**470 Author contributions based on CRediT authorship contribution**

471 Gwendal Coste: Data curation, Formal analysis, Investigation, Methodology,  
472 Validation, Visualization, Writing - original draft. Fabien Robin: Resources. Jonathan  
473 Chemouny: Resources, Visualization. Camille Tron: Formal analysis, Investigation,  
474 Methodology, Visualization, Writing - original draft, Writing - review & editing. Jérôme  
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485 Funding acquisition, Methodology, Project administration, Supervision, Visualization,  
486 Writing - review & editing. All authors have read and agreed to this version of the  
487 manuscript.

488

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604

605 **Figure captions:**

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

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

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

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

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

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



**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

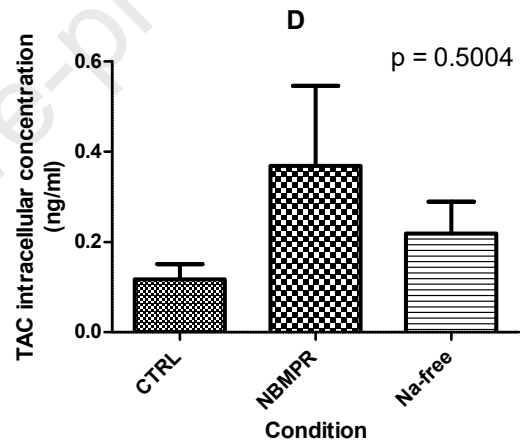
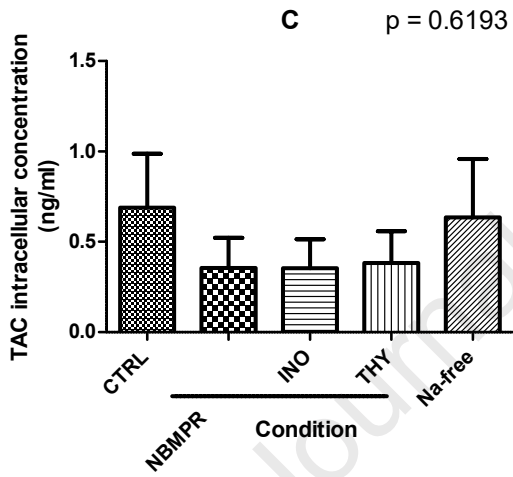
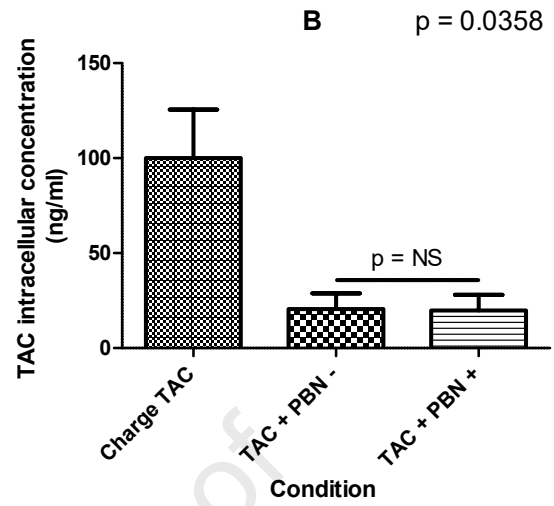
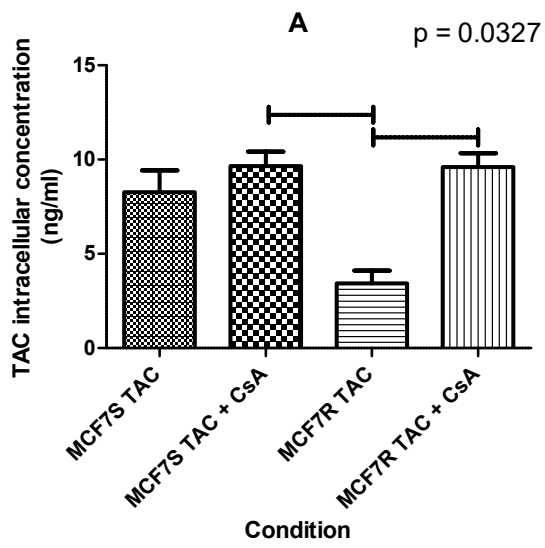
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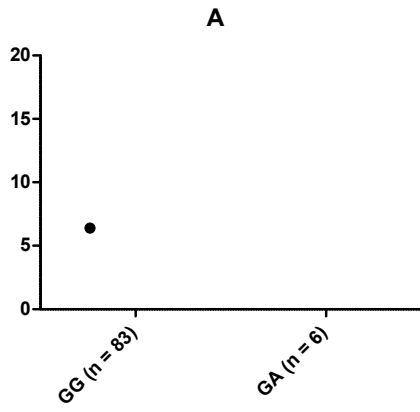
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	Liver transplant recipients	Kidney transplant recipients
Number	30	30
Age (years)	60.4 ± 7.6	52.6 ± 13.2
Weight (kg)	75.9 ± 15.6	77.3 ± 16.0
Male sex (%)	77	63
Post-operative months	2.4 ± 0.2	9.0 ± 5.8
TAC daily dose (mg)	4.6 ± 1.9	7.4 ± 4.3
TAC pharmaceutical 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/1.73m <sup>2</sup> )	77.5 ± 21.7	52 ± 20.3

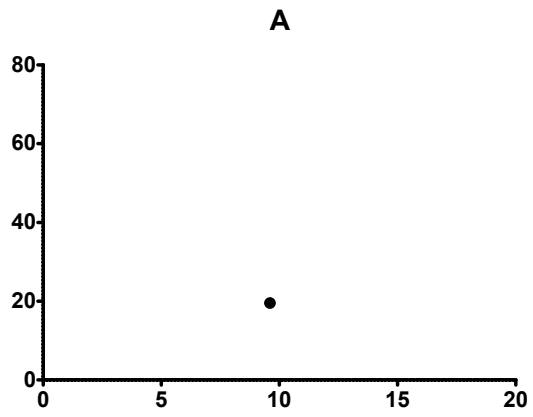
**Table 1** Caption: Baseline characteristics of the patients included in the TRANS-TAC study (impact of mRNA and protein expression of four transporters on the TAC distribution in PBMCs)

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

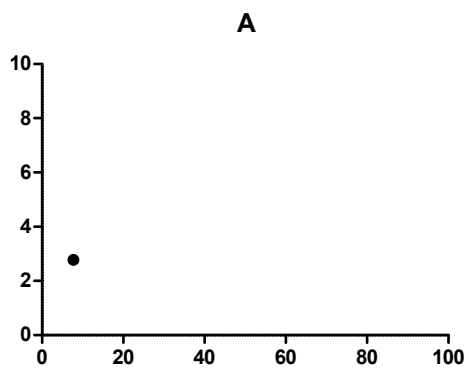




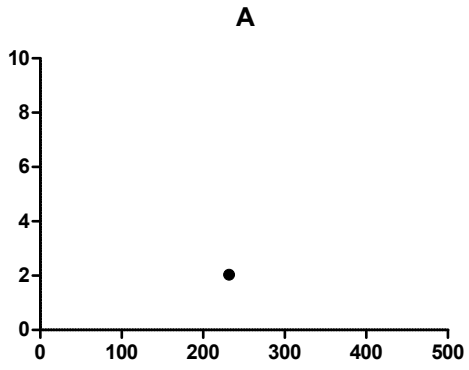
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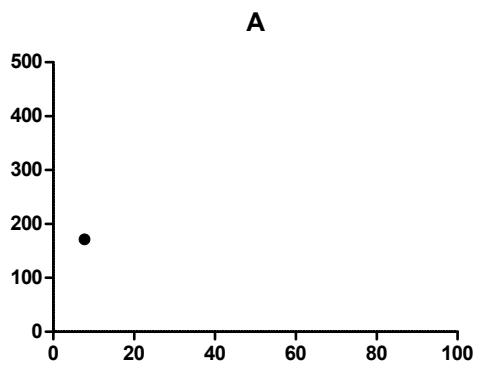
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