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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

Gwendal Coste, Fabien Robin, Jonathan Chemouny, Camille Tron, Jérôme Le Priol, Regis Bouvet, Marc Le Vée, Pauline Houssel-debry, Michel Rayar, Marie-Clémence Verdier, et al.

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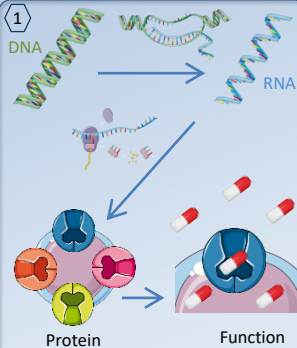
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2

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

6 **Author information** (first name – last name)

7 Gwendal Coste (0000-0002-5417-5629)^{a,b,c*}, Fabien Robin (0000-0002-1234-5351)^d,
8 Jonathan Chemouny (0000-0001-6309-3986)^{a,b,e}, Camille Tron (0000-0002-1030-
9 1750)^{a,b,c}, Jérôme Le Priol (0000-0002-6899-6655)^f, Régis Bouvet^g, Marc Le Vée^a,
10 Pauline Houssel-Debry (0000-0001-9271-9810)^{b,d}, Michel Rayar (0000-0003-3113-
11 2260)^{b,d}, Marie-Clémence Verdier (0000-0001-6595-8154)^{a,b,c}, Mikael Roussel (0000-
12 0002-9741-0668)^{f,h}, Marie-Dominique Galibert (0000-0003-0095-742X)^{g,i}, Edouard
13 Bardou-Jacquet (0000-0002-0784-0710)^{b,d}, Olivier Fardel (0000-0001-5657-4255)^{a,f},
14 Cécile Vigneau (0000-0002-1857-0656)^{a,e}, Karim Boudjema (0000-0002-3894-
15 8767)^{a,b,d}, Bruno Laviolle (0000-0002-9541-6708)^{a,b,c} and Florian Lemaitre (0000-
16 0002-0908-3629)^{a,b,c}.

17 a: Irset (Institut de Recherche en Santé, Environnement et Travail)—UMR S 1085,
18 EHESP, Inserm, CHU Rennes, Université Rennes 1, F-35000 Rennes, France

19 b: INSERM, Centre d'Investigation Clinique 1414, F-35000 Rennes, France

20 c: Service de pharmacologie biologique, Centre Hospitalier Universitaire de Rennes,
21 Université de Rennes 1, Rennes, France

22 d: Service de chirurgie hépato-biliaire et digestive, Centre Hospitalier Universitaire de
23 Rennes, Université de Rennes 1, Rennes, France

24 e: Service de néphrologie, Centre Hospitalier Universitaire de Rennes, Université de
25 Rennes 1, Rennes, France

26 f: Laboratoire d'hématologie, Centre Hospitalier Universitaire de Rennes, Université
27 de Rennes 1, Rennes, France

28 g: Laboratoire de génétique moléculaire et génomique médicale, Centre Hospitalier
29 Universitaire de Rennes, Université de Rennes 1, Rennes, France

30 h: Université de Rennes, Établissement Français du Sang (EFS) de Bretagne,
31 Inserm, MICMAC-UMR_S1236, Rennes, France

32 i: Université Rennes, CNRS, IGDR, UMR 6290, Rennes, France

33 *: Corresponding author. Email: gwendal.coste@univ-rennes1.fr. Postal address:
34 Laboratoire de pharmacologie biologique, Pôle biologie, CHU de Rennes, 2 rue Henri
35 Le Guilloux, 35033 Rennes CEDEX 9, France. Telephone: +33642088003

36

37 e-mail address of each author:

38 Bruno Laviolle: bruno.laviolle@chu-rennes.fr; Karim Boudjema:

39 karim.boudjema@univ-rennes1.fr; Cécile Vigneau: cecile.vigneau@univ-rennes1.fr;

40 Jonathan Chemouny: jonathan.chemouny@chu-rennes.fr; Fabien Robin:

41 fabien.robin@univ-rennes1.fr; Michel Rayar: michel.rayar@chu-rennes.fr; Olivier

42 Fardel: olivier.fardel@univ-rennes1.fr; Marc Le Vée: marc.levee@free.fr; Pauline

43 Houssel-Debry: pauline.houssel-debry@chu-rennes.fr; Mikael Roussel:

44 mikael.roussel@chu-rennes.fr; Jérôme Le Priol: Jerome.LE.PRIOL@chu-rennes.fr;

45 Marie-Dominique Galibert-Anne marie-dominique.galibert-anne@univ-rennes1.fr;

46 Bouvet Régis: Regis.BOUVET@chu-rennes.fr; Edouard Bardou-Jacquet:

47 edouard.bardou-jacquet@univ-rennes1.fr; Camille Tron: [48 \[rennes.fr\]\(mailto:rennes.fr\); Marie-Clémence Verdier: \[Marie-clemence.VERDIER@chu-rennes.fr\]\(mailto:Marie-clemence.VERDIER@chu-rennes.fr\);](mailto:Camille.TRON@chu-</p></div><div data-bbox=)

49 Florian Lemaitre: Florian.LEMAITRE@chu-rennes.fr; Gwendal Coste:

50 gwendal.coste@univ-rennes1.fr

51

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_{blood}) is mandatory in solid organ transplant
56 recipients. Using peripheral blood mononuclear cells concentrations (C_{PBMC}) could
57 improve patient outcomes. The poor correlation between C_{blood} and C_{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_{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

75 Statements and Declarations**76 Funding**

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_{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_{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_{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_{PBMC}), has already been suggested to be more directly linked to the
112 immunosuppressive effect of the drug compared to C_{blood} , in renal and hepatic

113 transplant recipients [5, 6]. C_{blood} and C_{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_{PBMC} , based on C_{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 24th, 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_{blood} and C_{PBMC} over C_{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_{blood} determination. Using the blood
176 remaining from this analysis, PBMCs were isolated and C_{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_{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_{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 ± 2.55 , and C_{PBMC} was 25.42 ± 16.67
269 $\text{pg}/10^6$ cells. rs2229109 variant (*ABCB1*) failed to influence $C_{\text{PBMC/blood}}$ and C_{PBMC}
270 (figure 1A and 1B). rs10868152 (*SLC28A3*) had no significant effect on $C_{\text{PBMC/blood}}$
271 and C_{PBMC} (figure 1C and 1D), even if a trend was visible for C_{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_{blood} was $8.9 \pm$
277 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
278 ng/mL, and C_{PBMC} was 14.7 ± 10.2 pg/ 10^6 cells. For all patients, C_{blood} and C_{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 ± 1.4 vs. 8.3 ± 2.3 ng/mL). Moreover, in MCF7S cells, the P-gp
315 inhibitor CsA did not significantly impair TAC intracellular concentration (8.3 ± 2.3 vs
316 9.7 ± 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 ± 1.4
318 vs. 9.6 ± 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 ± 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 ± 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 ± 0.07
340 vs. 0.37 ± 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_{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_{blood} and C_{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_{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 21st 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_{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
475 Le Priol: Data curation, Formal analysis, Investigation, Methodology, Validation,
476 Writing - original draft. Régis Bouvet: Formal analysis, Investigation, Methodology,
477 Validation, Writing - review & editing. Marc Le Vée: Data curation, Formal analysis,
478 Investigation, Methodology, Writing - review & editing. Pauline Housel-Debry:
479 Resources. Michel Rayar: Resources. Marie-Clémence Verdier: Writing - review &
480 editing. Mikael Roussel: Methodology, Supervision. Marie-Dominique Galibert:
481 Supervision. Edouard Bardou-Jacquet: Resources. Olivier Fardel: Formal analysis,
482 Methodology, Supervision, Writing - review & editing. Cécile Vigneau: Resources,
483 Writing - review & editing. Karim Boudjema: Resources. Bruno Laviolle: Funding
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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_{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_{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_{blood} versus concomitant C_{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_{blood} : blood trough TAC concentration; C_{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

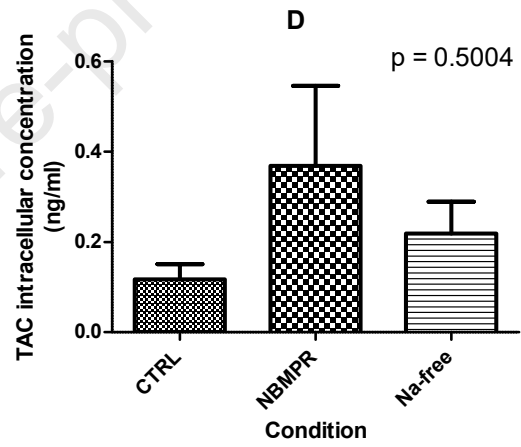
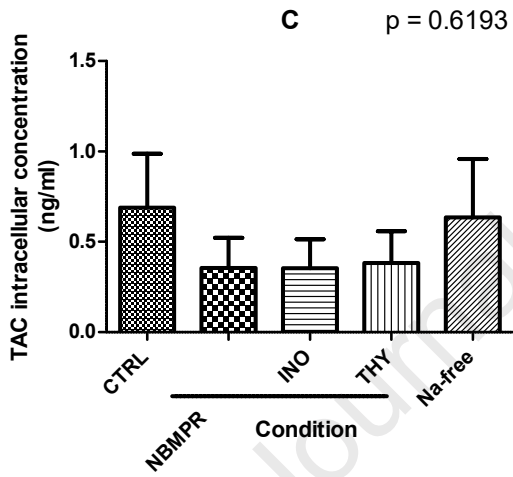
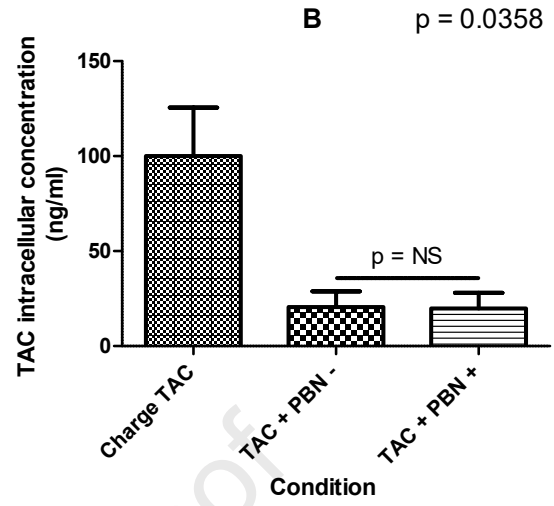
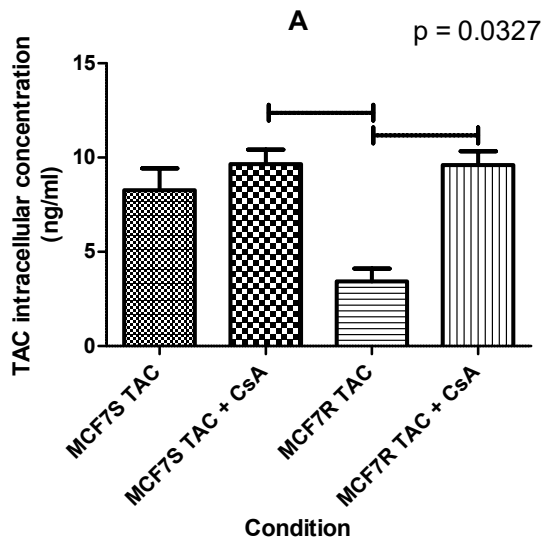
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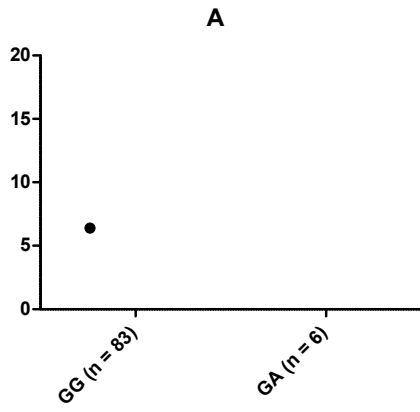
Corresponding author: Gwendal Coste; gwendal.coste@univ-rennes1.fr

	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 ²)	77.5 ± 21.7	52 ± 20.3

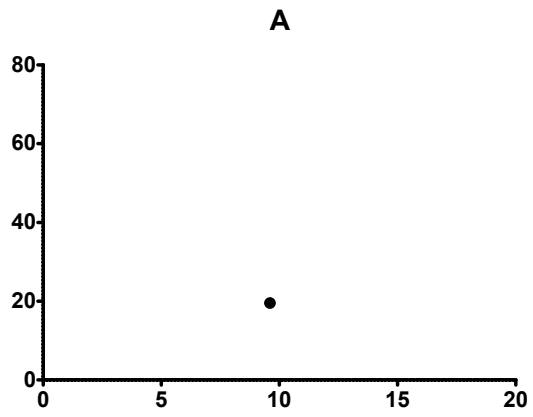
Table 1 Caption: Baseline characteristics of the patients included in the TRANS-TAC study (impact of mRNA and protein expression of four transporters on the TAC distribution in PBMCs)

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

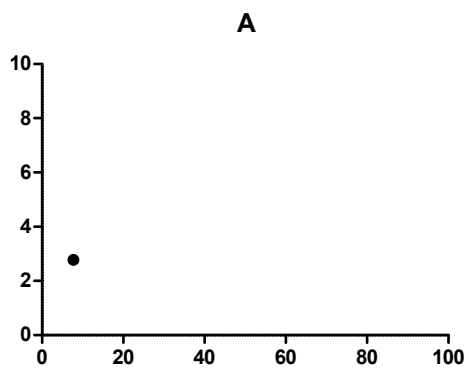




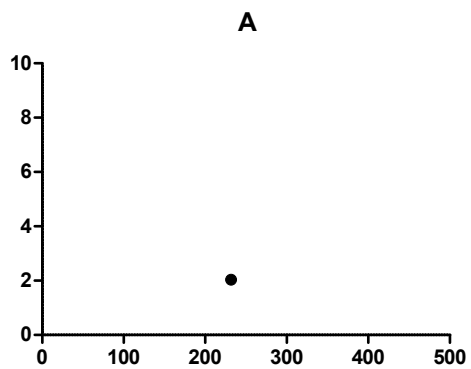
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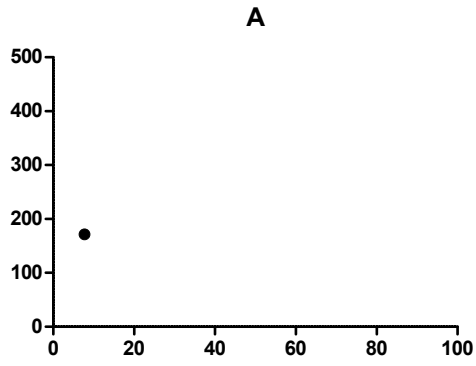
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