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Genetics of tacrolimus pharmacokinetics and kidney transplant outcomes

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1. **Hu R**, Barratt DT, Coller JK, Sallustio BC, Somogyi AA. *CYP3A5*3* and *ABCB1* 61A>G significantly influence dose-adjusted trough blood tacrolimus concentrations in the first three months post kidney transplantation. Basic Clin Pharmacol Toxicol. 2018; 123(3):320-326. doi:10.1111/bcpt.13016.
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Abstract

Tacrolimus (TAC) is the mainstay of current immunosuppressive therapy following kidney transplantation. However, TAC pharmacokinetics (PK) are highly variable and excessive high and low whole blood trough TAC concentrations (TAC C_0) have been associated with toxicity and acute rejection, respectively. Therefore, therapeutic drug monitoring (TDM), targeting TAC C_0 between 5-15 ng/mL or a slightly varied range, has been adopted in most transplant centres to maximise immunosuppression and minimise toxicity. Nonetheless, a TAC C_0-rejection relationship has not been adequately shown under TDM. In addition, TAC pharmacogenetic (PGx) and innate immunogenetic studies reported conflicting findings (except for *CYP3A5*3*) regarding their impact on dose-adjusted TAC C_0 (TAC C_0/D), acute rejection and kidney function. Therefore, this thesis hypothesised that: 1) TAC dispositional genes would affect TAC C_0/D inter-individual variability, biopsy-proven acute rejection (BPAR) and estimated glomerular filtration rate (eGFR); 2) there would be a temporal relationship between TAC C_0 and BPAR; and 3) innate immunogenetics would predict BPAR incidence.

Chapters 2 and 3 investigated if *CYP3A4/5*, *POR*, *ABCB1* and *NR1I2* genetics affect TAC C_0/D inter-individual variability, BPAR and eGFR in a retrospective cohort of 165 Australian kidney transplant recipients in the first 3 months post-transplantation. *CYP3A5* expressors (**1/*1 + *1/*3*) ($P = 5.5\times10^{-16}$) and *ABCB1* 61G allele carriers ($P = 0.001$) had lower log-transformed TAC C_0/D (56% and 26% lower geometric mean TAC C_0/D, respectively) and accounted for approximately 30% and 4%, respectively, of $\log_{10}$-transformed TAC C_0/D variability in the first 3 months post-transplantation. However, *CYP3A4*, *POR* and *NR1I2* genotypes did not significantly affect TAC C_0/D. In addition, none of these TAC PK genes significantly affected BPAR incidence in the first 14 days, or eGFR in the first 3 months, post-transplantation. Notably, an incidental

finding was that there was no significant difference in BPAR incidence between the groups with 1) TAC C_0 < and ≥ 8 ng/mL, or 2) TAC C_0 < and ≥ 5 ng/mL for 3 consecutive days in the first 14 days post-transplantation, prompting further investigation.

Chapter 4 explored the relationship between TAC C_0 and BPAR incidence in the first 14 days post-transplantation and confirmed that TAC C_0 (log_{10}-transformed) were lower on the day of (mean difference [95% confidence interval] = -0.13 [-0.24 to -0.025], post-hoc P = 0.013), and 1 day prior to (-0.13 [-0.21 to -0.048], post-hoc P = 0.002), BPAR. Adjusting for haematocrit variability assisted to identify this temporal TAC C_0-response relationship.

Chapter 5 found pro- and anti-inflammatory mediator (*CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *TGFB* and *TNF*) and MyD88-dependent TLR signalling pathway (*LY96*, *MYD88*, *TLR2* and *TLR4*) genetics did not significantly affect BPAR incidence in the first 14 days post-transplantation. Notably, *IL6* -6331C/C genotype had a higher incidence of BPAR compared to T/T genotype (Odds Ratio [95% confidence interval] = 6.6 [1.7 to 25.8], likelihood-ratio test P = 0.02), whereas it was non-significant after correction for multiple comparisons (P-value threshold = 0.0038).

In summary, this thesis provides the first evidence that *ABCB1* 61A>G, along with confirming *CYP3A5*3*, affects TAC C_0/D variability. However, the known single nucleotide polymorphisms (SNP) in TAC dispositional genes did not affect kidney transplant outcomes, likely due to TAC TDM substantially reducing the risk of sub- and supra-exposure of TAC. Notably, a temporal TAC C_0-rejection relationship was identified, and it was shown for the first time that accounting for haematocrit variability assisted in identifying this response relationship. None of the investigated innate immunogenetic factors predicted BPAR incidence in a relatively limited cohort of

TAC-treated kidney transplant recipients. Therefore, future studies are still needed to confirm current findings and explore novel factors to predict kidney transplant outcomes.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Rong Hu

5th August 2019

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Also, many words to my family. I dedicate this thesis to the two most important people in my life — my grandma, Mrs Aizhen Zhou, and my mum, Mrs Yunxia Zhu. I love you very much and I am very grateful for everything you have done for me. Also, many thanks to my dad, Mr Xinsheng Hu, and my beloved Pingan Ge. Thank you for your unconditional love, patience, companion and financial support.

Last but not least, for some words to myself, I would like to quote my favourite poem from a great Chinese poet named Zhi-Huan Wang, who left the verses "To see a thousand-mile view, go up another floor", when he was visiting the Stork Tower at the east bank of Yellow River in China about 1,300 years ago. Just as Wang encouraged to all, I would like to step higher to see further in genetics.

登鸛雀楼

白日依山尽， 黄河入海流。

欲穷千里目，更上一层楼。

— 王之涣 （唐 • 公元 688-742）

List of Abbreviations

ABCB1	ATP-binding cassette subfamily B member 1
AFR	African
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
AUC	area under curve
AZA	azathioprine
BKV	Thea Brennan-Krohn polyomavirus
BPAR	biopsy-proven acute rejection
CABG	coronary artery bypass grafting
CASP1	Caspase 1
C_{max}	peak whole blood TAC concentration
CMV	cytomegalovirus
CNI	calcineurin inhibitor
Con A	concanavalin A
CsA	ciclosporin A
CYP3A4/5	cytochromes P450 3A4/5
D	dose
DAMPs	damage/danger-associated molecular patterns
DGF	delayed graft function
DNR	donor
EAS	east Asian
eGFR	estimated glomerular filtration rate
EM	extensive metabolisers
ESRD	end-stage renal disease
Eth	ethnicity
EUR	European
FKBP-12	FK-506 binding protein 12
Gly	glycine
hour	h

HEK 293 cells	human embryonic kidney 293 cells
HLA	human leukocyte antigen
IBD	inflammatory bowel diseases
IL	interleukin
IM	intermediate metabolisers
IPT	intensive periodontal therapy
kg	kilogram
L	litre
LD	linkage disequilibrium
Leu	leucine
LPS	lipopolysaccharides
mg	milligram
MHC	major histocompatibility complex
mL	millilitre
MMF	mycophenolate mofetil
MPA	mycophenolate
MyD88 or *MYD88*	myeloid differentiation primary response 88
N or n	number of patients
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
ng	nanogram
NR	not reported
NR1I2	nuclear receptor subfamily 1, group I, member 2
OR	Odds ratio
P	point-wise P-value
PBL	peripheral blood lymphocyte
PBMCs	peripheral blood mononuclear cells
PD	pharmacodynamics
P-gp	P-glycoprotein
PGx	pharmacogenetics
PHA	phytohemagglutinin
PK	pharmacokinetics
PM	poor metabolisers

PMA	phorbol myristate acetate
POR	cytochrome P450 reductase
PRA	peak panel-reactive antibodies
Pro	proline
PRRs	pattern recognition receptors
PXR	pregnane X receptor
RPT	recipient
Ref	reference(s)
RR	relative risk
Ser	serine
sIL-6R	soluble IL-6 receptor
SNP	single nucleotide polymorphism
TAC	tacrolimus
TAC C_0	trough whole blood tacrolimus concentration
TAC C_0/D	dose-adjusted trough whole blood tacrolimus concentration
TDM	therapeutic drug monitoring
TGF-β	transforming growth factor-β
TIR	toll/IL-1 receptor domain
TLRs	Toll-like receptors
T_{max}	time to peak whole blood tacrolimus concentration
TNF-α	tumour necrosis factor-α
Val	valine

Chapter 1: Introduction

Kidney transplantation is the most effective treatment for end-stage renal disease, enabling patients' higher survival rates [1] and better quality of life than receiving dialysis alone. Immunosuppressive therapy is applied post-transplantation to suppress the recipient's immune system to prevent rejection. Nowadays, most recipients will receive oral maintenance therapy consisting of a calcineurin inhibitor (CNI; e.g. ciclosporin A (CsA) or tacrolimus (TAC)), mycophenolate mofetil (MMF) or enteric coated mycophenolate sodium salt (MPA) and a corticosteroid. Since the introduction of the CNIs, the first-year graft survival rate has been dramatically improved from around 60% to over 90% in the last 40 years [1, 2]. Although CsA is the first CNI, TAC has about 100 times higher immunosuppressive potency [3] and fewer rejection complications [4]. Therefore, TAC has been used as the first-choice CNI in over 80% Australian kidney transplant recipients since 2009 [5].

TAC is the backbone of maintenance therapy, however, there are still challenges in its clinical use, mainly the large inter-individual variability in TAC pharmacokinetics (PK) and the complications of acute rejection and kidney dysfunction. Single nucleotide polymorphisms (SNPs) in TAC dispositional genes can alter TAC metabolising enzymes and efflux transporter expression/activity [6-14]; therefore, they are likely to significantly contribute to the varied TAC PK and might affect kidney transplant outcomes via modifying TAC intracellular concentrations in T-cells and kidney cells. Trough whole blood TAC concentrations (TAC C_0) from 5 to 15 ng/mL have been recommended to maximise immunosuppression and minimise toxicity [15]. Whilst TAC therapeutic drug monitoring (TDM) has been applied in most transplant centres (TAC TDM range may vary slightly in different hospitals and at different times post-transplantation [16-19]), rejection episodes can still occur even within a target TAC TDM range [16-19]. In addition, the innate immune system assists T-cell differentiation,

proliferation and activation [20], and intensifies the severity of kidney tissue injury [21-23]. Therefore, any innate immune genetic variation which can alter pro- and anti-inflammatory mediator secretion in leukocytes, may also cause the difference in rejection incidence between kidney transplant recipients.

More than 50% of the inter-individual variability of dose-adjusted TAC C_0 (TAC C_0/D) cannot be explained by currently known TAC PK genetics [24-26], and conflicting findings also exist about their impact on TAC C_0/D. In addition, no reliable pharmacogenetic (PGx) or innate immunogenetic predictors of kidney transplant outcomes have been found. Therefore, the main purpose of this introductory chapter is to update the progress of genetic research in TAC C_0/D, acute rejection and kidney function and identify the research gaps in current TAC PGx and innate immunogenetic studies in kidney transplantation.

1.1 Kidney rejection and TAC

1.1.1 Kidney rejection post-transplantation

The first and foremost challenge post-transplantation is rejection, which is mediated by the recipient's immune system. Based on the time post-transplantation, kidney rejection can be classified into 4 sub-groups: 1) hyperacute rejection, which happens within minutes post-transplantation; 2) acute rejection, which occurs from days to weeks post-transplantation; 3) late acute rejection, which develops after 3 months post-transplantation; and 4) chronic rejection, which occurs months to years post-transplantation [21]. Kidney rejection can also be grouped into antibody-mediated and T-cell-mediated rejection, and the latter is the most common type of acute rejection [21].

The major histocompatibility complex (MHC) on recipients' antigen-presenting cells binds to the antigens derived from donors' cells to form MHC-peptide complex, which

then is recognised by recipients' T-cell receptor [21]. This binding triggers intracellular signalling transduction in T-cells (calcium mobilisation, etc.), resulting in the activation of transcription factors (e.g. nuclear factor of activated T cells (NFAT)) [27]. NFAT activation leads to the secretion of interleukin-2 (IL-2) [28], which drives T-cell differentiation, proliferation and activation [20]. Consequently, cytotoxic T-cells induce apoptosis and inflammation in the kidney cells [21].

An increased serum creatinine (or decreased estimated glomerular filtration rate (eGFR) calculated from serum creatinine) may indicate rejection, however, it is not diagnostic. The only gold-standard of kidney rejection diagnosis remains tissue biopsy. The 2018 Banff classification system [29] is the most updated clinical consensus of kidney graft pathology.

To prevent rejection, a *triple therapy* of TAC, MMF or MPA, and a corticosteroid is widely applied post-transplantation and TAC is the cornerstone of the immunosuppressive therapy.

1.1.2 Mechanism of action of TAC

The molecular and cellular mechanisms of TAC immunosuppression to prevent kidney rejection has been reviewed by Thomson *et al*. [30] in depth. Overall, TAC binds to FK-506 binding protein 12 (FKBP-12) to form a drug-immunophilin complex to inhibit calcineurin phosphatase [31]. IL-2 is a T-cell growth factor and *IL2* transcription is calcineurin-dependent [20]. In addition, calcineurin is a key rate-limiting enzyme of T-cell signalling transduction [32]. Therefore, calcineurin inhibition by TAC ultimately prevents *IL2* transcription and T-cell proliferation (see Figure 1). Consequently, T-cell mediated acute rejection is interrupted.

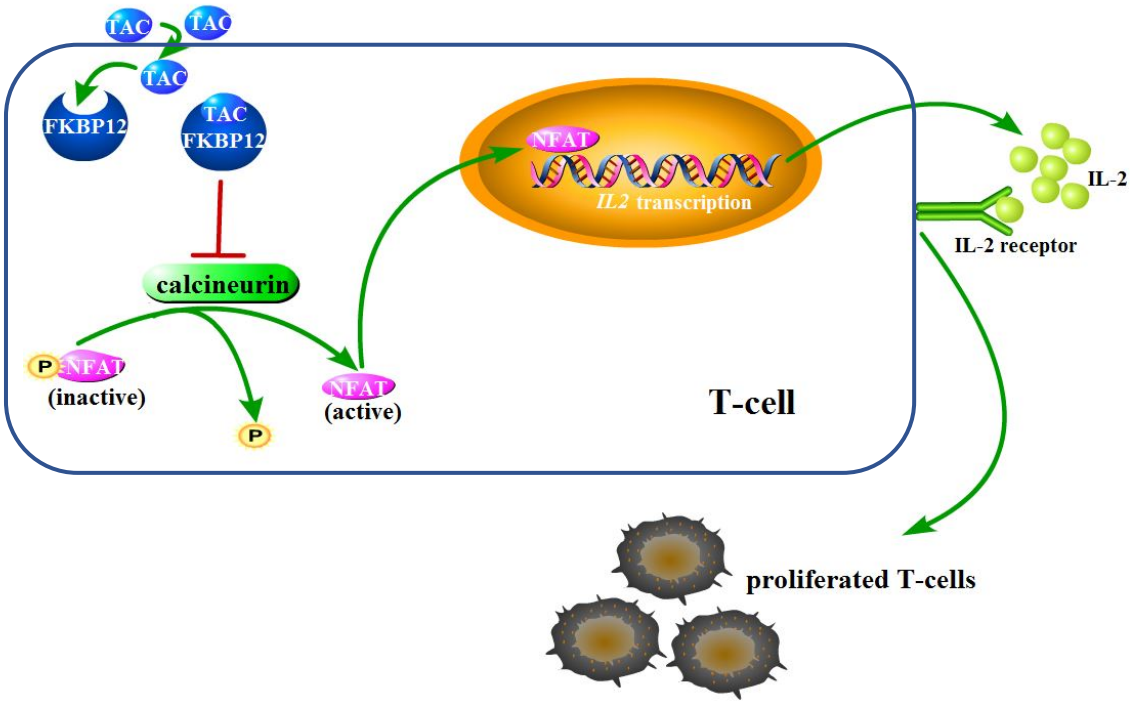

Figure 1. Mechanism of action of TAC. TAC binds to the immunophilin FKBP-12 to inhibit calcineurin activity. Therefore, the dephosphorylation of P-NFAT, to the transcription factor NFAT, is inhibited. NFAT is essential for T-cell signalling transduction, therefore, inhibition of P-NFAT dephosphorylation interrupts *IL2* transcription and T-cell differentiation, proliferation and activation.

1.2 TAC PK

1.2.1 TAC absorption, distribution, metabolism and excretion

TAC PK has been reviewed by Venkataramanan *et al*. [33] and Staatz *et al*. [34] in depth. Therefore, this section only gives a descriptive summary of TAC absorption, distribution, metabolism and excretion primarily based on these 2 systematic reviews. Figure 2 briefly depicts TAC disposition in recipient intestine and liver, and donor kidney cells.

- **Absorption**

TAC is dosed orally and its absorption is rapid. The time (T_{max}) to the peak whole blood TAC concentration (C_{max}) is usually within 2 hours (h) [33]. The bioavailability of TAC is low (mean bioavailability of 25%) and highly variable within and between patients (from 4 to 89%) [33]. The low bioavailability can be explained by the insolubility of TAC in aqueous media [35] and the extensive pre-systemic TAC metabolism in intestine [36]. In addition, TAC is a substrate of P-glycoprotein (P-gp; encoded by *ABCB1* (ATP-binding cassette subfamily B member 1)) [37] and the apical membrane of intestinal cells expresses P-gp [38], therefore, P-gp can decrease TAC absorption by pumping TAC back into the intestinal lumen.

- **Distribution**

TAC is lipophilic and undergoes extensive body distribution [35]. In rats, TAC concentrations after intramuscular administration range from high to low in the order of lung, spleen, heart, kidney, pancreas and liver [35]. P-gp can decrease TAC hepatic and intestinal accumulation as P-gp is also expressed on the apical membrane of hepatocytes and tubular epithelial cells [38]. The whole blood TAC concentration is about 15-fold higher (range 4- to 114-fold) than plasma TAC concentration as TAC is extensively bound to erythrocytes [33, 34]. The volume of distribution of TAC is only about 1 L/kg based on the whole blood TAC concentration but can be 30 L/kg based on the plasma TAC concentration [39]. In plasma, over 70% of TAC is bound to α1-acid glycoprotein and albumin [40]. Only the unbound TAC is therapeutically active. Whole blood has been chosen as the medium for TAC TDM as erythrocyte binding is temperature dependent; therefore, using whole blood reduces variability in TAC concentrations caused by variations in sample processing within and between

laboratories [41]. In addition, quantifying TAC whole blood concentrations mitigates sensitivity challenges as TAC concentrations are much higher in whole blood than plasma [41]. Haematocrit is the volume percentage of red blood cells in whole blood. A change of haematocrit can alter the distribution of TAC in whole blood, and more details are discussed in section 1.2.2.2 about the impact of haematocrit on TAC whole blood concentration.

- **Metabolism**

TAC undergoes extensive pre-systemic and systemic metabolism by the intestinal and hepatic cytochromes P450 3A4/5 (CYP3A4/5) [36, 42-44]. Less than 0.5% of TAC was found unchanged in urine [45]. More than 15 TAC metabolites have been found [33] but the demethylation of TAC to 13-O-demethyl-TAC is the predominant metabolic reaction [43]. The immunosuppressive activity of this major TAC metabolite is only about one-tenth compared with the parent drug [46]. Although CYP3A4 is the most abundant hepatic CYP3A enzyme, the catalytic efficiency of TAC to its major metabolite by CYP3A5 is over 60% higher than that by CYP3A4 [44].

- **Excretion**

More than 95% of the metabolites of TAC are excreted via the faeces or biliary route [35]. Renal clearance was responsible for < 3% of the total body clearance of TAC [45].

- **TAC PK parameters**

The mean elimination half-life of TAC is about 12 h (ranging from 8.7 to 32.5 h) [33]. Total body clearance of orally administered TAC in the whole blood varies from 2 to 4 L/h in kidney transplant recipients [33]. Intra- and inter-individual coefficients of variation for apparent whole blood TAC clearance range from 40 to 71% and 30 to 42%,

respectively [47]. Overall, TAC has highly variable PK and CYP3A4/5 and P-gp are major determinants of TAC disposition (see section 1.5.1.1).

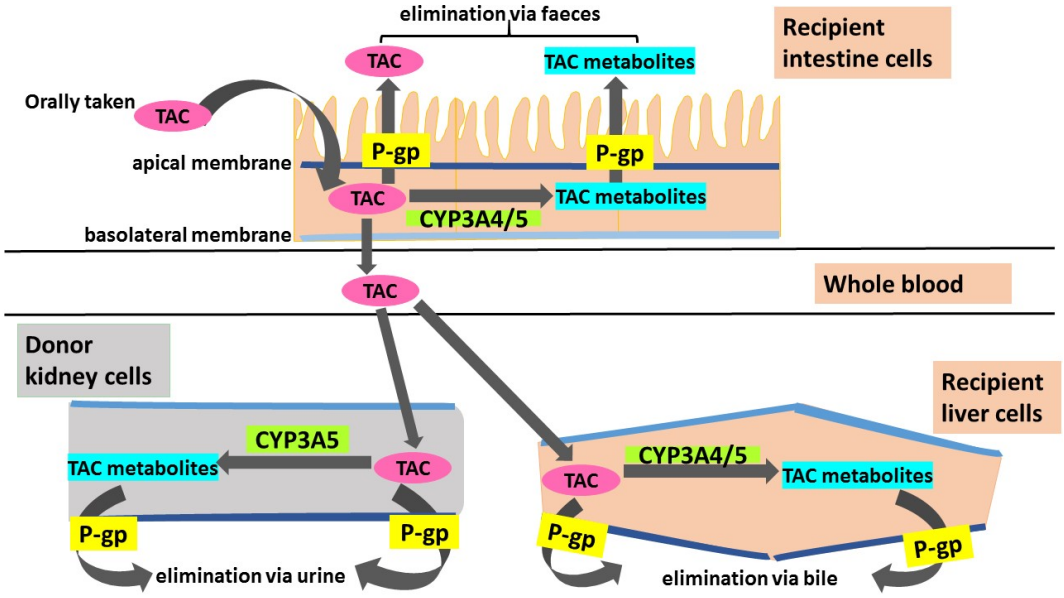

Figure 2. TAC disposition in recipient intestine and liver, and donor kidney cells. TAC undergoes extensive pre-systemic and systemic metabolism via intestinal and hepatic CYP3A4/5. Although kidney cells also express CYP3A5, the kidney is not the major metabolism organ of TAC systemic clearance. As TAC is a substrate of P-gp, and the apical membrane of intestine, liver and kidney cells all express P-gp, TAC absorption, hepatic and renal accumulation can decrease via pumping TAC out of these tissue cells. Dark and light blue lines in Figure 2 indicate the apical and basolateral membranes, respectively.

1.2.2 Clinical factors affecting TAC PK

Age, sex, haematocrit, liver function, food-drug and drug-drug interactions are potential clinical factors affecting TAC absorption, distribution and metabolism. Therefore, their impact on TAC PK intra- and/or inter-individual variability has been widely studied in kidney transplant recipients.

1.2.2.1 Age and sex

In adult kidney transplant recipients, some studies reported age did not affect TAC PK [24, 48], whilst other studies found age as a significant factor increasing TAC C_0 [49] or TAC C_0/D [50-52]. However, the contribution of age to TAC C_0/D is minor (~ 5%) based on multivariate analysis [50, 51]. In addition, most studies did not find sex affecting TAC PK [24, 49, 51, 52]. Altogether, these results suggest that age and sex are not the major clinical factors affecting TAC PK in adult kidney transplant recipients, therefore, TAC dosing is not age- or sex-based.

1.2.2.2 Haematocrit

Haematocrit is usually low due to kidney dysfunction pre-transplantation but can recover to normal in the weeks post-transplantation. However, transfusion, post-transplantation anaemia and erythrocytosis [53] can contribute to haematocrit intra- and inter-individual variability. TAC is highly bound to red blood cells [54] and TAC concentration is measured in whole blood, therefore, changes in haematocrit over time can cause fluctuations in TAC C_0 within and between patients. However, the therapeutically active unbound TAC concentration is not affected by haematocrit [55] as TAC is a low clearance drug with clearance equivalent to only 3% of liver blood flow [45]. If a recipient has a very high haematocrit (but "normal" whole blood clearance) and TAC C_0 is above the target TDM range, a dose reduction may not be appropriate as it can increase the risk of acute rejection. Conversely for a very low haematocrit and increased risk of toxicity. TAC population PK models and multivariate analysis have already identified haematocrit as a significant confounder of TAC C_0 inter-individual variability [49, 52, 55, 56]. However, haematocrit variations have not been accounted for in clinical interpretation of TAC C_0 under TDM. Although the

relationship between TAC C_0 and rejection is not well defined, the role of haematocrit has not been adequately assessed in this concentration-response relationship.

1.2.2.3 Liver dysfunction

TAC clearance and bioavailability are similar between healthy volunteer subjects (n = 6) and subjects with mild liver dysfunction (n = 8) [57]. However, severe liver dysfunction can substantially decrease TAC clearance by up to two thirds and increase the elimination half-life by up to 3-fold [58, 59]. In contrast, kidney function did not significantly affect TAC clearance [58, 60]. This can be explained by the fact that the intestine and liver but not the kidney are the major organs for TAC metabolism and excretion [33].

1.2.2.4 Food-drug interaction

Food, especially fatty food, and the relative time interval between food and TAC oral dosing can reduce TAC absorption and relative bioavailability [61, 62]. In a single-dose study in 15 healthy volunteers, subjects taking a high-fat meal had 10% lower mean area under TAC concentration-time curve (AUC), 35% lower C_{max} and 2-fold longer T_{max} than subjects taking a low-fat meal [61]. In another single-dose study of 16 healthy volunteers, compared to the fasting group or the group having TAC 1 h before meal, subjects showed about 35% decreased TAC AUC, 60% lower C_{max} and 1.8-fold longer T_{max} [62] in groups having TAC administration immediately, or 1.5 h, after a meal.

Grapefruit juice is a CYP3A4 inhibitor and it can decrease intestinal CYP3A4 expression by over 60% in 10 healthy volunteers [63]. A daily consumption of 500 mL grapefruit juice for 1 week increased TAC C_0 by 2-fold in 30 liver transplant recipients [64].

1.2.2.5 Drug-drug interaction

Since TAC is a CYP3A and P-gp substrate, any co-medications, which are CYP3A and/or P-gp inducers or inhibitors, may affect TAC PK. Liu *et al*. [65] and Kim *et al*. [66] have summarised the clinically common CYP3A and P-gp inhibitors and inducers which should be prescribed with caution when co-administered with TAC. The best examples are corticosteroids, well known inducers of both CYP3A [67, 68] and P-gp [69, 70]. In 2 groups of kidney transplant recipients in the first 2 weeks post-transplantation, TAC C_0 was about 20% lower in the group co-administered prednisolone than a non-corticosteroid group, even though the TAC dose was similar between the 2 groups [71]. A high-dose of corticosteroid > 0.25 mg/kg/day can increase TAC dose requirement by 40-80% to achieve the same target TAC C_0 when compared with a low corticosteroid dose regimen < 0.15 mg/kg/day in the first 3 months post-transplantation [72].

Herbal supplements can also cause drug-drug interactions with TAC. For example, St. John's Wort is an anti-depressant herbal product, an inducer of intestinal and hepatic CYP3A4, and intestinal P-gp [73]. St. John's Wort can reduce TAC AUC by 40-60% in healthy volunteers [74] and kidney transplant recipients [75] (n = 10 in both studies).

1.3 TAC TDM

Calcineurin inhibition by TAC not only contributes to immunosuppression but also induces toxicity (e.g. nephrotoxicity) [76]. In addition, as TAC PK is highly variable between patients (see section 1.2.1), TAC undergoes TDM to maximise immunosuppression and minimise toxicity.

A target TAC C_0 from 5 to 15 ng/mL was derived from an open-label, concentration-ranging trial of TAC in the first 42 days following kidney transplantation [15]. Ninety-

six kidney transplant recipients were randomised to target 3 different TAC C_0 ranges: 1) 5 to 14 ng/mL; 2) 15 to 25 ng/mL; and 3) 26 to 40 ng/mL. All recipients were co-administered antilymphocyte globulin, azathioprine and a corticosteroid. No toxicity event (e.g. kidney dysfunction) occurred when TAC C_0 was < 5 ng/mL, however, it was over 50% when TAC C_0 was > 15 ng/mL. Whilst biopsy-proven acute kidney rejection (BPAR) incidence was about 2- to 3-fold higher when TAC C_0 was below compared with above 5 ng/mL, BPAR occurred even when TAC C_0 was > 15 ng/mL. Therefore, the lower limit of TAC TDM to predict BPAR incidence remains under debate. In a cohort of 29 kidney transplant recipients treated with TAC, azathioprine and prednisolone, Staatz *et al*. [77] collected 349 TAC C_0 data in total and found all 12 rejectors and 10 out of 17 non-rejectors had a median trough concentration between 0 and 10 ng/mL, whereas the remaining 7 non-rejectors had a median concentration between 10 and 15 ng/mL. Therefore, they concluded "In order to minimise rejection in the first month after renal transplantation, trough concentrations greater than 10 ng/mL must be achieved". However, in a pooled-analysis of 3 randomised, open-label clinical trials [16-18], Bouamar *et al*. [19] found TAC C_0 1) below or above 5 ng/mL or 2) below or above 10 ng/mL was not significantly associated with BPAR incidence when patients were co-administered MMF and a corticosteroid in the first year post-transplantation. The ELITE-Symphony study (n = 1645) [16], a prospective clinical trial, recommended a target TAC C_0 from 3 to 7 ng/mL to decrease kidney dysfunction when recipients were also co-administered MMF and a corticosteroid in the first year post-transplantation. However, the relationship between TAC C_0 and BPAR was not formally investigated.

Although TAC TDM has been adopted to maximise immunosuppression, the relationship between TAC C_0 and rejection has not been adequately shown and

limitations exist in the previous studies [19, 77, 78]. Firstly, the published studies only classified recipients with and without BPAR into 2 groups and then compared TAC C_0 between groups on each day post-transplantation. However, importantly, patients can develop BPAR on different days, in addition, no previous studies compared TAC C_0 on the days prior to and on the day of BPAR within patients. Secondly, whilst haematocrit affects TAC C_0 intra- and inter-individual variability (see section 1.2.2.2), TAC C_0 was not adjusted for haematocrit. Overall, the relationship between TAC and BPAR under TDM still needs to be elucidated.

TAC TDM for long-term transplant outcomes remains extremely complicated, which is beyond the scope of this study. Noteworthy, the Collaborative Transplant Study has published registry data indicating graft failure with TAC C_0 < 5 ng/mL [79].

For new TAC TDM approaches, especially intracellular TAC concentration monitoring, the Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology has issued a most up-to-date consensus report [80]. This report has comprehensively reviewed the pharmacological justifications, clinical evidence and current analytical methods for future TAC concentration monitoring. More details are covered in Chapter 6 (see section 6.3) about intracellular TAC concentration monitoring in peripheral blood mononuclear cells (PBMCs) in kidney transplant recipients.

1.4 Kidney transplant outcomes

Although kidney rejection rate has now been reduced to about 20% in the first 6 months post-transplantation, the average lifespan of a transplanted kidney is still less than 15 years [1]. Acute rejection is the most common and serious early-stage complication post-transplantation and it affects kidney function and long-term graft survival [81]. In addition, a 30% decline of eGFR has been significantly associated with graft failure

[82]. Therefore, studies have been carried out to explore potential predictors (other than TAC C_0) for acute rejection and kidney dysfunction post-transplantation.

1.4.1 Factors affecting acute kidney rejection

Human leukocyte antigen (HLA) mismatches are routinely examined before all transplantation surgery to reduce the risk of rejection. Other clinical factors including induction therapy, donor type (living or deceased donor), retransplant and peak panel-reactive antibodies (PRA) scores, have been extensively studied for their impact on BPAR incidence in kidney transplant recipients receiving *triple therapy* in the last 10 years [19, 78, 83-89]. However, acute rejection still happens under TAC TDM.

Donor specific antibodies have been suggested as predicting biomarkers for antibody-mediated rejection as recently reviewed [90]. However, no biomarkers for T-cell mediated acute rejection (the most common type of acute rejection) have been found/accepted as recently reviewed [91], although metabolomic, proteomic and genomic studies keep exploring novel predictors to identify high risk recipients of T-cell mediated acute rejection and monitor pharmacodynamic (PD) response.

Since the immunosuppressive site of action of TAC is in T-cells [30], TAC intracellular exposure in T-cells may determine TAC PD response. Therefore, any factor decreasing intracellular TAC concentration in T-cells is worthwhile to be explored as a predictor for BPAR in the future.

1.4.2 Factors affecting kidney function post-transplantation

Although most kidney transplant recipients have improved kidney function post-transplantation, short- and long-term kidney function still differ between recipients. Acute kidney rejection, Thea Brennan-Krohn polyomavirus (BKV) infection [92] and delayed graft function [93] (DGF; dialysis in the first week post-transplantation due to

kidney dysfunction) can cause a decline in eGFR. Organs from deceased donors usually have longer cold ischaemia times than living donors. Due to the ischaemia/reperfusion injury to the kidney, cold ischaemia times > 20 h can increase DGF incidence by 50-100% compared with cold ischaemia times < 10 h [94].

TAC also plays an important role in decreasing kidney function. Over exposure of TAC in kidney cells is likely to cause acute kidney dysfunction due to endothelial cell injury and glomerular constriction by TAC [76]. The ongoing ischaemia by glomerular constriction can activate pro-fibrotic and pro-inflammatory pathways [95], leading to structural kidney damage. Whilst acute kidney dysfunction is reversible by reducing TAC dose or discontinuation of TAC, it increases the risk of BPAR [96]. In addition, the decline in eGFR by chronic allograft nephropathy is irreversible. Moreover, TAC drives new on-set diabetes and hypertension and these comorbidities can decrease kidney function [97]. Overall, any factor increasing intracellular TAC concentration in kidney cells is worthwhile exploring as a predictor for kidney function post-transplantation.

1.5 Genetic research in kidney transplantation

TAC PGx and innate immunogenetic studies have been widely carried out to explore potential genetic determinants of the inter-individual variabilities in TAC PK and/or kidney transplant outcomes, which are only partially explained to date (see sections 1.2 and 1.4). This section does not aim to be a comprehensive systemic review including all past relevant genetic research in TAC C_0/D, BPAR and eGFR, but a summary of the a) **accepted findings**, b) **conflicting findings** and c) **other research gaps** in TAC PGx and innate immunogenetic studies in kidney transplantation.

1.5.1 TAC dispositional genetics

SNPs in *CYP3A4/5* and *ABCB1* can alter CYP3A and P-gp expression and/or activity [6, 8-11], therefore, recipient *CYP3A4/5* and *ABCB1* genetics have been most widely investigated for their impact on TAC C_0/D as discussed in section 1.5.1.1. Since the immunosuppressive site of action of TAC is in T-cells [30] and P-gp is also expressed on lymphocytes (including T-cells) [98], recipient *ABCB1* genetics are also likely to affect BPAR incidence via modifying intracellular TAC concentrations in T-cells as discussed in section 1.5.1.2. In addition, TAC is nephrotoxic [76, 95] while CYP3A5 [99] and P-gp [38] are also expressed in/on donor kidney cells, therefore, donor *CYP3A5* and *ABCB1* genetics are likely to affect kidney function by altering TAC intracellular concentration in kidney cells as discussed in section 1.5.1.3. Figure 3 briefly depicts how recipient P-gp and donor CYP3A5 and P-gp can affect TAC local exposure in T-cells and kidney cells, respectively.

SNPs in cytochrome P450 reductase (*POR*) [13] and *NR1I2* (nuclear receptor subfamily 1, group I, member 2; encoding gene for pregnane X receptor (PXR)) [7, 12] regulate CYP3A4/P-gp expression and/or activity. However, neither recipient nor donor *POR* or *NR1I2* have been adequately studied for their genetic association with TAC C_0/D and kidney transplant outcomes, and the results are inconclusive.

The relevant key SNPs in *CYP3A*, *ABCB1*, *POR* and *NR1I2*, their functional consequences and allele frequencies (African, east Asian and European) are summarised in Table 1. Allele frequency data were collected from the Ensembl database (http://grch37.ensembl.org/Homo_sapiens/Info/Index (last accessed on 8th March 2019)).

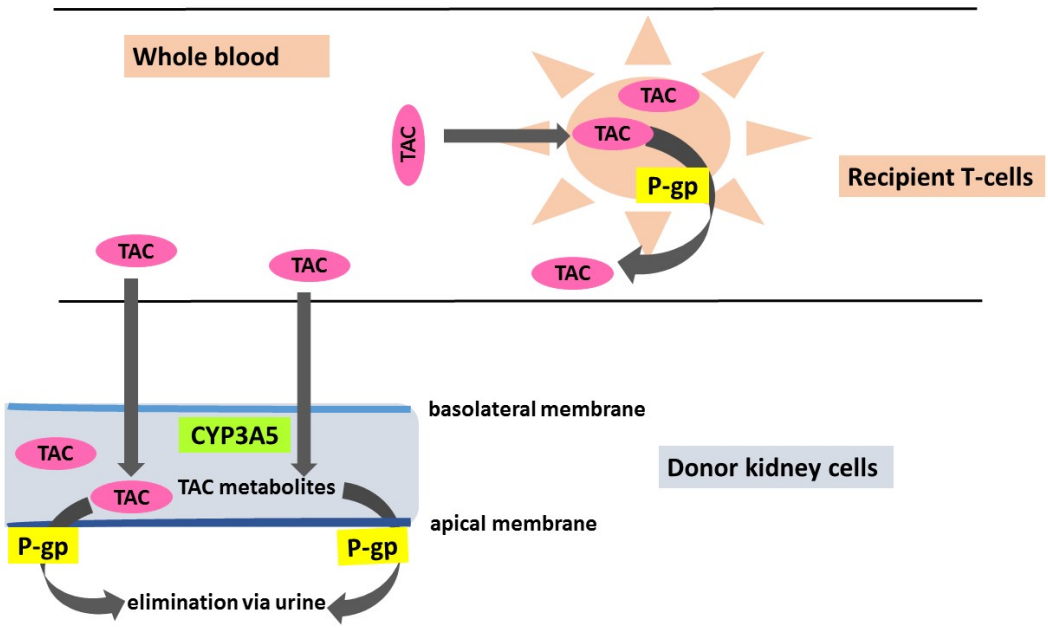

Figure 3. CYP3A5 and/or P-gp can determine TAC intracellular exposure in kidney cells and T-cells as P-gp is expressed on T-cell membranes while both CYP3A5 and P-gp are expressed in or on kidney cells. Dark and light blue lines in Figure 3 indicate the apical and basolateral membranes, respectively.

Table 1. Allele frequency (African, east Asian and Europeans) and functional consequences of key SNPs in *CYP3A*, *ABCB1*, *POR* and *NR1I2*

Genes	SNPs	Allele frequency (%)			Functional consequences	Ref
		AFR	EAS	EUR		
CYP3A5	rs776746 (**3*; 6986A>G)	18	71	94	**loss of functional CYP3A5**	[6]
CYP3A4	rs2740574 (**1B*; -392A>G)	77	-	3	****1B*** **↑ CYP3A4 expression** 1) **1B* promoter had 1.2- to 1.9-fold ↑ luciferase expression than **1* promoter in MCF-7 and HepG2 cells ($P < 0.0001$) 2) **1B* promoter had 1.5- and 1.9-fold ↑ CYP3A4 expression than **1* promoter in human hepatocytes ($P < 0.03$)	[8]
	rs35599367 (**22*; 15389C>T)	-	-	5	**T allele ↓ CYP3A4 expression and activity** 1) C/C had 1.7-fold ↑ CYP3A4 mRNA expression than T carriers in human hepatocytes ($P = 0.03$); 2) C/C had 2.5-fold ↑ CYP3A4 activity than C/T in human hepatocytes ($P = 0.04$)	[14]
ABCB1	rs9282564 (61A>G; Asn21Asp)	-	-	8	5 common European *ABCB1* SNPs are in strong LD, however, their genetics were associated with ↑, ↓ or ↔ P-gp efflux in *in vitro* or clinical studies with different substrates and/or in different ethnicities. Therefore, it has been suggested by the cited reviews that on balance **the variant genotypes/haplotypes of the 5 SNPs ↓ P-gp efflux in a substrate- and ethnicity-dependent manner**	[100-105]
	rs2229109 (1199G>A; Ser400Asn)	-	-	3		
	rs1128503 (1236C>T)	14	63	42		
	rs2032582 (2677G>T; Ala893Ser)	2	40	41		
	rs1045642 (3435C>T)	15	40	52		
POR	rs1057868 (**28*; Ala503Val)	17	37	30	****28*** **↑ CYP3A4 activity** **28* homozygotes had 1.6-fold ↑ CYP3A4 activity than **1* allele carriers by midazolam phenotyping test ($P = 0.004$)	[13]

NR1I2	rs3814055 (-25385C>T)	31	22	37	**T allele ↑ CYP3A4 activity** T/T had 2-fold ↑ CYP3A4 activity than C carriers in erythromycin breath test after rifampin treatment (P = 0.05)	[7]
	rs2276707 (8055C>T)	42	47	17	**T allele ↑ CYP3A4 expression** T allele carriers had 2-fold ↑ intestinal CYP3A4 mRNA expression than C/C after rifampin treatment (P = 0.04)	[7]
	rs2472677 (63396C>T)	37	62	66	**T allele ↑ CYP3A4 activity** T carriers had 3-fold ↑ CYP3A4 activity than C/C in testosterone 6β hydroxylation test in primary human hepatocytes (all CYP3A5 non-expressors; P = 0.006)	[12]

↑: increase; ↓: decrease; ↔: unchanged; AFR: African; Ala: alanine; Asn: asparagine; Asp: aspartic acid; EAS: east Asian; EUR: European; LD: linkage disequilibrium; Ref: reference(s); Ser: serine; SNPs: single nucleotide polymorphisms; Val: valine.

1.5.1.1 TAC dispositional genetics and TAC C_0/D

Recipient (RPT) genetics determine TAC systemic exposure as the donor (DNR) kidney is not the major metabolism or excretion organ of TAC (see sections 1.2.1 & 1.2.2.3). A large number of TAC dispositional genetic studies (RPT) have been carried out for their impact on TAC C_0/D. Accepted PGx findings on TAC C_0/D are supported by the cited review articles and/or meta-analyses while conflicting findings are shown in Table 2, whereas other research gaps are concisely summarised in section 1.5.1.1.3.

1.5.1.1.1 Accepted PGx findings on TAC C_0/D

- ***CYP3A5*3* — a major genetic factor affecting TAC C_0/D**

*CYP3A5*3* results in non-functional CYP3A5 protein [6], therefore, CYP3A5 phenotypes have been classified accordingly into extensive metabolisers (EM) – *CYP3A5*1* homozygotes (CYP3A5 expressors), intermediate metabolisers (IM) – *CYP3A5*3* heterozygotes (CYP3A5 expressors) and poor metabolisers (PM) – *CYP3A5*3* homozygotes (CYP3A5 non-expressors) [106]. It has been summarised by Staatz *et al*. [101] in a TAC PK-PGx review that there is "an approximate halving of the tacrolimus C_0/Dose and doubling of tacrolimus dose requirements in CYP3A5 expressors compared with CYP3A5 non-expressors."

- ***CYP3A4*1B* — a genetic factor dependent on *CYP3A5* to affect TAC C_0/D**

A meta-analysis reported that *CYP3A4*1/*1* had significantly higher TAC C_0/D than *CYP3A4*1B* carriers at 1, 6 and 12 months post-transplantation ($P < 0.001$, $P = 0.001$ and 0.01, respectively); however, this impact was dependent on *CYP3A5* genotypes (*CYP3A4*1B* is in strong LD with *CYP3A5*1*) [107]. Hesselink *et al*. [108] concluded in a PGx review that "Genotyping transplant patients for *CYP3A4*1B* therefore does

not appear to be meaningful from a clinical perspective."

- ***ABCB1* 1236C>T, 2627G>T and 3435C>T — no/limited impact on TAC C_0/D**

In terms of *ABCB1* SNPs, TAC PGx studies mainly focused on 1236C>T, 2677G>T and 3435C>T. Most studies found the genotypes of these 3 SNPs did not affect TAC C_0/D, whereas only a few but not all PGx studies found their haplotypes/diplotypes were statistically significant, but quantitively minor, contributors to TAC C_0/D inter-individual variability as reviewed by Staatz *et al*. [101]. *ABCB1* haplotypes may have a combined genetic impact on P-gp efflux [101]. However, Hesselink *et al*. [108] concluded in a PGx review that "most association studies have reported negative results and the effect of variation in *ABCB1* on tacrolimus pharmacokinetics, if any, is likely to be small and not clinically relevant."

1.5.1.1.2 Conflicting PGx findings on TAC C_0/D

- ***CYP3A4*22***

Inconsistent findings exist regarding the impact of *CYP3A4*22* on TAC C_0/D (see Table 2). Some studies found *CYP3A4*22* increases TAC C_0/D, however, others found *CYP3A4*22* did not affect TAC C_0/D unless adjusted for *CYP3A5*3* or combined with *CYP3A5*3* into the predicted EM/IM/PM phenotypes, suggesting *CYP3A4*22* may only be a minor factor to affect TAC C_0/D.

- ***POR*28***

It is still inconclusive if *POR*28* decreases or does not affect TAC C_0/D (with or without adjusting for *CYP3A5*3*) and if the impact is through enhanced CYP3A4 [109] or CYP3A5 [110] activity given that *POR*28* decreases TAC C_0/D (see Table 2). Since small sample size is a common limitation in most PGx studies, especially when

stratification analysis is within CYP3A5 expressors (< 20% of Europeans) and multiple comparisons at different time post-transplantation are carried out, both type I and type II errors are likely to occur. This may be the main reason causing the conflicting findings of the impact of *POR*28* on TAC C_0/D.

- ***NR1I2* -25385C>T and 8055C>T**

Only *NR1I2* -25385C>T and 8055C>T have been studied for their genetic impact on TAC C_0/D in kidney transplant recipients, however, neither SNP had a consistent impact on TAC C_0/D, with or without adjusting for *CYP3A5*3* (see Table 2). Different ethnicities between studies, small sample sizes and limited number of studies may contribute together to the inconsistent impact of *NR1I2* genetics on TAC C_0/D.

1.5.1.1.3 Other gaps in PGx research on TAC C_0/D

- ***ABCB1* 61A>G and 1199G>A**

Two *ABCB1* SNPs found in Europeans, 61A>G and 1199G>A, have not been adequately studied for their effect on TAC C_0/D. No study yet has investigated if 61A>G affects TAC C_0/D in kidney transplant recipients but only one study explored its impact, along with another 43 genetic variants, on TAC C_0 under TDM as dose data were not reported [111]. Although 61A>G did not significantly affect TAC C_0, *CYP3A5*3* had not been adjusted for in that study (n = 1560; P = 1.0). In addition, only one study explored the relationship between 1199G>A and TAC C_0/D in 96 kidney transplant recipients, however, the result was non-significant (P > 0.05) but also without adjusting for the impact of *CYP3A5*3* on TAC C_0/D [112].

- ***NR1I2* 63396C>T**

NR1I2 63396C>T increases CYP3A4 activity (see Table 1), however, it has not been studied for its impact on TAC C_0/D.

1.5.1.1.4 Future directions in PGx research on TAC C_0/D

Future studies (European cohort) should take *ABCB1* 61A>G and 1199G>A, along with 1236C>T, 2677G>T and 3435C>T, and their haplotypes into account for TAC C_0/D inter-individual variability as these 5 SNPs have not been investigated together in kidney transplant recipients. Moreover, the impact of *CYP3A4*22*, *POR*28* and *NR1I2* genetics on TAC C_0/D needs further elucidation in a large cohort adjusting for the impact of *CYP3A5*3* and the other potential cofounders (e.g. haematocrit and co-medication) on TAC C_0/D.

1.5.1.2 TAC dispositional genetics and BPAR

TAC dispositional genes (RPT & DNR) have been investigated for their impact on BPAR incidence, however, none of the genetic factors investigated have been identified as a reliable predictor for BPAR under TAC TDM due to minor contribution and poor reproducibility. Accepted PGx findings on BPAR incidence are supported by the cited review articles while conflicting findings are shown in Table 3, whereas other research gaps are concisely described in section 1.5.1.2.3.

1.5.1.2.1 Accepted PGx findings on BPAR incidence

- ***CYP3A5*3*** **(RPT),** ***CYP3A4*1B*** **(RPT),** ***CYP3A4*22*** **&** ***ABCB1*** **genetics (RPT & DNR) — limited/no impact on BPAR incidence**

Most studies did not find an association between recipient *CYP3A5*3*, *CYP3A4*1B* and *ABCB1* 1236C>T, 2677G>T and 3435C>T and BPAR incidence in kidney transplant recipients undergoing TDM as reviewed by Staatz *et al*. [113] and Shuker *et al*. [114]

in depth. In addition, *CYP3A4*22* (RPT & DNR) [52, 115] and *ABCB1* 3435C>T (DNR) [116] did not significantly affect BPAR incidence, although the number of studies and sample sizes were both limited. TAC TDM has been widely applied to reduce the risk of sub-exposure of TAC and this may explain why TAC dispositional genes did not affect BPAR incidence. Overall, Hesselink *et al*. [108] concluded in a TAC PGx review that "Although some authors have reported an increased incidence of rejection in association with certain genotypes, the additional risk posed by one's *CYP3A* or *ABCB1* genotype appears to be small and is unlikely to be clinically useful."

1.5.1.2.2 Conflicting findings on BPAR incidence

- ***CYP3A5*3*** **and** ***CYP3A4*1B*** **(DNR)**

Glowacki *et al*. [116] reported *CYP3A5*3* (DNR) did not affect BPAR incidence in kidney transplant recipients, however, Gervasini *et al*. (2018) [115] recently found *CYP3A5*3* along with *CYP3A4*1B* (DNR) predicted increased BPAR incidence (see Table 3). Not all recipients were treated with TAC (some were treated with CsA) and HLA mismatches were not adjusted for BPAR incidence in the later study. Notably, even the authors could not justify the mechanism behind the genetic association between *CYP3A* (DNR) and BPAR.

1.5.1.2.3 Other gaps in PGx research on BPAR incidence

- ***ABCB1*** **61A>G and 1199G>A (RPT & DNR)**

ABCB1 1199G>A increases TAC concentration in PBMCs from kidney transplant recipients by 1.4-fold (P = 0.001) [112], whereas the impact of 61A>G on PBMCs TAC concentration is still unknown. However, neither 61A>G nor 1199G>A (RPT & DNR), nor the haplotypes consisting of the 5 *ABCB1* SNPs, have been investigated for their

impact on BPAR incidence.

- ***POR*28*** **(RPT & DNR)**

Only recipient but not donor *POR*28* has been investigated for its impact on BPAR incidence, although no significant associations were found [110, 117]. However, if *CYP3A* (DNR) genetics predicted BPAR incidence, *POR*28* (DNR) would be worthwhile investigating as it is essential for CYP3A (DNR) activity.

- ***NR1I2*** **genetics (RPT & DNR)**

Whilst PXR regulates CYP3A and P-gp expression [118], no published studies have investigated if *NR1I2* genetics (RPT & DNR) affected BPAR incidence.

1.5.1.2.4 Future directions in PGx research on BPAR incidence

Future studies (European cohort) should take *ABCB1* 61A>G and 1199G>A (RPT & DNR), along with 1236C>T, 2677G>T and 3435C>T, and their haplotypes into account for BPAR incidence as these 5 SNPs have not been investigated together in kidney transplant recipients. Moreover, *POR*28* (DNR) and *NR1I2* genetics (RPT & DNR) are worthwhile investigating in the future for their impact on BPAR incidence. Importantly, confounding factors (HLA mismatches, retransplant, etc.) should be adjusted when exploring the relationship between the genetic factors and BPAR.

1.5.1.3 TAC dispositional genetics and eGFR

TAC dispositional genes (RPT & DNR) have also been investigated for their impact on eGFR, an indicator to easily evaluate kidney dysfunction, which can be caused by factors such as BPAR, DGF and TAC-induced nephrotoxicity. However, similarly to BPAR results, no PGx study to date has identified a reliable predictor for eGFR due to minor contribution and/or poor reproducibility. Accepted PGx findings on eGFR are

supported by the consistent individual studies while conflicting findings are shown in Table 4, whereas other research gaps are concisely described in section 1.5.1.3.3.

1.5.1.3.1 Accepted PGx findings on eGFR

- ***CYP3A*3*** **and** ***ABCB1*** **genetics (RPT) — no impact on eGFR**

Recipient *CYP3A5*3* and *ABCB1* genetics (1236C>T, 2677C>T and 3435C>T) did not affect eGFR [51, 52, 116, 119-124], probably because TDM has substantially reduced the risk of supra-exposure of TAC.

1.5.1.3.2 Conflicting PGx findings on eGFR

- ***CYP3A5*3*** **and** ***ABCB1*** **3435C>T (DNR)**

No consistent associations between *CYP3A5*3* and *ABCB1* 3435C>T (DNR), and eGFR have been identified in kidney transplant recipients (see Table 4). This inconsistency may be caused by varied and limited sample sizes (n = 50 – 237), different ethnicities and time post-transplantation (day 7 to 12 months). In addition, none of these studies were adjusted for the impact of clinical factors (e.g. BPAR and DGF) on eGFR.

1.5.1.3.3 Other gaps in PGx research on eGFR

- ***CYP3A4*1B*** **and** ***CYP3A4*22*** **(RPT)**

No published studies have investigated if *CYP3A4*1B* or *CYP3A4*22* (RPT) affect eGFR. Notably, *CYP3A4* genetics (DNR) has not been investigated for its impact on eGFR, probably because CYP3A4 is not expressed in kidney cells.

- ***ABCB1*** **1236C>T, 2677G>T (DNR) and 61A>G and 1199G>A (RPT & DNR)**

Although donor 1236C>T and 2677G>T were not associated with eGFR, the number of studies and the sample sizes were limited [51, 120, 122]. The 1199G allele was

associated with decreased TAC accumulation in HEK293 and K562 recombinant cell lines [125]. However, neither 61A>G nor 1199G>A (RPT & DNR), nor the haplotypes consisting of the 5 *ABCB1* SNPs (RPT & DNR), have been investigated for their impact on eGFR.

- ***POR*28*** **(RPT & DNR)**

Only recipient but not donor *POR*28* has been investigated for its impact on eGFR, although no significant results were reported [110, 117]. However, if *CYP3A5*3* (DNR) predicted eGFR, *POR*28* (DNR) would be worthwhile investigating as it is essential for CYP3A (DNR) activity.

- ***NR1I2*** **genetics (RPT & DNR)**

Whilst PXR regulates CYP3A and P-gp expression [118], no published study has investigated its genetic impact (RPT & DNR) on eGFR.

1.5.1.3.4 Future directions in PGx research on eGFR

Future studies (European cohort) should take *ABCB1* 61A>G and 1199G>A (RPT & DNR), along with 1236C>T, 2677G>T and 3435C>T, and their haplotypes into account for eGFR as these 5 SNPs have not been investigated together in kidney transplant recipients. Moreover, *POR*28* (DNR) and *NR1I2* genetics (RPT & DNR) are worthwhile investigating in the future for their impact on eGFR. Importantly, confounding factors (e.g. BPAR and DGF) should be adjusted for when exploring the relationship between the genetic factors and eGFR.

Table 2. Conflicting PGx findings of *CYP3A4*22*, *POR*28* and *NR1I2* genetics on TAC C_0/D in kidney transplant recipients

Genes	SNPs	Ref	N	Eth	Comedication	Time (months)	Differences of TAC C_0/D between genetic groups
CYP3A4	15389C>T **22*	[25]	223	EUR	NR	0-16.5±29.4	**22* carriers *vs* non-carriers (adjusted P-value threshold = 0.01) (1) German cohort (n = 10 *vs* 126): **1.5-fold** ↑ TAC C_0/D (**P = 0.02**); (2) Denmark cohort (n = 8 *vs* 79): **P > 0.2**; (3) Combined cohort (n = 18 *vs* 205): **1.5-fold** ↑ TAC C_0/D (**P = 0.01**)
		[50]	49	EUR	MMF or AZA & Steroid[1]	0-38.6±44.0	(1) **22* carriers *vs* non-carriers (n = 6 *vs* 43): **2-fold** ↑ TAC C_0/D (lowest **P = 0.02**); (2) CYP3A PM *vs* IM & EM (n = 6 *vs* 43): **1.6- to 4-fold** ↑ TAC C_0/D (**P < 0.001**)
		[52]	272	NR	MMF & Steroid[1]	0-12	*(1) *22* carriers *vs* non-carriers (n = 20-24 *vs* 200-237): at day 5-7 to 6 months post-transplantation: **1.6- to 2-fold** ↑ TAC C_0/D (lowest **P = 0.006**); at 12 months 12 post-transplantation: **P = 0.2**; (2) CYP3A PM *vs* EM (n = 23 *vs* 41) and PM *vs* IM (n = 23 *vs* 198): **1.9- to 3.3-fold** and **1.1- to 1.6-fold** ↑ TAC C_0/D, respectively (**P < 0.0001**)
		[109]	1407	Mixed[1]	MMF & Steroid[2]	0-6	**P = 0.3**
		[126]	241	EUR	MMF & Steroid[1]	0-12	**22* carriers *vs* non-carriers (n = 13 *vs* 218): (1)**1.3-fold** ↑ TAC C_0/D at month 3 post-transplantation (**P = 0.02**); (2) lowest **P = 0.1** at day 7, month 1, 6 or 12 post-transplantation

POR	**28*	[25]	223	EUR	NR	0-16.5±29.4	**1/*1 vs *1/*28 vs *28/*28*: (1) German cohort (total n = 136): **P = 0.1**; (2) Denmark cohort (total n = 87): **P = 0.7**
		[52]	272	NR	MMF & Steroid[1]	0-12	lowest **P = 0.2**
		[109]	1429	Mixed[1]	MMF & Steroid[2]	0-6	(1) *POR*28* carriers *vs* non-carriers when adjusting for: *CYP3A5*1*: **P > 0.05**; *CYP3A5*1* & clinical factors*: **5%** ↓ geometric mean TAC C_0/D (**P = 0.04**); (2) *POR*28* carriers *vs* non-carriers in: CYP3A5 non-expressors: **5.6%** ↓ TAC C_0/D when also adjusting for clinical factors* (**P = 0.03**); CYP3A5 expressors: **P = 0.7**
		[110]	184	Mixed[2]	MMF & Steroid[3]	0-12	(1) **1/*1 vs *1/*28 vs *28/*28*: lowest **P = 0.2**; (2) **28* carriers *vs* non-carriers in: CYP3A5 expressors: lowest **P = 0.07**; CYP3A5 non-expressors: lowest **P = 0.6** (3) **28/*28 vs *1* carriers in CYP3A5 non-expressors: **24.1%** ↓ TAC C_0/D (**P = 0.02**); (4) *CYP3A5*3/*3-POR*1* carriers had **1.3- to 1.8-fold** ↑ TAC C_0/D than *CYP3A5*3/*3-POR*28/*28*, *CYP3A5*1* carriers-*POR*1/*1* and *CYP3A5*1* carriers-*POR*28/*28* (lowest **P = 0.04**)
		[127]	229	Mixed[3]	MMF & Steroid[3]	0-3	**28* carriers *vs* non-carriers in: (1) the whole cohort: **P = 0.2**; (2) CYP3A5 expressors: **P = 0.1**
NR1I2	-25385C>T	[26]	159	EAS	MMF & Steroid[2]	day 7	T/T *vs* C allele carriers (P threshold = 0.007) in:

						(1) whole cohort: **P = 0.06**; (2) CYP3A5 expressors: **P = 0.8**; (3) CYP3A5 non-expressors: **P = 0.03**
	[48]	142	EAS	MMF & Steroid[1]	NR	**P = 0.6**
	[128]	240	EUR	MMF & Steroid[1]	0-6	(1) T allele carriers *vs* C/C (n = 42 *vs* 98): **~ 1.5-fold** ↑ TAC C_0/D (**P = 0.005**); (2) T allele carriers *vs* C/C in: CYP3A5 non-expressors (n = 39 *vs* 183): **~ 1.2-fold** ↑ TAC C_0/D (**P = 0.004**) CYP3A5 expressors (n = 3 *vs* 15): **P = 0.4**
8055C>T	[25]	223	EUR	NR	NR	T allele carriers *vs* non-carriers (after adjusting for *CYP3A5*3*): (1) German cohort (n = 42 *vs* 94): **30-50%** ↓ TAC C_0/D (**P = 0.01**); (2) Danish cohort (n = 30 *vs* 57): **P = 0.3**; (3) combined cohort (n = 72 *vs* 151): **P = 0.2**
	[128]	240	EUR	MMF & Steroid[1]	0-6	T allele carriers *vs* C/C in: (1) whole cohort: **P = 0.4**; (2) CYP3A5 expressors: **P = 1**; (3) CYP3A5 non-expressors: **P = 0.3**

↑: increase; ↓: decrease; AZA: azathioprine; clinical factors*: time post-transplantation, age, comorbidity and comedication; CYP3A EM: *CYP3A5*1-CYP3A4*1/*1* carriers; CYP3A IM: *CYP3A5*3/*3-CYP3A4*1/*1* carriers; CYP3A PM: *CYP3A5*3/*3-CYP3A4*22* carriers; CYP3A5 expressors: *CYP3A5*1* allele carriers; CYP3A5 non-expressors: *CYP3A5*3* homozygotes; EAS: east Asian; Eth: ethnicity; EUR: European; Mixed[1]: African-American, Asian, European, Hawaiian/Pacific islander, Native American/Aleutian Islander and other ethnicities;

Mixed[2]: African, Asian and European and other ethnicities; Mixed[3]: European and other ethnicities; MMF: mycophenolate mofetil; N or n: number of patients; NR: not reported; P: point-wise P-value; PGx: pharmacogenetic; Ref: reference(s); SNPs: single nucleotide polymorphisms; Steroid[1]: methylprednisolone and prednisolone; Steroid[2]: prednisolone; Steroid[3]: corticosteroid but details not given; TAC C_0/D: dose corrected tacrolimus trough whole blood concentration.

Table 3. Conflicting PGx findings of donor *CYP3A5*3* on BPAR incidence

SNPs	Ref	N	Eth	Immunosuppressants	Time (month)	Differences of BPAR incidence between genetic groups
CYP3A5*3 (6986A>G) **&** ***CYP3A4*1B*** (392A>G)	[115]	137 (RPT) + 137 (DNR)	EUR	TAC or CsA, MMF & Steroid[1]	0-12	RPT: lowest **P = 0.7**; DNR (adjusted P-value threshold = 0.017): (1) CYP3A5 expressors *vs* CYP3A5 non-expressors (n = 21 *vs* 116): ↑ BPAR incidence (**OR = 3.4**; **P = 0.04**) (2) **1B* carriers *vs* non-carriers (n = 13 *vs* 124): ↑ BPAR incidence (**OR = 6.3**; **P = 0.008**); (3) *CYP3A4*1B-CYP3A5*1* homozygotes *vs* non-carriers (n = 13 *vs* 116): ↑ BPAR incidence (**OR = 6.2**; **P = 0.007**)
CYP3A5*3 (6986A>G)	[116]	203 (RPT) + 201 (DNR)	EUR	TAC, MMF & Steroid[2]	0-21.8±9	RPT & DNR: **P > 0.05**

↑: increase; BPAR: biopsy-proven acute rejection; CsA: ciclosporin A; CYP3A5 expressors: *CYP3A5*1* allele carriers; CYP3A5 non-expressors: *CYP3A5*3/*3* carriers; DNR: donor; Eth: ethnicity; EUR: European; MMF: mycophenolate mofetil; N or n: number of patients; OR: Odds ratio; P: point-wise P-value; PGx: pharmacogenetic; RPT: recipient; Ref: reference(s); Steroid[1]: methylprednisolone and prednisolone; Steroid[2]: corticosteroid but details not reported; SNPs: single nucleotide polymorphisms; TAC: tacrolimus.

Table 4. Conflicting PGx findings of *CYP3A5*3* and *ABCB1* 3435C>T (R & D) on eGFR

Genes	SNPs	Ref	N	Eth	Immunosuppressants	Time (month)	Differences of eGFR between genetic groups
CYP3A5	**3* (6986A>G)	[116]	203 (RPT) + 201 (DNR)	EUR	TAC, MMF & Steroid[1]	0-21.8±9	RPT & DNR: **P > 0.05**
		[121]	90 (RPT) + 65 (DNR)	EUR	TAC, MMF & Steroid[3]	0-12	RPT: lowest **P = 0.1**; D: lowest **P = 0.3**
		[122]	120 (RPT) + 120 (DNR)	EAS	TAC, MMF & Steroid[2]	0-10	RPT & DNR: **P > 0.05**
		[124]	237 (RPT) + 232 (DNR)	Mixed	TAC, MMF & Steroid[3]	0-3	RPT: **P > 0.05**; D: lowest **P = 0.2**
		[129]	50(RPT)+ 50 (DNR)	EAS	TAC, MMF & Steroid[3]	12 & 36	RPT: lowest **P = 0.5** and **0.06** on month 12 and 36 post-transplantation, respectively; DNR: CYP3A5 non expressors *vs* expressors (n = 29 *vs* 21) (1) **18%** ↓ eGFR at month 12 post-transplantation (**P = 0.005**) (2) **P = 0.3** at month 36 post-transplantation
ABCB1	3435C>T	[116]	202 (RPT) + 195 (DNR)	EUR	TAC, MMF & Steroid[1]	0-21.8±9	RPT & DNR: **P > 0.05**
		[121]	90 (RPT) + 65 (DNR)	EUR	TAC, MMF & Steroid[3]	0-12	RPT: lowest **P > 0.2**; DNR: T allele carriers *vs* C/C (n = 67 *vs* 23): (1) **P = 0.35** in the first 2 weeks post-transplantation (2) **4-26%** ↓ eGFR at month 1, 3, 6 and 12 post-transplantation (lowest **P = 0.01**)
		[122]	120 (RPT) + 120 (DNR)	EAS	TAC, MMF & Steroid[2]	0-6	RPT: **P > 0.05**; DNR: T allele carriers *vs* non-carriers (n = 69-79 *vs* 37-41):

				(1) 10-26% ↑ eGFR on day1, 2, 3, 7, 14, 21 and at 1 month post-transplantation (lowest **P = 0.04**) (2) lowest **P = 0.06** at month 2, 3, 4, 5 and 6 post-transplantation

↑: increase; ↓: decrease; CYP3A5 expressors: *CYP3A5*1* allele carriers; CYP3A5 non-expressors: *CYP3A5*3/*3*; DNR: donor; eGFR: estimated glomerular filtration rate; EAS: east Asian; Eth: ethnicity; EUR: European; Mixed: African, Asian and European and other ethnicities; MMF: mycophenolate mofetil; N or n: number of patients; P: point-wise P-value; PGx: pharmacogenetic; RPT: recipient; Ref: reference(s); SNPs: single nucleotide polymorphisms; Steroid[1]: corticosteroid without details; Steroid[2]: methylprednisolone and prednisolone; Steroid[3]: prednisolone; TAC: tacrolimus.

1.5.2 Innate immunogenetics and acute kidney rejection

1.5.2.1 Adaptive and innate immune system in acute kidney rejection

The T-cell driven adaptive immune system plays a major role in acute kidney rejection, with a fast immune-response of antigen presentation and recognition, T-cell proliferation, differentiation, migration and infiltration into the allograft, finally causing kidney tissue cell apoptosis [21]. However, an acute rejection event cannot happen without the involvement of the innate immune system, which can assist T-cell proliferation and differentiation, and intensifies the severity of kidney tissue injury [21-23].

In the innate immune system, pattern recognition receptors (PRRs) can not only recognise pathogen-associated molecules [130] but also self-molecules from the apoptotic or necrotic cells [131]. Damage/danger-associated molecular patterns (DAMPs) are the biomolecules from the damaged tissue cells or chemical stress from ischaemia-reperfusion injury. Toll-like receptors (TLRs) belong to PRRs and they can recognise DAMPs [132]. Extracellular DAMPs bind to membrane-bound TLR2 and TLR4, with recruitment of intracellular myeloid differentiation primary response 88 (MyD88), leading to subsequent signal transduction by Toll/IL-1 receptor domain (TIR) pathway [133]. Consequently, transcription factors, e.g. nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), are translocated into the nucleus to activate the transcription and subsequent release of pro-inflammatory mediators (see Figure 4) [134]. These pro-inflammatory mediators, including ILs and tumour necrosis factor-α (TNF-α), can drive T-cell differentiation, proliferation and activation [20] and intensify inflammation in the transplanted kidney [21-23]. In contrast, the anti-inflammatory effect from IL-10 and transforming growth factor-β (TGF-β) can decrease the release of pro-inflammatory cytokines [135], with the potential to attenuate rejection risk.

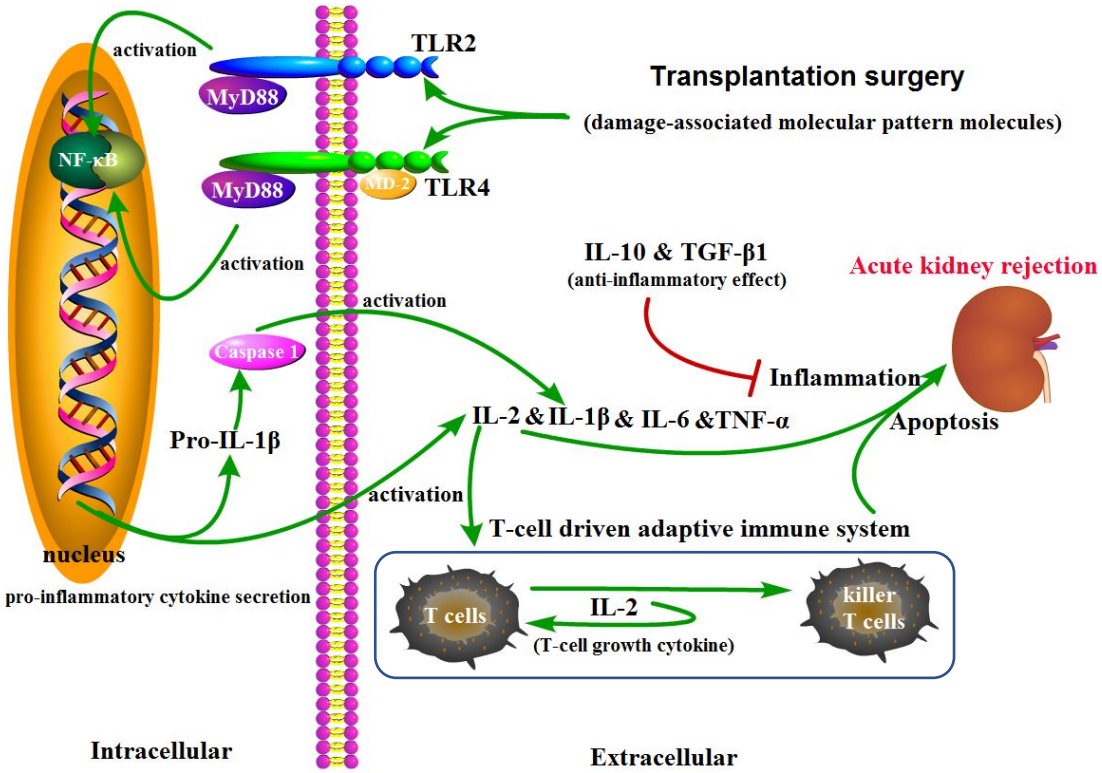

Figure 4. Joint contribution of the innate and adaptive immune system to acute kidney rejection. Damage-associated molecular patterns (DAMPs) caused by transplantation surgery can cause nuclear translocation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) via myeloid differentiation primary response 88 (MyD88)-dependent toll-like receptor (TLR) signalling pathway. MD-2 (encoded by *LY96*) is required as a co-factor binding with TLR4 for NF-κB nuclear translocation. Translocated NF-κB can activate the secretion of pro-inflammatory ILs and TNF-α. Caspase 1 coverts pro-IL-1β into mature IL-1β. These pro-inflammatory mediators assist T-cell proliferation and differentiation and intensify the severity of kidney tissue injury. In contrast, anti-inflammatory effect from IL-10 and TGF-β1 can decrease the pro-inflammatory cytokine release, with the potential to attenuate rejection risk.

1.5.2.2 Innate immunogenetics and BPAR

Relevant key SNPs in pro- and anti-inflammatory mediators (*CASP1*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *TGFB*, *TNF* and *CRP*) and MyD88-dependent TLR signalling pathway (*MYD88*, *TLR2*, *TLR4* and *LY96*) are associated with increased or decreased cytokine secretion or immune activation as summarised in Table 5. Allele frequencies (African, East Asian and European) of these SNPs are collected from the Ensembl database (http://grch37.ensembl.org/Homo_sapiens/Info/Index (last accessed on 8th March 2019)).

Accepted innate immunogenetic findings on BPAR incidence are supported by the cited meta-analyses or consistent individual studies while conflicting findings are shown in Table 6, whereas other research gaps are concisely summarised in section 1.5.2.2.3.

Table 5. Allele frequency and functional consequences or associations with immunological response/risk of key SNPs in pro- and anti-inflammatory mediators and MyD88-dependent TLR signalling pathway

Genes	SNPs	Allele Frequency (%)			Functional consequences or associations with immunological response/risk	Ref
		AFR	EAS	EUR		
CASP1	rs580253 (5352G>A)	9	1	18	**↓ IL-1β protein production *in vitro***	[136]
	rs554344 (10643G>C)	9	1	18	5352A and 10643C ↓ IL-1β protein production by **2-3%** (log-transformed) in LPS-treated whole blood samples from elderly population with (increased risk of) vascular diseases (**P = 0.008** and **0.009**, respectively)	
CRP	rs2794521 (-717T>C)	13	20	29	**↓ *CRP* transcriptional activity in reporter gene assay**	[137]
					T allele *vs* C allele: **2- to 3-fold** ↑ luciferace acitivy in HepG2 cells (**P < 0.005**)	
IL1B	rs16944 (-511C>T)	57	47	35	**↑ IL-1β protein production *in vitro***	[138]
	rs1143627 (-31T>C)	63	48	35	-511T and -31C ↑ IL-1β protein production by **2- to 3-fold** in LPS-treated whole blood samples from healthy volunteers and rheumatoid arthritis patients (**P = 0.008** and **0.002**, respectively)	
	rs1143634 (3954C>T)	12	2	25	**↓ Serum IL-1β concentration** in infliximab-treated IBD patients	[139]
					T allele carriers *vs* C/C: **97%** ↓ serum IL-1β concentration (**P = 0.03**)	

IL2	rs2069762 (-330T>G)	3	32	29	↑ **IL-2 protein production *in vitro*** G/G *vs* T allele carriers: over **3-fold** ↑ IL-2 protein production in anti-CD3/CD28-stimulated PBL from healthy volunteers (**P < 0.001**)	[140]
IL6	rs10499563 (-6331T>C)	23	16	23	↓ **Plasma/serum IL-6 concentration** in an acute inflammatory state *in vivo* (1) C/C *vs* G/G: **42%** ↓ plasma IL-6 concentration at 6 h post-CABG surgery (**P = 0.02**); (2) C/C *vs* G/G: **74-84%** ↓ serum IL-6 concentration at 24 h and 1 week after IPT (**P < 0.0001** and **P = 0.02**, respectively)	[141]
	rs1800795 (-174G>C)	2	0	42	↓ **plasma IL-6 concentration** in healthy volunteers G/G *vs* C/C: **2-fold** ↑ plasma IL-6 concentration (**P = 0.02**)	[142]
IL6R	rs2228145* (48892A>C; Asp358Ala)	9	32	36	↑ **Serum sIL-6R concentration** in healthy volunteers C allele carriers *vs* A/A: **25-67%** ↑ serum sIL6-R concentration (**P < 0.0001**)	[143]
IL10	rs1800896 (-1082G>A)	69	99	55	↓ **IL-10 protein production *in vitro*** A allele carriers *vs* G/G: **25%** ↓ IL-10 protein production in ConA stimulated PBL from healthy volunteers	[144]
	rs1800871 (-819C>T)	44	68	24	↔ **IL-10 protein production *in vitro***	[144]
	rs1800872 (-592C>A)	44	68	24	both in LD with -1082G>A	

TGFB	rs1800469 (-509C>T)	22	55	31	↑ **plasma TGF-β1 concentration** in healthy female volunteers (1) C/T *vs* C/C: **1.3-fold** ↑ plasma TGF-β1 concentration (**P = 0.04**) (2) T/T *vs* C/C: **2-fold** ↑ plasma TGF-β1 concentration (**P = 0.002**)	[145]
	rs1800470 (869T>C; Leu10Pro)	41	55	38	↑ **TGF-β1 protein production *in vitro*** C allele *vs* T allele: **2.4-fold** ↑ (mean; P not reported) TGF-β1 protein production in CMV-promoter transfected HeLa cells	[146]
	rs1800471 (915G>C; Arg25Pro)	94	100	93	↓ **TGF-β1 protein production *in vitro*** G/C *vs* G/G: **33%** ↓ TGF-β1 protein production in PHA and PMA-stimulated PBL from healthy volunteers (**P < 0.02**)	[147]
TNF	rs1800629 (-308G>A)	12	6	13	↑ ***TNF* transcriptional activity in reporter gene assay** A allele *v*s G allele: **1.7-fold** (**P < 0.05**) and **2.1-fold** (**P < 0.01**) ↑ luciferace acitivy in PMA-stimulated Jurkat and U937 cells, respectively	[148]
MYD88	rs6853 (1593A>G)	29	2	13	↓ **vaccine response *in vivo*** G/G *vs* A allele carriers: **~80%** ↓ dose-related measles-specific antibody response (**P = 0.001**)	[149]
TLR2	rs3804100 (1350T>C)	5	24	6	↓ **vaccine response and** ↑ **susceptibility to infection *in vivo*** (1) T/C *vs* T/T: **26%** ↓ dose-related measles-specific antibody response (**P = 0.002**) (2) frequency of C/C genotype *vs* T/T genotype or T allele carriers in paediatric patients infected with congenital CMV: **OR = 11.7** (**P = 0.02**) and **9.5** (**P = 0.01**), respectively	[150, 151]

TLR4	rs4986790 (896A>G; Asp299Gly)	7	0	6	↑ **vaccine response and inflammation *in vivo*** (1) A/G *vs* A/A: **2-fold** ↑ dose-related measles-specific IL-4 response (**P = 0.01**) (2) A/G *vs* A/A: **1.4-fold** ↑ lymphocyte count in ESRD patients (**P = 0.01**)	[149, 152]
	rs4986791 (1196C>T; Pro399Leu)	1	0	6	↑ **vaccine response *in vivo*** C/T *vs* C/C: **2-fold** ↑ dose-related measles-specific IL-4 response (**P = 0.009**)	[149]
LY96	rs11466004 (379C>T; Ser157Pro)	0	0	2	↓ **vaccine response *in vivo*** C/T *vs* C/C: **~87%** ↓ dose-related measles-specific IL-10 response (**P = 0.03**)	[149]

↑: increase; ↓: decrease; ↔: unchanged; AFR: African; Ala: alanine; Arg: arginine; Asp: aspartic acid; CABG: coronary artery bypass grafting; ConA: concanavalin A; CMV: cytomegalovirus; EAS: east Asian; ESRD: end-stage renal disease; EUR: European; Gly: glycine; hour: h; IBD: inflammatory bowel diseases; IPT: intensive periodontal therapy; Leu: leucine; LD: linkage disequilibrium; LPS: lipopolysaccharides; OR: Odds ratio; PBL: peripheral blood lymphocyte; PHA: phytohemagglutinin; PMA: phorbol myristate acetate; Pro: proline; rs2228145*: previously known as rs8192284; Ref: reference(s); Ser: serine; sIL-6R: soluble IL-6R; SNPs: single nucleotide polymorphisms; TLR: Toll-like receptor.

1.5.2.2.1 Accepted innate immunogenetic findings on BPAR incidence

- ***IL2*, *IL6*, *IL10*, *TGFB* and *TNF* genetics (RPT or RPT & DNR) — no impact on BPAR incidence**

Recent meta-analyses reported *IL2* -330T>G (RPT) [153], *IL6* -174G>C (RPT & DNR) [154], *IL10* -1082G>A, -819C>T and -592C>A (RPT) [153, 154], *TGFB* 869T>C and 915G>C (RPT & DNR) [157, 158] and *TNF* -308G>A (RPT & DNR) [159] did not affect BPAR incidence in (European) kidney transplant recipients.

- ***IL1B* -511C>T (RPT) — no impact on BPAR incidence**

IL1B -511C>T (RPT) [160-164] did not affect BPAR incidence in kidney transplant recipients.

1.5.2.2.2 Conflicting innate immunogenetic findings on BPAR incidence

- ***TLR4* 869A>G & 1196C>T (RPT & DNR)**

Inconsistent results exist on whether *TLR4* genetics affect BPAR incidence (see Table 6). Notably, not all studies adjusted for confounding factors (e.g. HLA mismatches) when associating *TLR4* genetics with BPAR incidence. In addition, different immunosuppressive protocols, limited sample size (n = 122 - 238) and low 896G and 1196T frequencies (see Table 5) may contribute together to the conflicting impact of *TLR4* genetics on BPAR incidence.

1.5.2.2.3 Other gaps in innate immunogenetic research on BPAR incidence

- **Other *IL1B* and *IL6* genotypes/diplotypes (RPT)**

In a study of 200 kidney transplant recipients and their donors, recipient but not donor *IL1B* 3954C/T carriers had higher BPAR incidence than C/C carriers (OR = 3.1, P =

0.045), however, there was no significant difference between C/C and T/T carriers (P = 0.4) [162]. No other published studies investigated the relationship between 3954C>T and BPAR incidence in kidney transplant recipients. In addition, no published studies yet have explored if *IL1B* -31T>C or *IL1B* diplotypes (-511C>T and -31T>C) affect BPAR incidence.

Although *IL6* -6331T>C decreases plasma/serum IL-6 concentration in an acute inflammatory state *in vivo* (see Table 5), it has not been studied for the impact on BPAR incidence.

- ***TGFB* -509C>T (RPT & DNR)**

Two studies were carried out respectively for an association between recipient (n = 209) or donor (n = 145) *TGFB* -509C>T and BPAR incidence [160, 161], although the relationship was non-significant (both P = 0.9). No other studies have investigated the relationship between -509C>T (R & D) and BPAR incidence.

- ***CASP1*, *CRP*, *IL6R*, *LY96*, *MYD88* and *TLR2* genetics (RPT & DNR)**

The associations between *CASP1*, *CRP*, *IL6R*, *LY96*, *MYD88* and *TLR2* SNPs and immunological response/risk are summarised in Table 5, however, no study has explored if these SNPs affect BPAR incidence.

1.5.2.2.4 Future directions in innate immunogenetic research on BPAR incidence

Currently, no innate immunogenetic studies have been carried out in TAC-treated kidney transplant recipients only (previous studies were either in a CsA-treated cohort or a mixed cohort of CsA/TAC). Therefore, it is still worthwhile investigating if all these immunogenetic factors mentioned above could affect BPAR incidence in a TAC-treated kidney transplant cohort.

Table 6. Conflicting innate immunogenetic findings of *TLR4* genetics (RPT & DNR) on BPAR incidence

SNPs	Ref	N	Eth	Immunosuppressants	Time (month)	Differences of BPAR incidence between genetic groups
896A>G (Asp299Gly) & 1196C>T (Pro399Leu)	[165]	238 (RPT)	EUR	(1) CsA, AZA & Steroid[1] or (2) CsA, MMF & Steroid[1] or (3) TAC, MMF & Steroid[1] or (4) MMF & Steroid[1]	95±29	896A/A-1196C/C *vs* other (n = 211 *vs* 27): (1) 72% ↓ BPAR incidence (**P = 0.02**) (2) **RR = 0.4**; **P = 0.01** (Cox regression analysis)
	[166]	122 (RPT) + 122 (DNR)	EUR	CsA or TAC, MMF & Steroid[2]	36±15	896A/A-1196C/C *vs* other (RPT: n = 104 *vs* 18; DNR: n = 102 *vs* 20) RPT: 20% *vs* 22% (**P = 0.8**); DNR: 16% *vs* 0% (**P = 0.04**)
	[167]	200 (RPT) + 186 (DNR)	BRAZILIAN	NR	108±85	896A/A-1196C/C *vs* other (RPT: n = 183 *vs* 17; DNR: n = 167 *vs* 19) RPT: 39.5% *vs* 17.6% (**P = 0.4**); DNR: **P > 0.05**

↓: decrease; Asp: aspartic acid; AZA: azathioprine; BPAR: biopsy-proven acute rejection; CsA: ciclosporin A; DNR: donor; Eth: ethnicity; EUR: European; Gly: glycine; Leu: leucine; MMF: mycophenolate mofetil; N or n: number of patients; NR: not reported; P: point-wise P-value; Pro: proline; RPT: recipient; Ref: reference(s); RR: relative risk; SNPs: single nucleotide polymorphisms; Steroid[1]: prednisolone; Steroid[2]: methylprednisolone and prednisolone; TAC: tacrolimus.

1.6 Research gaps and aims

There are 4 main gaps in the current genetic research on the inter-individual variabilities in TAC C_0/D, BPAR incidence and eGFR:

1) if TAC dispositional genetics (other than *CYP3A5*3* (RPT)) determine TAC C_0/D inter-individual variability in kidney transplant recipients;
2) if TAC dispositional genetics account for BPAR incidence and eGFR in kidney transplant recipients;
3) if a temporal response relationship exists between TAC C_0 and BPAR under TAC TDM;
4) if innate immunogenetics affect BPAR incidence in kidney transplant recipients.

To bridge these research gaps, I aimed to assess:

1) **the impact of recipient *CYP3A5*3*, *CYP3A4*22*, *POR*28*, *ABCB1* 61A>G, 1199G>A, 1236C>T, 2677G>T and 3435C>T, and *NR1I2* 8055C>T and 63396C>T genotypes/haplotypes on TAC C_0/D in 165 Australian kidney transplant recipients in the first 3 months post-transplantation.** This included confirmation of the major impact of *CYP3A5*3* on TAC C_0/D and, for the first time in kidney transplant recipients, the investigation of the 5 most common European *ABCB1* SNPs together with their haplotypes for their impact on TAC C_0/D — addressed in Chapter 2.
2) **the impact of recipient (n = 165) and donor (n = 129) *CYP3A5*3*, *CYP3A4*22*, *ABCB1* 61A>G, 1199G>A, 1236C>T, 2677G>T and 3435C>T, *POR*28* and *NR1I2* 8055C>T and 63396C>T genotypes/haplotypes on BPAR incidence in the first 14 days, and eGFR in the first 3 months, post-**

transplantation. This included investigating, for the first time, the impact of *NR1I2* genetics (RPT & DNR) and the 5 most common European *ABCB1* SNPs together with their haplotypes on kidney transplant outcomes in organ transplantation — addressed in Chapter 3.

3) **the temporal relationship between TAC C_0 and BPAR incidence (n = 38) in the first 14 days post-transplantation.** This included investigating the TAC C_0 and haematocrit immediately preceding BPAR and on the day of BPAR for the first time in a TAC concentration-response relationship study. Also, haematocrit has been included for the first time in TAC C_0-rejection relationship — addressed in Chapter 4.
4) **the genetics of pro- and anti-inflammatory mediators, and MyD88-dependent TLR signalling pathway, and their impact on BPAR incidence in 165 Australian kidney transplant recipients in the first 14 days post-transplantation.** Nineteen immunogenetic SNPs (RPT & DNR) were included: *CASP1* 5352G>A and 10643G>C, *CRP* -717T>C, *IL1B* -511C>T, -31T>C and 3954C>T, *IL2*-330T>G, *IL6* -6331T>C, *IL6R* 48892A>C, *IL10*-1082G>A and -819C>T, *LY96* 379C>T, *MYD88* 1593A>G, *TGF* -1287G>A and -509C>T; *TLR2* 1350T>C, *TLR4* 896A>G and 1196C>T and *TNF* -308G>A. It is the first time that *CASP1*, *CRP*, *IL6R*, *TLR2*, *LY96* and *MYD88* genotypes/diplotypes have been studied for their impact on BPAR incidence in kidney transplant recipients receiving TAC as the only calcineurin inhibitor — addressed in Chapter 5.

Notably, to exclude the potential impact from clinical and non-clinical factors (e.g. HLA mismatch) on TAC C_0/D, acute rejection and kidney function (see factors

summarized in sections 1.2.2 and 1.4), the following factors went into the study design:

1) age, sex and haematocrit were adjusted for PGx impact on TAC C_0/D in the linear mixed effects regression analysis in Chapter 2.
2) HLA mismatches, induction therapy, kidney transplant number, peak PRA scores and living donor were adjusted for PGx impact on BPAR incidence in the generalised linear mixed effects regression analysis in Chapter 3.
3) BPAR incidence, DGF and living donor were adjusted for PGx impact on kidney function in the linear mixed effects regression analysis in Chapter 3.
4) HLA mismatches, induction therapy, kidney transplant number, peak PRA scores and living donor were adjusted for innate immunogenetic impact on BPAR incidence in the generalised linear mixed effects regression analysis in Chapter 5.

Chapter 2: Relationship between tacrolimus dispositional genetics and dose-adjusted trough whole blood tacrolimus concentration

Statement of Authorship			
Title of Paper	*CYP3A5*3* and *ABCB1* 61A>G significantly influence dose-adjusted trough blood tacrolimus concentrations in the first three months post kidney transplantation.		
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style		
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Name of Principal Author (Candidate)	Rong Hu		
Contribution to the Paper	Study design, isolated, quantified and genotyped DNA samples, collected the clinical data, performed statistical analysis, interpreted the results, wrote the first manuscript draft and acted as the corresponding author		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14th May 2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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*CYP3A5*3* and *ABCB1* 61A>G Significantly Influence Dose-adjusted Trough Blood Tacrolimus Concentrations in the First Three Months Post-Kidney Transplantation

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Abstract: Tacrolimus (TAC) is a first-line immunosuppressant used to prevent organ rejection after kidney transplantation. There is large inter-individual variability in its pharmacokinetics. Single nucleotide polymorphisms (SNPs) in genes encoding TAC metabolizing enzymes cytochromes P450 3A4/5 (*CYP3A4/5*), P-glycoprotein efflux transporter (*ABCB1*), their expression regulator pregnane X receptor (*NR1I2*) and CYP3A co-factor cytochrome P450 reductase (*POR*) have been studied for their effects on tacrolimus disposition. However, except for *CYP3A5*3*, controversies remain about their roles in predicting dose-adjusted trough blood TAC concentrations (C_0/D). This study aimed to investigate the effects of *ABCB1* (61A>G, 1199G>A, 1236C>T, 2677G>T and 3435C>T), *CYP3A4*22*, *CYP3A5*3*, *NR1I2* (8055C>T, 63396C>T and -25385C>T) and *POR*28* SNPs on TAC C_0/D. In total, 165 kidney transplant recipients were included in this study. SNPs were genotyped by probe-based real-time polymerase chain reaction. Associations between log-transformed whole blood TAC C_0/D (measured at 1 and 3 months post-transplant) and genotypes/haplotypes were assessed by linear mixed effects analysis, controlling for age, sex and haematocrit. It was observed that *CYP3A5* expressors (**1/*1 + *1/*3*) ($p = 5.5 \times 10^{-16}$) and *ABCB1* 61G allele carriers ($p = 0.001$) had lower log-transformed TAC C_0/D (56% and 26% lower geometric mean TAC C_0/D, respectively) and accounted for approximately 30% and 4%, respectively, of log-transformed TAC C_0/D variability in the first 3 months post-transplant. In conclusion, *CYP3A5*3* is a major, and *ABCB1* 61A>G is a novel, although minor, genetic factor affecting TAC C_0/D in kidney transplant recipients.

End stage renal disease is a substantial health and economic burden worldwide. Compared with dialysis, kidney transplantation remains the most effective treatment for such patients, and post-surgery immunosuppressive therapy has increased the first-year graft survival rate to over 90% [1]. Tacrolimus (TAC) is one of the first-line immunosuppressants widely used to prevent organ rejection after kidney transplantation; however, it has a narrow therapeutic index [2] and large inter-individual dose and trough blood concentration (C_0) variability [3].

Tacrolimus undergoes extensive intestinal and hepatic metabolism whilst renal clearance accounts for less than 1% of total body clearance [4]. Its pharmacokinetics (PK) is mainly determined by its metabolizing enzymes cytochromes P450 (CYP) 3A5 (encoded by *CYP3A5*) and 3A4 (encoded by *CYP3A4*) [5,6], and the efflux transporter P-glycoprotein (P-gp, encoded by *ABCB1*) [7]. Cytochrome P450 reductase (encoded by *POR*) is essential for CYP3A activity [8] and the pregnane X receptor (encoded by *NR1I2*) regulates CYP3A4/5 and P-gp expression [9]; with the potential for these genes to affect TAC PK. However, only *CYP3A5*3* [the most common *CYP3A5* single nucleotide polymorphism (SNP)] significantly affects TAC PK across different studies [10–20]. The *CYP3A5*3* allele (6986A>G, rs776746) leads to non-functional CYP3A5 [21], and consequently, transplant recipients with *CYP3A5*3/*3* genotype (termed 'non-expressors') exhibit two times higher TAC C_0/D than *CYP3A5 *1/*1* and **1/*3* genotypes (collectively termed 'expressors') [11]. *ABCB1* 1236C>T (rs1128503), 2677G>T/A (rs2032582) and 3435C>T (rs1045642) SNPs have also been widely studied for their effects on TAC PK; however, most studies report no significant effect on TAC C_0/D [11–13,16,18,20]. Other relevant SNPs, that is *CYP3A4*22* (rs35599367), *NR1I2* -25385C>T (rs3814055) and *POR*28* (rs1057868), have been less frequently studied, with contradictory findings [14,17,18,20,22–24]; thus, their contributions to TAC PK variability are currently unclear.

We hypothesised that *CYP3A5*, *CYP3A4*, *ABCB1*, *POR* and *NR1I2* genotypes significantly affect TAC C_0/D. Therefore, we aimed to assess their effect on TAC C_0/D in the first 3 months post-kidney transplantation. This included investigating the five most common Caucasian *ABCB1* SNPs (61A>G (rs9282564), 1199G>A (rs2229109), 1236C>T, 2677G>T and 3435C>T) and their haplotypes together for the first time in kidney transplant recipients.

Materials and Methods

Study participants. This retrospective study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). All procedures performed were in accordance with the Declaration of Helsinki and institutional

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and/or national research committee ethical standards. Kidney transplant recipients, aged over 18 years taking TAC (Prograf®) as the only calcineurin inhibitor for immunosuppressive therapy (together with mycophenolic acid and prednisolone), were recruited for this study. Those with combined organ transplant or severe liver dysfunction were excluded. One hundred and sixty-five kidney transplant recipients were included. Informed consent was obtained from all participants. According to clinical practice guidelines, TAC was given twice daily and TAC daily dose (D) was adjusted to achieve whole blood target C_0 (8–15 ng/ml) using therapeutic drug monitoring.

Participant demographic and clinical data collection. Patient demographics and clinical data were obtained from clinical case notes, including pre-transplant measures of alanine aminotransferase, aspartate aminotransferase and haematocrit. Tacrolimus C_0 for therapeutic drug monitoring was quantified by LC-MS/MS [25]. Intra- and inter-assay imprecision and inaccuracy were <12% over the concentration range of 2.4–48.2 ng/ml. TAC D and haematocrit were collected at 1 and 3 months post-transplant.

Genetic analysis. Whole blood samples were collected, and genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen Pty Ltd, Melbourne, VIC, Australia) or Maxwell® 16 Blood DNA Purification kit (Promega Corporation, Sydney, NSW, Australia). DNA purity and concentrations were determined by absorbance using a Synergy Mx microplate reader (Biotek Instruments Inc., Winooski, VT, USA). *CYP3A4*22* and *CYP3A5*3* were genotyped by commercially available TaqMan® SNP genotyping assays (C_59013445_10 and C_26201809_30, respectively; Thermo Fisher Scientific Australia Pty Ltd, Adelaide, SA, Australia) and assay conditions were described previously [26]. Probe-based allelic discrimination assays were developed for *ABCB1* (61A>G, 1199G>A, 1236C>T, 2677G>T and 3435C>T), *NR1I2* (8055C>T, -25385C>T and 63396C>T) and *POR*28* genotyping. The A allele of 2677 G>T/A was not assessed due to its low frequency (~2%) in Caucasians [27]. All primers and probes are described in Table S1. Three different multiplex assays, *ABCB1* 61A>G and 3435C>T, *ABCB1* 1199G>A and 2677G>T, and *NR1I2* 8055C>T and -25385C>T, were established because their annealing temperatures were compatible (Table S1). Reactions for each single or multiplex assay contained 1× iTaq Universal Probes Supermix (Bio-Rad Laboratories Pty Ltd., Sydney, NSW, Australia), 150–225 nM primers (Integrated DNA Technologies Pty Ltd, Sydney, NSW, Australia), 150 nM probes (Sigma-Aldrich™, Sydney, NSW, Australia), 20 or 40 ng of DNA for single or multiplex assays, respectively, and nuclease-free water (Thermo Fisher Scientific Australia Pty Ltd, Adelaide, SA, Australia) to a 20 µl total reaction volume. Thermocycling conditions and fluorescence detection performed with a CFX96 real-time PCR system (BioRad) were described previously [26], with annealing temperatures as described in Table 1. Each assay run of samples included two no-template controls (nuclease-free water) and positive controls of each genotype previously confirmed by Sanger sequencing [26].

Statistical analysis. Statistical analyses were conducted in R version 3.3.1 [28] unless stated otherwise. Genotype Hardy–Weinberg equilibrium (HWE) was tested by chi-squared test or Fisher's exact test (package::function, genetics [29]::HWE.chisq and HWE.exact). Pairwise D' and r^2 were calculated to estimate linkage disequilibrium (LD) between loci within a gene (genetics [29]::LD). *ABCB1* haplotypes were inferred using PHASE 2.1 [30,31] as previously described [32]; individuals were excluded from further haplotype analysis if their haplotype pairs had low confidence predictions (predicted probability <0.8) due to missing genotype data or rare genotype combinations. Common haplotypes were defined based on a frequency of greater than 0.05. If there were fewer than five homozygotes for an allele or a haplotype, then they were combined with heterozygotes for statistical analysis as follows: *CYP3A5*3* grouped as **3/*3* or **1/*1 + *1/*3*; *ABCB1* 61A>G grouped as A/A or A/G + G/G (G allele carriers); *NR1I2* 8055C>T grouped as C/C or C/T + T/T (T allele carriers); and *ABCB1* 61-1199-1236-2677-3435 AGCGT and GGTTT haplotypes grouped as 0 copies or 1 + 2 copies (i.e. AGCGT carrier and GGTTT carrier, respectively). Normality of continuous variables (age, haematocrit and TAC C_0/D) was checked using histograms and quantile–quantile plots (graphics [28]::hist, stats [28]::qqnorm and qqline). Log transformation (base 10) of TAC C_0/D was required to normalize distribution prior to further analysis. All data are expressed as median and interquartile range or range.

Table 1.
Demographic, clinical and TAC C_0/D data for kidney transplant recipients.

Characteristic	Value
Number of recipients (n)	165
Age (years)	55 (18–73)[1]
Sex (n)	99 M/66 F
Ethnicity (n, %)	
Caucasian	138 (84)
Aboriginal Australian	5 (3)
Asian	4 (2)
Unknown	18 (11)
Living donors (n, %)	58 (35)
Number of kidney transplants (n, %)	
1	135 (82)
2	26 (16)
3	4 (2)
Primary kidney disease (n, %)	
Diabetic nephropathy	9 (5)
Glomerulonephritis	41 (25)
Mesangial proliferative nephropathy	27 (16)
Polycystic kidney disease	23 (14)
Reflux nephropathy	11 (7)
Others	25 (14)
Unknown	29 (18)
ALT (U/l)	19 (2–225)[1]
AST (U/l)	22 (7–360)[1]
Haematocrit	
Pre-transplant	0.35 (0.14–0.49)[1]
1-month	0.35 (0.24–0.47)[1]
3-month	0.38 (0.23–0.53)[1]
TAC C_0/D (ng/ml per mg/day)	
1-month (n = 147)	1.35 (0.35–6.78)[1]
3-month (n = 133)	1.45 (0.28–5.75)[1]

ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; M, male; TAC C_0/D, dose-adjusted whole blood trough TAC concentration.
[1]Values are median (range).

Linear mixed effects (LME) analysis (lme4 [33]::lmer) was used to identify potential genetic predictors of log-transformed TAC C_0/D. Haematocrit, age and sex have been reported to significantly impact TAC C_0 [34,35]; therefore, genotypes/haplotypes were treated as fixed effects adjusted by age, sex and haematocrit whilst patient and time (1 or 3 months) were treated as random effects on intercept. Diagnostic plot (stats [28]::resid) was applied to check the normality and homoscedasticity of model residuals. Using a forward selection procedure, all SNPs and haplotypes were tested individually controlling for age, sex, haematocrit and random effects. In each selection run, the genetic factor with the lowest *p*-value which also met the Bonferroni-

adjusted threshold ($\alpha = 0.05/N$) was retained, until no further genetic factors met this criterion. To aid with interpretation of LME findings, the total and relative contributions (model and partial R^2, respectively (relaimpo [36]::calc.relimp)) of significant genetic and relevant non-genetic factors to log-transformed TAC C_0/D variability were determined by multiple linear regression (stats [28]::lm) of 1 and 3 months post-transplant data separately. Figures were drawn by GraphPad Prism v7 (GraphPad Software, San Diego, CA, USA).

Results

All 165 kidney transplant recipients had at least one TAC C_0/D measurement at 1 or 3 months post-transplant. Table 1 summarizes recipients' demographic, clinical and TAC C_0/D data.

Genotype call rates for most SNPs were >98% except for *NR1I2* -25385C>T (<80%), thus -25385C>T data were excluded from further analysis. Consequently, 134 patients had data for all SNPs, whilst for 28 patients there were 1–5 missing SNPs and for three patients 6–9 missing SNPs because of limited amount of DNA. Recipient genotype, allele and haplotype frequencies are shown in table 2. Genotype frequencies did not deviate from HWE ($p > 0.1$) except for *CYP3A5*3* (point-wise $p = 0.02$), with an excess of *CYP3A5*1/*1* (table 2). To rule out errors in genotyping, all *CYP3A5*1/*1* genotypes were confirmed by Sanger sequencing [26]. Further, this deviation was not a result of mixed-ethnicity in the recipients, as recipient *CYP3A5*3* genotype frequencies were similar when considering only Caucasians (2%, 13% and 85% for *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3*, respectively).

NR1I2 8055C>T and *63396C>T* were not in significant LD ($p > 0.7$). *ABCB1* 1236C>T, 2677G>T and 3435C>T variant alleles were all in strong LD with each other (D' = 0.7–0.9, $r^2 > 0.4$, $p < 2 \times 10^{-16}$), and 61A>G and 1199G>A variant alleles were in strong LD with 1236C>T, 2677G>T and 3435C>T variant alleles (D' = 0.9–1.0, $r^2 > 0.07$, $p < 0.005$). However, there was no LD between *ABCB1* 61A>G and 1199G>A alleles (D' = 1, $r^2 = 0.004$, $p > 0.2$). Four common *ABCB1* haplotypes were predicted as follows: AGCGC (wild-type for all SNPs), AGCGT, AGTTT and GGTTT. Consequently, a multiple testing-adjusted *p*-value threshold for initial LME inclusion was determined at 0.004 ($\alpha = 0.05/14$ tests, 10 SNPs and four haplotypes).

Differences in TAC C_0/D between genotype/haplotype groups at 1 and 3 months post-transplant are summarized in Table S2. Residual normality and homoscedasticity confirmed by diagnostic plot. Forward selection LME analysis identified *CYP3A5*3* and *ABCB1* 61A>G genotype as the only significant genetic predictors of log-transformed TAC C_0/D, which was lower in *CYP3A5* expressors (mean difference [95% CI] = −0.36, [−0.45 to −0.28]; $p = 5.5 \times 10^{-16}$) and *ABCB1* 61A>G variant (G) carriers (−0.13, [−0.21 to −0.05]; $p = 0.001$), equivalent to 56% and 26% lower geometric mean TAC C_0/D, respectively. There was no interaction between *CYP3A5*3* and *ABCB1* 61A>G genotypes ($p = 0.54$). *CYP3A5*3* and *ABCB1* 61A>G genotype differences in unadjusted TAC C_0/D at 1 and 3 months post-transplant are shown in figs 1 and 2, respectively. When considered together, *CYP3A5*3*, *ABCB1* 61A>G, age, sex and haematocrit accounted for 32.0% and 38.3% of log-transformed TAC C_0/D variability at 1 and 3 months post-transplant, respectively. In terms of their relative contributions (partial R^2), *CYP3A5*3* explained 25.1% and 30.5%, *ABCB1* 61A>G 3.0% and 4.3%, and non-genetic variables (combined) 3.9% and 3.5%, of log-transformed TAC C_0/D variability in these 1 and 3 months post-transplant multiple regression models, respectively.

Table 2.
ABCB1, *CYP3A4/5*, *NR1I2* and *POR* genotype, allele and *ABCB1* haplotype data (n, %).

Gene	SNPs/ Haplotypes	Genotypes	Alleles/ Haplotypes	HWE P-value
ABCB1	61A>G	A/A (134, 82%) A/G (27, 17%) G/G (2, 1%)	A (295, 90%) G (31, 10%)	0.64
	1199G>A	G/G (148, 92%) G/A (12, 8%)	G (308, 96%) A (12, 4%)	1
	1236C>T	C/C (44, 27%) C/T (77, 47%) T/T (42, 26%)	C (165, 51%) T (161, 49%)	0.54
	2677G>T	G/G (47, 29%) G/T (78, 48%) T/T (36, 22%)	G (172, 53%) T (150, 47%)	0.76
	3435C>T	C/C (41, 25%) C/T (69, 43%) T/T (52, 32%)	C (151, 47%) T (173, 53%)	0.08
	AGCGC	0 copies (65, 41%) 1 copy (71, 45%) 2 copies (21, 13%)	AGCGC (113, 36%)	
	AGCGT	0 copies (127, 81%) 1 copy (29, 18%) 2 copies (1, 1%)	AGCGT (31, 10%)	
	AGTTT	0 copies (73, 46%) 1 copy (64, 41%) 2 copies (20, 13%)	AGTTT (104, 33%)	
	GGTTT	0 copies (132, 84%) 1 copy (23, 15%) 2 copies (2, 1%)	GGTTT (27, 9%)	
CYP3A4	**22*	**1/*1* (141, 93%) **1/*22* (10, 7%)	**1* (292, 97%) **22* (10, 3%)	1
CYP3A5	**3*	**1/*1* (4, 3%) **1/*3* (19, 12%) **3/*3* (129, 85%)	**1* (27, 9%) **3* (277, 91%)	0.02
NR1I2	8055C>T	C/C (102, 71%) C/T (39, 27%) T/T (3, 2%)	C (243, 84%) T (45, 16%)	1
	63396C>T	C/C (28, 18%) C/T (71, 46%) T/T (54, 35%)	C (127, 42%) T (179, 58%)	0.62
POR	**28*	**1/*1* (71, 50%) **1/*28* (62, 44%) **28/*28* (9, 6%)	**1* (204, 72%) **28* (80, 28%)	0.41

HWE, Hardy–Weinberg Equilibrium.

Discussion

Although the effect of *ABCB1* genetic variability on TAC PK has been extensively studied [11–13,15,16,18,20,37–39], to our knowledge, this is the first TAC pharmacogenetic study in solid organ transplantation patients to identify a significant

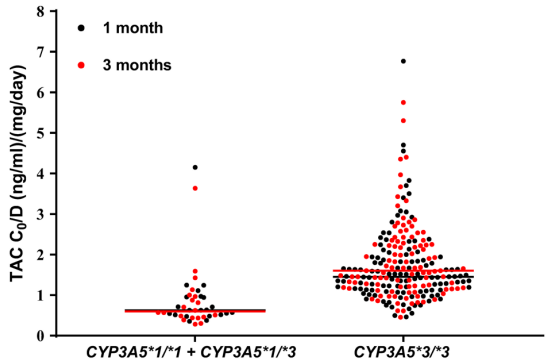

Fig. 1. Influence of *CYP3A5*1/*1 + CYP3A5*1/*3* and *CYP3A5*3/*3* genotypes on TAC C_0/D. Black and red dots and lines indicate individual recipient TAC C_0/D and medians at 1 and 3 months post-transplant, respectively.

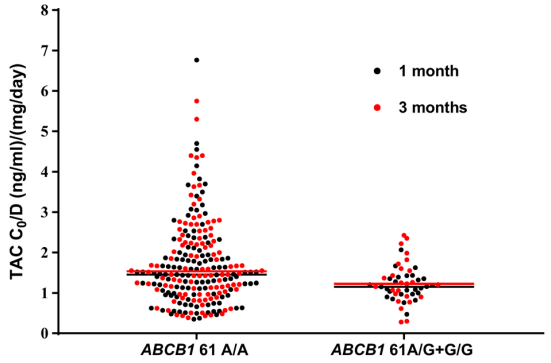

Fig. 2. Influence of *ABCB1* A61G A/A and A/G + G/G genotypes on TAC C_0/D. Black and red dots and lines indicate individual recipient TAC C_0/D and medians at 1 and 3 months post-transplant, respectively.

impact of *ABCB1* 61A>G on TAC C_0/D in the first 3 months post-transplant. A LME model was used to analyse genetic determinants of log-transformed TAC C_0/D, which allowed for assessment of repeated measurements, accounted for confounding non-genetic effects and adjusted for the expected major effect of *CYP3A5*3* genotype in identifying additional genetic factors, such as *ABCB1* 61A>G. However, since this study was conducted predominantly in Caucasians, the significance of 61A>G is unknown in other ethnicities.

ABCB1 61A>G is a non-synonymous SNP (Asn21>Asp) that does not lead to a significant functional change based on studies of P-gp-mediated transport of fluorescent substrates [40]. However, the mechanism underlying the association observed in this study is not entirely clear because the functional consequences of *ABCB1* polymorphisms appear to be substrate-dependent [41]. Whilst Jordan de Luna *et al.* [37] and Sánchez-Lázaro *et al.* [38] found no significant effect of 61A>G on TAC blood concentrations, both studies were in heart transplant recipients and had very small sample sizes (15 and 24 patients receiving TAC therapy, respectively). Very recently, Oetting *et al.* [20] validated 44 reported SNPs associated with TAC C_0/D in a cohort of 1560 European-American kidney transplant recipients and reported no significant effect of 61A>G on TAC C_0/D ($p = 0.97$). Considering the major effect of *CYP3A5*3* on TAC C_0/D, it is possible that Oetting *et al.* [20] may have underestimated the effect of 61A>G, as the *CYP3A5*3* genotype effect was not adjusted for in their LME modelling. In contrast, our results showed 61A>G significantly decreased TAC C_0/D in kidney transplant recipients, but only after adjusting for *CYP3A5*3* genotype and the effect was small relative to *CYP3A5**3 genotype, and will need replication.

Of the other *ABCB1* SNPs included in this study, 1199G>A decreases P-gp efflux and increases TAC intracellular accumulation *in vitro* [42,43]; however, similar to our observations, it was not associated with TAC C_0/D previously [12,20]. *ABCB1* 3435C>T reduces *ABCB1* mRNA stability and P-gp expression [44,45], and is in strong LD with 1236C>T and 2677G>T [18,39]. These three *ABCB1* SNPs, along with their haplotypes, are frequently studied in TAC PK; however, their impact remains uncertain. Although some associations have been reported [15,39], most studies, including ours, have found no effect of these *ABCB1* genotypes/haplotypes on TAC C_0/D [11–13,16,18,20]. Further, two meta-analyses of the impact of 3435C>T SNP [46,47] (n = 1386 and 1327, respectively) were conducted, and both suggested an inconsistent effect on TAC C_0/D that was dependent on the time post-transplantation.

This study confirmed that *CYP3A5*3* non-expressors have higher TAC C_0/D than *CYP3A5*3* expressors. *CYP3A5*3* genotype frequencies deviated from HWE (P = 0.02) in our study, which has been found in other studies [3,48]. This may be due to individuals not being from a truly random population (kidney transplant recipients), or simply by chance given 10 HWE tests were carried out in our study. Importantly, *CYP3A5*1/*1* genotypes were confirmed by sequencing, and this assay has been used without deviation from HWE in other populations [25], indicating systematic genotyping errors are unlikely to explain the deviation.

We did not observe any effect of *CYP3A4*22* or *POR*28* on TAC C_0/D. Theoretically, the *CYP3A4*22* and *POR*28* SNPs can potentially affect TAC PK. *In vitro* studies have found that *CYP3A4*22* decreases *CYP3A4* mRNA expression and activity by 40–60% [49], whilst the *POR*28*/*28 genotype increases *CYP3A* activity by 60% [8]. Oetting *et al.* [20] reported *CYP3A4*22* was significantly associated with increased TAC trough blood concentrations ($p = 4.8 \times 10^{-19}$) in 1560 European-American kidney transplant recipients. Two independent kidney transplant studies ($n = 241$ and 49) [16,23] also reported that *CYP3A4*22* carriers had higher TAC C_0/D than non-carriers (30% and 100%, respectively); whereas Santoro *et al.* [14] ($n = 140$) and Pulk *et al.* [19] (n = 1407) reported no effect of *CYP3A4*22* on TAC C_0/D. In a study of 298 kidney transplant recipients, De Jonge *et al.* [22] identified that *POR*28* carriers (n = 23) had statistically significant lower TAC C_0/D, but only in the *CYP3A5*3*

expressor subgroup. This effect was subsequently confirmed by Elens *et al.* [50] (n = 184) and Pulk *et al.* [19] (n = 1429), however, of these significant results (point-wise *p*-values = 0.03 and 0.04, respectively) only Elens *et al.* [50] adjusted for multiple comparisons. In contrast, Jannot *et al.* [24] (n = 229) and Oetting *et al.* [20] (n = 1560) found no effect of *POR*28* on TAC C_0/D in kidney transplant recipients. In other studies, the *CYP3A4*22* and *POR*28* SNPs were analysed in combination with *CYP3A5*3* to generate fast/intermediate/slow metabolizer groups [51,52] or to calculate *CYP3A4/5* scores [23,39]; thus, the independent effect of *CYP3A4*22* or *POR*28* on TAC PK was not reported. More research and meta-analyses are therefore needed to address these discrepancies regarding the roles *CYP3A4*22* and *POR*28* play in TAC PK variability.

The *NR1I2* 8055T allele is associated with two times increased intestinal CYP3A inducibility [53], and the 63396 C/C genotype is associated with three times lower basal and rifampin-inducible CYP3A4 activity [54]. However, there were no effects of 8055C>T or 63396C>T genotypes on TAC C_0/D in this study. Whilst we are the first to investigate 63396C>T in this setting, two previous studies have similarly reported no effect of 8055C>T on TAC$_0$/D [17,20]. The *NR1I2* -25385T allele is also associated with two times increased intestinal CYP3A inducibility [53]. In three independent kidney transplant recipient cohorts (n = 142, 159 and 1923, respectively) [13,18,20], no difference in TAC C_0/D was reported between -25385C>T genotype groups. In contrast, Kurzawski *et al.* [55] recently reported that -25385C/C genotype carriers had significantly lower TAC C_0/D ($p = 0.005$) in 240 Caucasian kidney transplant recipients. Unfortunately, we were unable to investigate the effect of -25385C>T on TAC C_0/D due to poor assay performance with our samples, and insufficient DNA for re-analysis. Hence, it remains unclear what effect -25385C>T has on TAC PK.

As with any study, there were some notable limitations that need to be considered. For example, our study had a relatively limited sample size, and some homozygous genotypes (i.e. *ABCB1* 61G/G, *NR1I2* 8055 T/T) needed to be combined with heterozygote genotypes for statistical analysis or were not observed (*CYP3A4*22/*22*, *ABCB1* 1199A/A). Therefore, we cannot rule out effects of these homozygous variant genotypes. Additionally, we had no record of recipients' co-medications and so could not assess potential drug–drug interactions with TAC. Finally, our current model explains less than 40% of TAC C_0/D variability, which although similar to the 18–42% variability found by other investigators [15,17,18,20], indicates novel genetic and non-genetic factors (i.e. co-administration of CYP3A inducers and/or inhibitors) need to be examined in future studies.

In conclusion, this study investigated potential genetic causes of inter-individual variability in TAC C_0/D by studying SNPs in the genes encoding TAC metabolizing enzymes, transporter, co-factor and regulator. Our approach has enabled us to confirm the significant effect of *CYP3A5*3* and to detect the novel although minor effect of *ABCB1* 61A>G on TAC C_0/D for the first time.

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Conflict of Interest

All authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Sequences and concentrations of primers and probes, annealing temperatures and product sizes for probe-based allelic discrimination genotyping assays.

Table S2. Median [interquartile range] TAC C_0/D for different genotypes and haplotypes in the first 3 months post-transplant.

Appendix tables: Table 1. Sequences and concentrations of primers and probes, annealing temperatures and product sizes for probe-based allelic discrimination genotyping assays

Genes	SNPs	Annealing temperature	Product size (bp)	Primer/ Probe	Sequence
ABCB1	61A>G	70.0°C	253	F	5'-ATCTTGAAGGGGACCGCAATGGAGGAG-3'
				R	5'-CATATGCTGTGCTCCACTCAGCCAACA-3'
				W	[ROX]AAGCTAGTTACCTTTTAT[+T][+G][+T][+T][+C][+A]G[BHQ2]
				V	[Q670]AAGCTAGTTACCTTTTAT[+C][+G][+T][+T][+C][+A]G[BHQ3]
	1199G>A	68.0°C	241	F	5'-TGACAGCTATTCGAAGAGTGGGCACAA-3'
				R	5'-GGCAATTCACAGACACAGGATATAGGAACTGA-3'
				W	[FAM]ATGTTCACT[+T][+C][+A][+G][+T][+T]ACCCATCTCG[BHQ1]
				V	[HEX]ATGTTCACT[+T][+C][+A][+A][+T][+T]ACCCATCTCG[BHQ1]
	1236C>T	71.5°C	168	F	5'-TCCTGTGTCTGTGAATTGCCTTGAAGTTT-3'
				R	5'-CTGTGGGGTCATAGAGCCTCTGCATCA-3'
				W	[FAM]CCTTCAGGTTC[+A][+G][+G][+C][+C]CTTCAAGAT[BHQ1]
				V	[HEX]CCTTCAGGTTC[+A][+G][+A][+C][+C]CTTCAAGAT[BHQ1]
	2677G>T	68.0°C	279	F	5'-CCCATCATTGCAATAGCAGGAGTTGTTGA-3'
				R	5'-TGAGTCCAAGAACTGGCTTTGCTACTTTCTG-3'
				W	[FAM]TCACCTTCCC[+A][+G][+C][+A][+C]CTTCTAGTTC[BHQ1]
				V	[HEX]TCACCTTCCC[+A][+G][+A][+A][+C]CTTCTAGTTC[BHQ1]
	3435C>T	70.0°C	199	F	5'-GTCCCAGGAGCCCATCCTGTTTGACT-3'
				R	5'-TATAGGCCAGAGAGGCTGCCACATGCT-3'
				W	[FAM]CAGGAAGAGA[+T][+C][+G]TGAGGGCAGCAA[BHQ1]
				V	[CalFluor540]CAGGAAGAGA[+T][+T][+G]TGAGGGCAGCAA[BHQ1]
NR1I2	8055C>T	68.5°C	150	F	5'-GCTACGCCAGGATATGCAGG-3'
				R	5'-TTGCTGGAAGCCACCTGTG-3'
				W	[FAM]AGCTGCCCCTCCAT[+C]CTGTTACCAT[BHQ1]

				V	[HEX]AGCTGCCCCTCCAT[+T]CTGTTACCAT[BHQ1]
	-25385 C>T	68.5°C	75	F	5'-ACC TGA AGA CAA CTG TGG TCA T-3'
				R	5'-GGA GAC CAC GAT TGA GCA AAC-3'
				W	[TxRd]CA ATC CCA G[+G][+T][+T][+C][+T][+C]TT TTC TAC[BHQ2]
				V	[Cy5]CA ATC CCA G[+G][+T][+T][+T][+T][+C]TT TTC TAC[BHQ2]
	63396C>T	64.0°C	361	F	5'-TGGTCATTCATAGCTTCTTTGG-3'
				R	5'-ACTGGTGGTTGGTAAGACAG-3'
				W	[FAM]CTTTTTTGTGCCATATTTT[+T][+T][+C][+T]G[BHQ1]
				V	[HEX]CTTTTTTGTGCCATATTTT[+T][+T][+T][+T]G[BHQ1]
POR	*28	70.0°C	133	F	5'-TGCGGTGGTTGTGGAGTAC-3'
				R	5'-GGACTTGCGCACGAACATG-3'
				W	[FAM]TTCTCCCCG[+G]CAGGCTCCTT[BHQ1]
				V	[HEX]CGTTCTCCCCG[+A]CAGGCTCCTT[BHQ1]

BHQ = Black Hole Quencher; bp: base pairs; CalFluor540 = CalFluor Gold 540; Cy5: Cyanine 5; F: forward primer; FAM: 6-carboxy-fluorescein; HEX: hexachloro-6-carboxy-fluorescein; Q670 = Quasar 670; R: reverse primer; ROX: 6-Carboxyl-X-Rhodamine; TxRd: Texas red; V: variant probe; W: wild-type probe; "+" precedes locked nucleic acid bases.

Appendix tables: Table 2. Median [interquartile range] TAC C_0/D for different genotypes and haplotypes in the first 3 months post-transplant

Genes	Genotypes/ Haplotypes		TAC C_0/D (ng/ml per mg/day)	
			1 month	3 month
ABCB1	61A>G	A/A	1.45 [0.98-1.94]	1.53 [1.09-2.34]
		A/G+G/G	1.15 [0.99-1.35]	1.23 [0.92-1.60]
	1199G>A	G/G	1.33 [0.97-1.81]	1.45 [0.99-2.24]
		G/A	1.46 [1.16-1.93]	1.75 [1.20-2.46]
	1236C>T	C/C	1.44 [1.09-1.73]	1.55 [0.97-2.35]
		C/T	1.35 [0.95-1.83]	1.42 [0.93-2.18]
		T/T	1.28 [1.08-1.83]	1.48 [1.16-2.33]
	2677G>T	G/G	1.35 [1.08-1.76]	1.60 [0.96-2.41]
		G/T	1.34 [0.94-1.86]	1.42 [0.98-2.10]
		T/T	1.31 [1.08-1.80]	1.46 [1.17-2.23]
	3435C>T	C/C	1.44 [0.99-1.83]	1.57 [0.97-2.59]
		C/T	1.39 [1.01-1.78]	1.55 [1.05-2.25]
		T/T	1.15 [0.90-1.79]	1.25 [1.02-1.90]
	AGCGC	0 copies	1.30 [1.00-1.80]	1.43 [1.13-2.03]
		1 copy	1.43 [0.97-1.67]	1.42 [0.93-2.24]
		2 copies	1.37 [1.14-1.85]	1.70 [1.22-2.72]
	AGCGT	0 copies	1.35 [0.99-1.83]	1.45 [1.05-2.29]
		1+2 copies	1.35 [0.86-1.66]	1.43 [0.83-2.22]
	AGTTT	0 copies	1.27 [1.00-1.55]	1.40 [0.90-2.23]
		1 copy	1.44 [0.90-2.07]	1.53 [1.11-2.19]
		2 copies	1.59 [1.08-2.17]	1.57 [1.19-2.74]
	GGTTT	0 copies	1.44 [0.97-1.88]	1.53 [1.03-2.37]
		1+2 copies	1.12 [0.99-1.34]	1.24 [0.93-1.59]
CYP3A4	**22*	**1/*1*	1.33 [0.97-1.71]	1.44 [1.06-2.20]
		**1/*22*	1.34 [0.97-1.79]	1.45 [1.05-2.23]
CYP3A5	**3*	**1/*1+*1/*3*	0.63 [0.51-0.95]	0.59 [0.45-0.97]
		**3/*3*	1.45 [1.12-1.86]	1.60 [1.20-2.29]
NR1I2	8055C>T	C/C	1.35 [0.95-1.67]	1.45 [0.91-1.88]
		C/T+T/T	1.33 [1.09-1.92]	1.35 [1.20-2.11]
	63396C>T	C/C	1.22 [0.80-1.52]	1.31 [0.88-2.18]
		C/T	1.45 [1.12-1.85]	1.55 [1.16-2.22]
		T/T	1.58 [1.07-2.13]	1.59 [1.17-2.08]
POR	**28*	**1/*1*	1.28 [0.93-1.65]	1.48 [1.10-2.22]
		**1/*28*	1.37 [1.02-1.85]	1.45 [0.97-2.23]
		**28/*28*	1.35 [1.20-2.11]	1.68 [1.18-2.33]

TAC C_0/D: dose-adjusted trough blood tacrolimus concentration.

Chapter 3: Relationship between tacrolimus dispositional genetics and short-term kidney transplant outcomes

Statement of Authorship			
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Principal Author

Name of Principal Author (Candidate)	Rong Hu		
Contribution to the Paper	Study design, collected clinical data, performed statistical analysis, interpreted the results, wrote the first manuscript draft and acted as the corresponding author		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Effect of tacrolimus dispositional genetics on acute rejection in the first 2 weeks and estimated glomerular filtration rate in the first 3 months following kidney transplantation

Rong Hu[a], Daniel T. Barratt[a], Janet K. Coller[a], Benedetta C. Sallustio[a,b] and Andrew A. Somogyi[a,c]

Background **CYP3A4/5 and P-glycoprotein (P-gp, *ABCB1*) affect tacrolimus (TAC) exposure in T cells and kidney cells. Genetic variability of these genes has been widely studied for effects on acute rejection and kidney function after transplantation, but findings remain contradictory. In addition, cytochrome P450 reductase (*POR*) is important for CYP3A4/5 activity, and the pregnane X receptor (*NR1I2*) regulates CYP3A4/5 and P-gp expression. However, the relationship between *POR* and *NR1I2* genetics and acute rejection and kidney function has not been extensively investigated.**

Objective **The aim of this study was to investigate the effect of *ABCB1* (61A > G, 1199G > A, 1236C > T, 2677G > T, 3435C > T), *CYP3A4*22*, *CYP3A5*3*, *NR1I2* (8055C > T, 63396C > T) and *POR*28* genotypes/haplotypes on acute rejection and kidney function in the first 3 months after transplant.**

Participants and methods **The study included 165 kidney transplant recipients, who received TAC, mycophenolate and prednisolone, and 129 donors. TAC dose was adjusted to target trough blood concentrations of 8–15 ng/ml by therapeutic drug monitoring. Recipient and donor genotype/haplotype differences in acute rejection incidence within the first 2 weeks after transplant were assessed by logistic regression, adjusting for induction therapy, human leucocyte antigen mismatches, kidney transplant number, peak panel-reactive antibodies and donor type. Recipient and donor genotype/haplotype differences in estimated glomerular filtration rate in the first 3 months after transplant were assessed by linear mixed effects analysis, adjusting for acute rejection, delayed graft function and donor type.**

Results **No genetic factors significantly affected acute rejection or estimated glomerular filtration rate after correction for multiple comparisons ($P > 0.004$).**

Conclusion **Recipient and donor dispositional genetics had no significant effect on short-term clinical outcomes in kidney transplant patients receiving TAC therapeutic drug monitoring. *Pharmacogenetics and Genomics* 29:9–17**

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Keywords: ***ABCB1*, acute rejection, *CYP3A4*22*, *CYP3A5*3*, donor, estimated glomerular filtration rate, kidney transplant recipient, *NR1I2*, *POR*28*, tacrolimus**

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Introduction

Acute rejection and delayed graft function (DGF) are adverse clinical outcomes that result in decreased kidney function and contribute to long-term graft loss following kidney transplantation [1,2].

To minimize acute rejection, maintenance immunosuppression, typically comprising tacrolimus (TAC), mycophenolate and prednisolone, is routinely used. Of these agents, only TAC currently undergoes therapeutic drug monitoring (TDM) by most transplant centres, targeting trough blood concentrations (TAC C_0) between 5 and 15 ng/ml [3] to reduce under-immunosuppression or TAC-induced nephrotoxicity. TAC is a direct glomerulo-constrictor [4], and increased TAC C_0 (>15 ng/ml) is significantly associated with decreased kidney function (indicated by increased serum creatinine) [5]. However, ~10% of patients still develop acute rejection even when TAC C_0 is above 5 ng/ml in the first 2 weeks after transplant [6]. Induction therapy, human leucocyte antigen (HLA) mismatches, kidney transplant numbers and peak panel-reactive antibodies (PRA) have been associated with acute rejection [7–10].

Pharmacogenetic studies have also been conducted to examine predictors of acute rejection and TAC-related kidney dysfunction. Genotypes of TAC's key metabolizing enzyme cytochrome P450 3A5 (encoded by *CYP3A5*) and efflux transporter P-glycoprotein (P-gp, encoded by *ABCB1*) not only determine TAC C_0 [11–13]

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but also TAC intracellular exposure in cells that express CYP3A5 and/or P-gp [14–16], such as patient peripheral blood mononuclear cells (PBMCs, which include T cells) [17] and kidney (HEK293) cells *in vitro* [18]. As the immunosuppressive site of action of TAC is in T cells [19], recipient *ABCB1* genetics may have the greatest effect on acute rejection by modifying T-cell TAC concentrations rather than TAC C_0. In addition, donor and recipient *CYP3A5* and *ABCB1* genetics may affect TAC-induced kidney graft dysfunction by altering TAC accumulation within kidney tubular epithelial cells either directly (donor graft) or indirectly by effects on C_0 (recipient). Despite several studies to date, it is still unclear if recipient genotypes [*CYP3A5*3* (rs776746), *ABCB1* 1236C > T (rs1128503), 2677G > T (rs2032582) or 3435C > T (rs1045642)] affect acute rejection and/or kidney function [20–24]. Donor *CYP3A5*3* and/or *ABCB1* genetics may influence acute rejection and/or kidney function [25–27]; however, this has not been replicated [28,29]. In addition, no research has investigated the relationship between recipient/donor *ABCB1* 61A > G (rs9282564) and 1199G > A (rs2229109) genotypes and acute rejection or kidney function.

Cytochrome P450 3A4 (encoded by *CYP3A4*) is another key metabolizing enzyme of TAC [11], and cytochrome P450 reductase (encoded by *POR*) modulates CYP3A4/5 activity [30]. Recipient *CYP3A4*22* (rs35599367) and *POR*28* (rs1057868) genotypes do not influence acute rejection [31,32], but these findings need confirmation because of the limited number of studies. Furthermore, the effects of donor *CYP3A4*22* and *POR*28* genotypes on acute rejection and kidney function have not been previously examined. Finally, although pregnane X receptor (encoded by *NR1I2*) regulates CYP3A4/5 and P-gp expression [33], no studies have investigated the effect of recipient or donor *NR1I2* genetic variability on acute rejection or kidney function in kidney transplant patients receiving TAC.

We previously demonstrated that recipient *CYP3A5*1* and *ABCB1* 61G alleles were associated with lower dose-corrected TAC C_0 in a cohort of predominantly White kidney transplant recipients [34]. Therefore, in the same cohort of recipients and their donors, this study aimed to assess the effect of genes associated with TAC disposition on acute rejection in the first 2 weeks and kidney function in the first 3 months after transplant. We hypothesized that both recipient and donor *ABCB1*, *CYP3A*, *NR1I2* and *POR* genetics would affect these short-term clinical outcomes in kidney transplant recipients receiving TAC. Importantly, this study is the first to include all five most common white *ABCB1* single nucleotide polymorphisms (SNPs, 61A > G, 1199G > A, 1236C > T, 2677G > T and 3435C > T) together with their haplotypes, and the *NR1I2* 8055C > T (rs2276707) and 63396C > T (rs2472677) SNPs, in the investigation of short-term clinical outcomes.

Participants and methods

Study participants

As described in our previous study [34], 165 adult kidney transplant recipients (≥18 years) and their respective donors were recruited for this study (not every donor sample was available, $n = 129$). Eleven donors each provided kidneys for two recipients; these were counted only once in the Hardy–Weinberg equilibrium (HWE) analysis but were treated independently for outcome associations. Recipients with multiple-organ transplantation or severe liver dysfunction [34] were excluded. Anti-CD-25 antibody induction (Basiliximab; Novartis Pharmaceuticals Australia, North Ryde, Australia) was given to 93% of recipients. All recipients received maintenance immunosuppression with TAC [(Prograf; Astellas Pharma Australia Pty Ltd, Macquarie Park, Australia) twice daily], mycophenolate mofetil [(Cellcept; Roche Products Pty Limited, Sydney, Australia) fixed dose of 0.72–1 g, twice daily] and prednisolone (initial dose of 20–30 mg, followed by a corticosteroid tapering regimen). Informed consent was obtained from all recipients and living donors. For deceased donors, consent was obtained from respective recipients to use excess donor tissue blood vessels for genotyping. This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number: 2 008 178). All procedures met the Declaration of Helsinki and institutional research committee ethical standards.

Demographics, genotyping and clinical data

Participants' demographics, DNA extraction and genotyping protocols have been described previously [34,35]. TAC C_0 (quantified by LC–MS/MS [36]) data from TDM were collected every day for the first 2 weeks after transplant. TAC dose was adjusted to a target C_0 of 8–15 ng/ml by TDM. DGF (the use of dialysis within the first week after transplant), the number of HLA mismatches (HLA-A, HLA-B and HLA-DR antigens) between recipients and donors, induction therapy, kidney transplant number, peak PRA scores (%) assessed by serum lymphocytotoxicity assay [10] and donor type were all recorded from patients' case notes. Biopsy-confirmed acute rejection [37] was recorded within the first 2 weeks after transplant. Kidney function was evaluated by estimated glomerular filtration rate (eGFR) calculated by the Chronic Kidney Disease Epidemiology Collaboration equation [38], before transplant and 1 and 3 months after transplant. All clinical data are summarized in Table 1.

Statistical analysis

Statistical analyses were carried out in R, version 3.5.0 [39] unless stated otherwise. HWE was tested by χ^2 or Fisher's exact test (R package::function, genetics [40]::HWE.chisq and HWE.exact). Linkage disequilibrium (LD) between loci within a gene was examined by pairwise D' and r^2 (genetics [39]::LD). *ABCB1* haplotypes were inferred by PHASE 2.1 [41–43]. When low frequencies were observed,

Table 1 Clinical data for 165 kidney transplant recipients before transplant and in the first 3 months after transplant

Characteristics	Value
Acute rejection[a] (Y) [*n* (%)]	38 (23)
DGF (Y) [*n* (%)]	21 (13)
eGFR [median (range)] (ml/min/1.73 m^2)	
Before transplant	8 (3–33)
1 month after transplant	55 (12–111)
3 months after transplant	53 (18–115)
Recipient ethnicity [*n* (%)]	
White	138 (84)
Aboriginal Australian	5 (3)
Asian	4 (2)
Unknown	18 (11)
HLA mismatches [*n* (%)]	
A (0/1/2)	15/87/63 (9/53/38)
B (0/1/2)	7/57/101 (4/35/61)
DR (0/1/2)	12/70/83 (7/42/50)
Induction therapy (Y) [*n* (%)]	153 (93)
Kidney transplant number [*n* (%)]	
1	135 (82)
2	26 (16)
3	4 (2)
Living donors [*n* (%)]	58 (35)
Peak PRA [*n* (%)]	
≤10%/>10%	135 (82)/29 (17)
NA	1 (1)

Percentages may not sum to 100% because of rounding.
DGF, delayed graft function, the use of dialysis within the first week after transplant; eGFR, estimated glomerular filtration rate; HLA mismatches, human leucocyte antigens (HLA-A, HLA-B and HLA-DR) mismatches; NA, not available; peak PRA, peak panel-reactive antibodies scores assessed by serum lymphocytotoxicity assay; Y, yes.
[a]Acute rejection in the first 2 weeks after kidney transplant.

Table 2 Distribution of acute rejection relative to TAC C_0 in the first 2 weeks after transplant

TAC C_0	No rejection [*n* (%)]	Rejection [*n* (%)]	OR	95% CI	*P*
≥8 ng/ml[a]	84 (51)	24 (15)	1.1	0.5–2.4	0.7
<8 ng/ml[a]	43 (26)	14 (8)			

95% CI, 95% confidence interval; OR, odds ratio; TAC C_0, trough blood tacrolimus concentrations; TDM, therapeutic drug monitoring.
[a]Kidney transplant recipients were grouped according to whether or not their daily TAC C_0 were <8 ng/ml (the lower limit of the TDM target range of TAC in this study) for at least 3 consecutive days.

some genotypes/haplotypes were combined for statistical purposes: *CYP3A5* nonexpressors (*CYP3A5*3/*3*) versus expressors (**1/*1 + *1/*3*); *ABCB1* 61G allele carriers (A/G + G/G) versus noncarriers (A/A); AGCGT or GGTTT haplotypes as 0 copies versus 1 + 2 copies; and *NR1I2* 8055T allele carriers (C/T + T/T) versus noncarriers (C/C).

Normality of eGFR was assessed using histograms and quantile–quantile plots (graphics [39]::hist; stats [39]::qqnorm and qqline). To reduce non-normality of data, $\log_{10}$ transformation of eGFR (log eGFR) was performed according to a power transformation test (MASS [44]::boxcox). To identify recipient and donor genotype/haplotype differences in incidence of acute rejection, genetic factors were examined individually by logistic regression (generalized linear modelling by lme4 [45]::glm), adjusting for induction therapy [yes/no (Y/N)], HLA mismatches (<3 or ≥3), kidney transplant number (1 or ≥2), peak PRA (≤10 or >10%) and living donor (Y/N). The effect of all individual factors on acute rejection was assessed by odds ratio (OR), 95% confidence interval (CI) and point-wise *P* values (stats [39]::exp, coef and confint; car [46]::Anova).

Recipients were also grouped according to whether or not their daily TAC C_0 was less than 8 ng/ml (the lower limit of TDM target range of TAC in this study) for at least 3 consecutive days in the first 2 weeks after kidney transplant, and the incidence of acute rejection was compared by OR, 95% CI and point-wise *P* values (stats [39]::fisher.test).

Linear mixed effects regression analysis (lme4 [45]::lmer; car [46]::Anova) was applied to assess the effect of recipient and donor genetics on repeated measurements of log eGFR at 1 and 3 months after transplant. Acute rejection (Y/N), DGF (Y/N), living donor (Y/N), and recipient and donor genotypes/haplotypes, were treated as fixed effects, with genotypes/haplotypes tested individually, whereas recipient/donor ID and time (1 or 3 months after transplant) were random effects on intercept. The effect of all individual factors on log eGFR was assessed by mean difference and point-wise *P* values (stats [39]::summary, car [46]::Anova).

For each outcome, *P*-value thresholds for genotype/haplotype significance were corrected by Bonferroni-adjustment ($\alpha = 0.05/N$, where N equalled the number of genotype/haplotype tests carried out in the recipient or donor cohort).

Results

In total, 38 (23%) patients developed acute rejection; 19 (50%) within the first week, 14 (37%) between 1 and 2 weeks and five (13%) in both the first and second week after transplant. The distribution of acute rejection relative to TAC C_0 of greater than or equal to 8 ng/ml or less than 8 ng/ml in the first 2 weeks after the transplant is shown in Table 2. There was no significant difference in acute rejection between the groups with TAC C_0 of less than 8 ng/ml and greater than or equal to 8 ng/ml [OR (95% CI) = 1.1 (0.5–2.4); point-wise $P = 0.7$]. The effects of induction therapy, HLA mismatches, kidney transplant number and peak PRA scores and donor type on acute rejection, and of acute rejection, DGF and donor type on kidney function ($\log_{10}$ eGFR), are summarized in Tables 3 and 4, respectively. None of these factors had a statistically significant effect on these short-term outcomes (point-wise $P > 0.05$).

Genetic variability in kidney transplant recipients and donors

Recipient genotype, allele and haplotype frequencies, and LD have been reported by us previously [34]. Donor genotype, allele and common haplotype frequencies (>5%) are shown in Table 5. All donor genotypes were in HWE

Table 3 Effect of human leucocyte antigen mismatches, induction therapy, kidney transplant number, peak panel-reactive antibody scores and donor type on acute rejection in the first 2 weeks after transplant

	OR	95% CI	*P*
HLA mismatches ($n \geq 3$)	2.1	0.8–6.8	0.2
Induction therapy (Y)	0.5	0.1–2.0	0.3
Kidney transplant number ($n \geq 2$)	1.9	0.7–5.0	0.2
Peak PRA (>10%)	1.1	0.4–3.0	0.8
Living donor (Y)	1.3	0.6–2.9	0.5

95% CI, 95% confidence interval; HLA mismatches, human leucocyte antigens (HLA-A, HLA-B and HLA-DR) mismatches; OR, odds ratio; *P*, point-wise *P* value; peak PRA, peak panel-reactive antibodies scores assessed by serum lymphocytotoxicity assay; Y, yes.

Table 4 Effect of acute rejection, delayed graft function and donor type on estimated glomerular filtration rate [repeated measurement of estimated glomerular filtration rate (log_{10} transformed) at 1 and 3 months after transplant] in the first 3 months after transplant

	Estimate	SE	*P*
Intercept	1.7	0.02	$<2\times10^{-16}$
Acute rejection (Y)	−0.0008	0.03	1
DGF (Y)	−0.06	0.03	0.07
Living donor (Y)	−0.00008	0.02	1

DGF, delayed graft function, the use of dialysis within the first week after transplant; eGFR, estimated glomerular filtration rate; *P*, point-wise *P* value; Y, yes.

($P > 0.1$). Similar to recipients [34], donor *NR1I2 8055C>T* and *63396C>T* were not in significant LD ($P > 0.9$), whereas *ABCB1* 1236T, 2677T and 3435T were all in strong LD with each other ($D' = 0.8$–0.9, $r^2 > 0.5$, $P < 2\times10^{-16}$). Donor 61G and 1199A were in strong LD with 1236T, 2677T and 3435T ($D' = 0.9$–1.0, $r^2 > 0.03$, $P < 0.01$); however, there was no LD between *ABCB1* 61G and 1199A ($D' = 0.07$, $r^2 = 0.0009$, $P > 0.2$). Furthermore, as in recipients [34], four common *ABCB1* haplotypes were observed in donors: AGCGC (wild-type for all SNPs), AGCGT, AGTTT and GGTTT. Consequently, a multiple testing-adjusted *P*-value threshold was determined as 0.004 ($\alpha = 0.05/14$, 10 SNPs and four haplotypes within recipients and donors, respectively).

Complete genotype data for all variants were available for 81 recipient/donor pairs but only 2% had the same genotype across the 10 SNPs. For individual SNPs, 77% of recipient/donor pairs had the same genotypes for *CYP3A5*3*, 89% for *CYP3A4*22*, 52% for *POR*28*, 41–92% for 1 *ABCB1* SNP (17% for all five *ABCB1* SNPs), and 52 and 46% for *NR1I2 8055C>T* and *63396C>T* SNPs, respectively.

Genetic effect on acute rejection and kidney function

Recipient and donor genotype/haplotype differences in acute rejection in the first 2 weeks after transplant, adjusting for induction therapy, HLA mismatches, kidney transplant number, peak PRA scores and donor type, are summarized in Table 6. No acute rejection occurred in patients receiving a kidney from *CYP3A5* expressor donors ($n = 13$). However, donor *CYP3A5*3* genotype was not a statistically significant predictor of acute rejection after correction for multiple comparisons (threshold $P < 0.004$), with OR and 95% CI unable to be estimated (fitted probability numerically 0 for donor *CYP3A5* expressors). Differences of log_{10} eGFR in the recipient and the donor genotype/haplotype groups at 1 and 3 months after transplant, respectively, are summarized in Table 7. For all genetic factors, no significant effect on acute rejection or kidney function was found (threshold $P < 0.004$).

Discussion

To our knowledge, this is the first TAC pharmacogenetic study to investigate both recipient and donor *ABCB1*, *CYP3A4/5*, *NR1I2* and *POR* genotypes/haplotypes for their effects on the short-term outcomes of acute rejection in the first 2 weeks and kidney function in the first 3 months following kidney transplantation. None of these TAC disposition genes significantly affected acute rejection or kidney function.

So far, there is no consistent association between recipient *ABCB1* genetics or *CYP3A5*3* and acute rejection in the context of TAC TDM. Bandur *et al.* [22] found *ABCB1* 1236C–2677G–3435T haplotype carriers had 1.4-fold higher risk of acute rejection ($P = 0.04$) in white kidney transplant recipients; however, only half of the population ($n = 425$) was treated with TAC whereas the other half ($n = 407$) was treated with cyclosporin. A meta-analysis [47] ($n = 772$) found *CYP3A5* expressors had a higher risk of acute rejection than *CYP3A5* nonexpressors at 1 month ($P = 0.002$), but not at 3 or 12 months ($P = 0.5$) after transplant. In contrast, most research [20,21,24, 32,48], including the present study which only focused on the early post-transplant period, has failed to identify any significant effect of recipient *CYP3A5*3* or *ABCB1* genotypes/haplotypes on acute rejection.

There are also conflicting results regarding the effect of donor genetic variability on acute rejection and kidney function in the context of TAC TDM. Glowacki *et al.* [28] ($n = 209$) reported donor *ABCB1* 3435C>T and *CYP3A5*3* did not affect acute rejection, whereas Gervasini *et al.* [27] ($n = 137$) found donor *CYP3A5* expressors had a higher risk of acute rejection than *CYP3A5* nonexpressors at 1 year after transplant [OR (95% CI) = 3.42 (1.06–11.01), $P = 0.039$]. However, this finding did not meet the multiple testing correction unless analysed together with *CYP3A4*1B* [OR (95% CI) = 6.24 (1.60–24.33), $P = 0.007$]. Tavira *et al.* [25] observed lower ($n = 65$, $P < 0.01$), whereas Yan *et al.* [26] reported higher eGFR ($n = 120$, $P < 0.05$) in donor *ABCB1* 3435T allele carriers. In contrast, Yang *et al.* [29] recently failed to find any statistically significant effect of living donor *ABCB1* 3435C>T or *CYP3A5*3* genotype

Table 5 Frequencies of donor[a] *ABCB1*, *CYP3A4/5*, *NR1I2* and *POR* genotypes, alleles and *ABCB1* haplotypes

Genes	SNP/haplotype (*n*)	Genotype [*n* (%)]	Allele/haplotype [*n* (%)]	HWE *P* value
ABCB1	61A > G (117)	A/A [90 (77)]	A [206 (88)]	1
		A/G [26 (22)]	G [28 (12)]	
		G/G [1 (1)]	–	
	1199G > A (118)	G/G [111 (94)]	G [229 (97)]	1
		G/A [7 (6)]	A [7 (3)]	
		A/A [0 (0)]	–	
	1236C > T (119)	C/C [25 (21)]	C [113 (47)]	0.5
		C/T [63 (53)]	T [125 (53)]	
		T/T [31 (26)]	–	
	2677G > T (114)	G/G [28 (25)]	G [116 (51)]	0.6
		G/T [60 (53)]	T [112 (49)]	
		T/T [26 (23)]	–	
	3435C > T (115)	C/C [18 (16)]	C [96 (42)]	0.5
		C/T [60 (52)]	T [134 (58)]	
		T/T [37 (32)]	–	
	AGCGC (124)	0 copies [50 (40)]	AGCGC [88 (35)]	–
		1 copy [60 (48)]	–	
		2 copies [14 (11)]	–	
	AGCGT (124)	0 copies [105 (85)]	AGCGT [20 (8)]	–
		1 copy [18 (15)]	–	
		2 copies [1 (1)]	–	
	AGTTT (124)	0 copies [51 (41)]	AGTTT [84 (34)]	–
		1 copy [62 (50)]	–	
		2 copies [11 (9)]	–	
	GGTTT (124)	0 copies [97 (78)]	GGTTT [29 (12)]	–
		1 copy [25 (20)]	–	
		2 copies [2 (2)]	–	
CYP3A4	**22* (101)	**1/*1* [92 (91)]	**1* [193 (96)]	1
		**1/*22* [9 (9)]	**22* [9 (4)]	
		**22/*22* [0 (0)]	–	
CYP3A5	**3* (105)	**1/*1* [0 (0)]	**1* [12 (6)]	1
		**1/*3* [12 (11)]	**3* [198 (94)]	
		**3/*3* [93 (89)]	–	
NR1I2	8055C > T (99)	C/C [67 (68)]	C [161 (81)]	0.3
		C/T [27 (27)]	T [37 (19)]	
		T/T [5 (5)]	–	
	63396C > T (102)	C/C [19 (19)]	C [79 (39)]	0.1
		C/T [41 (40)]	T [125 (61)]	
		T/T [42 (41)]	–	
POR	**28* (102)	**1/*1* [48 (47)]	**1* [141 (69)]	0.7
		**1/*28* [45 (44)]	**28* [63 (31)]	
		**28/*28* [9 (9)]	–	

HWE, Hardy–Weinberg Equilibrium; SNP, single nucleotide polymorphism.
[a]For donors whose kidneys went to two recipients ($n = 11$), these were only counted once in HWE test, therefore, the numbers under each donor genotype/haplotype are different from those for clinical outcomes in Table 6.

on eGFR in the first 3 months after transplant. Our results also indicated that donor *ABCB1* genetics and *CYP3A5*3* do not significantly affect acute rejection or kidney function during the early post-transplantation period in the context of TAC TDM.

It is still unclear if *CYP3A4*22*, *POR*28* and *NR1I2* genetics affect TAC C_0 [31,32,34] and their effect on TAC intracellular concentrations is unknown. To date, no significant effect of *CYP3A4*22* and *POR*28* on acute rejection has been reported [31,32]. We confirmed these findings in our cohort; in addition, we found *CYP3A4*22* and *POR*28* were not associated with eGFR in the first 3 months after transplant. We are the first to investigate *NR1I2* genetic effects on TAC short-term outcomes, with no significant effects found.

Induction therapy, HLA mismatches, kidney transplant number, peak PRA scores and donor type did not significantly affect acute rejection in the first 2 weeks after transplant in this study ($P > 0.1$), nor did acute rejection, DGF and donor type significantly affect eGFR in the first 3 months after transplant ($P > 0.08$). However, this might be because of a relatively limited sample size in this study, with their nonsignificant effects at least nominally in agreement with previous studies [7–10]. In addition, most kidney transplant recipients [153 (93%)] received induction therapy to reduce immunological risk. Therefore, the factors above may have a lesser effect on acute rejection and kidney function in this cohort ($P > 0.08$).

Although TAC dispositional genes can have a significant effect on dose-corrected TAC C_0 [17,20,21,23,24,28,31, 32,34,48], most pharmacogenetic studies to date have found they do not affect the incidence of acute rejection [20,21,24,28,32,48] or kidney function [27,28]. It is also noteworthy that several studies have found acute

Table 6 Recipient and donor *ABCB1*, *CYP3A4/5*, *NR1I2* and *POR* genotype/haplotype differences in acute rejection in the first 2 weeks after transplant, adjusting for human leukocyte antigen mismatches, induction therapy, kidney transplant number, peak panel-reactive antibody scores and donor type

	Recipients					Donors[a]				
Genes	Genotypes/haplotypes (*n*)		AR (*n*)	OR (95% CI)	*P*	Genotypes/haplotypes (*n*)		AR (*n*)	OR (95% CI)	*P*
ABCB1	61A > G (163)	A/A (134)	33	Reference	0.3	61A > G (128)	A/A (99)	24	Reference	0.6
		A/G + G/G (29)	5	0.6 (0.2–1.7)			A/G + G/G (29)	6	0.8 (0.3–2.1)	
	1199G > A (160)	G/G (148)	33	Reference	0.1	1199G > A (129)	G/G (122)	26	Reference	0.3
		G/A (12)	5	0.3 (0.1–1.2)			G/A (7)	3	0.4 (0.1–2.3)	
	1236C > T (163)	C/C (44)	12	Reference	0.8	1236C > T (130)	C/C (27)	9	Reference	0.1
		C/T (77)	17	0.8 (0.3–1.9)			C/T (71)	18	0.6 (0.2–1.6)	
		T/T (42)	9	0.7 (0.3–2.0)			T/T (32)	4	0.3 (0.1–1.0)	
	2677G > T (161)	G/G (47)	14	Reference	0.4	2677G > T (124)	G/G (31)	9	Reference	0.4
		G/T (78)	16	0.6 (0.2–1.4)			G/T (67)	15	0.6 (0.2–1.8)	
		T/T (36)	8	0.7 (0.2–2.1)			T/T (26)	4	0.4 (0.1–1.5)	
	3435C > T (162)	C/C (41)	10	Reference	0.6	3435C > T (126)	C/C (20)	6	Reference	0.4
		C/T (69)	18	1.1 (0.4–3.1)			C/T (68)	16	0.7 (0.2–2.6)	
		T/T (52)	10	0.7 (0.3–2.1)			T/T (38)	6	0.4 (0.1–1.7)	
	AGCGC (157)	0 copies (65)	13	Reference	0.6	AGCGC (124)	0 copies (50)	9	Reference	0.5
		1 copy (71)	16	1.2 (0.5–2.9)			1 copy (60)	14	1.5 (0.5–4.3)	
		2 copies (21)	7	1.9 (0.5–6.6)			2 copies (14)	5	2.4 (0.5–10.1)	
	AGCGT (157)	0 copies (127)	31	Reference	0.4	AGCGT (124)	0 copies (105)	24	Reference	0.9
		1 + 2 copies (30)	5	0.6 (0.2–1.7)			1 + 2 copies (19)	4	0.9 (0.2–3.1)	
	AGTTT (157)	0 copies (73)	15	Reference	0.7	AGTTT (124)	0 copies (51)	14	Reference	0.4
		1 copy (64)	17	1.4 (0.6–3.2)			1 copy (62)	12	0.5 (0.2–1.4)	
		2 copies (20)	4	1.1 (0.3–3.6)			2 copies (11)	2	0.5 (0.1–2.4)	
	GGTTT (157)	0 copies (132)	32	Reference	0.4	GGTTT (124)	0 copies (97)	23	Reference	0.5
		1 + 2 copies (25)	4	0.6 (0.2–1.8)			1 + 2 copies (27)	5	0.7 (0.2–2.0)	
CYP3A4	**22* (151)	**1/*1* (141)	33	Reference	0.3	**22* (110)	**1/*1* (101)	21	Reference	0.2
		**1/*22* (10)	1	0.4 (0.02–2.3)			**1/*22* (9)	3	2.7 (0.5–12.9)	
CYP3A5	**3* (152)	**1/*1* + **1/*3* (23)	6	Reference	0.6	**3* (114)	**1/*3* (13)	0	Reference	0.02
		**3/*3* (129)	28	0.8 (0.3–2.3)			**3/*3* (101)	26	NA	
NR1I2	8055C > T (144)	C/C (102)	28	Reference	0.06	8055C > T (109)	C/C (73)	15	Reference	0.4
		C/T + T/T (42)	5	0.4 (0.1–1.0)			C/T + T/T (36)	9	1.5 (0.5–4.2)	
	63396C > T (153)	C/C (28)	7	Reference	0.7	63396C > T (112)	C/C (19)	5	Reference	0.5
		C/T (71)	15	0.6 (0.2–2.0)			C/T (46)	12	1.3 (0.4–5.4)	
		T/T (54)	12	0.9 (0.3–2.7)			T/T (47)	9	0.7 (0.2–3.0)	
POR	**28* (142)	**1/*1* (71)	20	Reference	0.4	**28* (112)	**1/*1* (53)	11	Reference	0.9
		**1/*28* (62)	13	0.6 (0.3–1.4)			**1/*28* (49)	12	1.1 (0.4–3.1)	
		**28/*28* (9)	1	0.3 (0.02–2.1)			**28/*28* (10)	2	0.8 (0.1–3.8)	

AR, acute rejection; HWE, Hardy–Weinberg Equilibrium; NA, not available, as fitted probability numerically 0 or 1 (perfect estimation) and/or unable to estimate confidence interval (fitted probability near 1); OR (95% CI), adjusted odds ratio (95% confidence interval); *P*, point-wise *P* value.
[a]Donors whose kidneys went to two recipients ($n = 11$) were treated independently when associated with acute rejection of the individual recipients, therefore, the numbers under each donor genotype/haplotype are different from those in HWE in Table 5.

rejection is not associated with interindividual TAC C_0 variability between patients under TDM [6,27]. In this analysis, we chose 3 consecutive days of TAC C_0 below the lower limit of 8 ng/ml on the basis of the Symphony trial [49] and investigated its relationship with acute rejection. However, 26% of the recipients did not develop acute rejection even though their TAC C_0 values were below 8 ng/ml. A similar analysis using a TAC C_0 of 5 ng/ml also failed to show a significant relationship between low TAC exposure and acute rejection (data not shown). This finding confirms a lack of significant relationship between TAC C_0 (< 5 or <8 ng/ml) and acute rejection under TAC TDM. We expect that TDM reduces interindividual variability in TAC C_0, leaving its effect on acute rejection and consequently that of TAC dispositional genetics less apparent. Of note, in liver transplant patients under TAC TDM, Capron *et al.* [50] found acute rejection was significantly correlated with PBMC TAC concentration at day 7 after transplant ($P = 0.01$) but not TAC C_0. Although the same group failed to replicate their finding in kidney transplant patients ($P = 0.09$) [17], this was probably because of the small number of acute rejection episodes ($n = 6$). Therefore, the relationship between TAC PBMC concentration and acute rejection is worthwhile exploring in the future.

Our results indicate no significant effect of the common TAC dispositional genes investigated on short-term kidney transplant outcomes; however, some limitations should be noted when interpreting these findings. First, this study had a relatively limited sample size, and therefore, it might be of insufficient power to support the negative findings. For example, the study had 80% power to detect a statistically significant OR greater than 6.1 ($P < 0.004$) for acute rejection among *CYP3A5* expressors (OR > 3.8 for $P < 0.05$). Insufficient power is a common issue for individual pharmacogenetic studies, but when taken together, these can offer valuable information for future meta-analyses. Second, we could not examine the

Table 7 Log_{10} transformed estimated glomerular filtration rate (mean ± SD, ml/min/1.73 m^2) in recipient and donor genotypes/haplotypes at 1 and 3 months after transplant

			Recipients				Donors[a]			
Genes	Genotypes/Haplotypes		n[b]	1 Month	n[b]	3 Months	n[b]	1 Month	n[b]	3 Months
ABCB1	61A > G	A/A	133	1.73 ± 0.15	127	1.73 ± 0.14	98	1.73 ± 0.14	95	1.73 ± 0.13
		A/G + G/G	29	1.74 ± 0.15	29	1.73 ± 0.13	29	1.70 ± 0.14	27	1.70 ± 0.14
	1199G > A	G/G	147	1.73 ± 0.15	141	1.73 ± 0.13	121	1.72 ± 0.14	116	1.72 ± 0.13
		G/A	12	1.73 ± 0.16	12	1.68 ± 0.14	7	1.79 ± 0.11	7	1.72 ± 0.17
	1236C > T	C/C	44	1.68 ± 0.14	44	1.70 ± 0.12	26	1.67 ± 0.14	26	1.70 ± 0.13
		C/T	77	1.76 ± 0.13	75	1.74 ± 0.14	71	1.73 ± 0.14	69	1.72 ± 0.14
		T/T	41	1.72 ± 0.17	37	1.71 ± 0.13	32	1.73 ± 0.13	29	1.73 ± 0.12
	2677G > T	G/G	47	1.68 ± 0.14	47	1.69 ± 0.13	30	1.69 ± 0.15	30	1.72 ± 0.14
		G/T	78	1.75 ± 0.14	76	1.74 ± 0.14	67	1.74 ± 0.13	65	1.72 ± 0.13
		T/T	35	1.72 ± 0.17	31	1.72 ± 0.13	26	1.72 ± 0.15	24	1.70 ± 0.13
	3435C > T	C/C	41	1.71 ± 0.16	41	1.72 ± 0.15	19	1.71 ± 0.17	19	1.74 ± 0.15
		C/T	69	1.75 ± 0.14	68	1.72 ± 0.13	68	1.73 ± 0.13	66	1.72 ± 0.13
		T/T	51	1.72 ± 0.17	46	1.73 ± 0.14	38	1.71 ± 0.14	35	1.70 ± 0.12
	AGCGC	0 copies	64	1.73 ± 0.16	59	1.73 ± 0.14	50	1.73 ± 0.14	47	1.71 ± 0.12
		1 copy	71	1.73 ± 0.14	70	1.72 ± 0.14	60	1.73 ± 0.13	58	1.73 ± 0.14
		2 copies	21	1.70 ± 0.13	21	1.71 ± 0.12	13	1.67 ± 0.17	13	1.70 ± 0.14
	AGCGT	0 copies	126	1.73 ± 0.14	121	1.72 ± 0.13	104	1.73 ± 0.14	99	1.73 ± 0.14
		1 + 2 copies	30	1.71 ± 0.17	29	1.73 ± 0.15	19	1.67 ± 0.13	19	1.67 ± 0.08
	AGTTT	0 copies	73	1.70 ± 0.15	73	1.71 ± 0.14	50	1.71 ± 0.15	49	1.71 ± 0.14
		1 copy	64	1.77 ± 0.12	62	1.74 ± 0.12	62	1.72 ± 0.13	59	1.72 ± 0.12
		2 copies	19	1.69 ± 0.19	15	1.70 ± 0.15	11	1.78 ± 0.14	10	1.73 ± 0.15
	GGTTT	0 copies	122	1.72 ± 0.15	116	1.72 ± 0.14	96	1.73 ± 0.14	93	1.73 ± 0.13
		1 + 2 copies	25	1.74 ± 0.14	25	1.73 ± 0.12	27	1.71 ± 0.14	25	1.69 ± 0.14
CYP3A4	*22	*1/*1	140	1.73 ± 0.14	136	1.72 ± 0.14	100	1.73 ± 0.15	95	1.73 ± 0.14
		*1/*22	9	1.73 ± 0.14	4	1.72 ± 0.13	8	1.73 ± 0.15	3	1.73 ± 0.14
CYP3A5	*3	*1/*1 + *1/*3	23	1.77 ± 0.12	23	1.77 ± 0.12	13	1.76 ± 0.17	12	1.74 ± 0.16
		*3/*3	128	1.72 ± 0.15	124	1.72 ± 0.14	100	1.72 ± 0.14	96	1.72 ± 0.13
NR1I2	8055C > T	C/C	101	1.72 ± 0.15	98	1.73 ± 0.14	72	1.73 ± 0.15	69	1.72 ± 0.14
		C/T + T/T	42	1.74 ± 0.13	40	1.71 ± 0.12	36	1.74 ± 0.12	34	1.74 ± 0.13
	63396C > T	C/C	28	1.71 ± 0.17	26	1.73 ± 0.13	19	1.69 ± 0.16	19	1.69 ± 0.13
		C/T	71	1.74 ± 0.13	69	1.73 ± 0.13	45	1.74 ± 0.14	43	1.74 ± 0.13
		T/T	53	1.72 ± 0.16	52	1.72 ± 0.14	47	1.72 ± 0.13	44	1.72 ± 0.14
POR	*28	*1/*1	70	1.73 ± 0.16	68	1.72 ± 0.14	52	1.73 ± 0.14	51	1.73 ± 0.14
		*1/*28	62	1.73 ± 0.13	62	1.74 ± 0.13	49	1.72 ± 0.14	47	1.72 ± 0.14
		*28/*28	9	1.67 ± 0.10	7	1.62 ± 0.08	10	1.74 ± 0.17	8	1.77 ± 0.11

Log_{10} eGFR, log_{10} transformed estimated glomerular filtration rate.
[a]For donors whose kidneys went to 2 recipients ($n = 11$), these were treated independently when associated with clinical outcome of individual recipients.
[b]Number of recipients or donors in different genotype/haplotype groups whose log_{10} eGFR data were available for analysis at 1 and 3 months after transplant.

effect of certain rare homozygous genotypes or haplotypes because they had to be combined with heterozygotes for statistical purposes (e.g. *CYP3A5*1/*1 + CYP3A5*1/*3*) or were not observed in our cohort (e.g. *CYP3A4*22/*22*). Our sample size similarly precluded the investigation of additional rarer variants (frequency < 1%). Third, we did not have T-cell/PBMC or kidney tissue TAC concentration data; therefore, the relationship between TAC intracellular concentrations and acute rejection and kidney function, as well as the genetic effect on TAC intracellular concentrations, was not investigated. Fourth, as only 13% ($n = 21$) of the recipients had DGF, and low minor allele frequencies for some important SNPs (e.g. *CYP3A5*1*, *ABCB1* 61A > G) were found, the statistical approximation was inappropriate in logistic regression. Therefore, we cannot fully assess these known TAC dispositional genetics for their effect on DGF. Finally, this study only considered short-term clinical outcomes; therefore, the effect of these TAC dispositional genes on long-term outcomes (i.e. graft loss) in our patients remains unknown.

Conclusion

This study found no statistically significant effect of the recipient or the donor *ABCB1*, *CYP3A4/5*, *NR1I2* or *POR* genetics on acute rejection and eGFR in the first 3 months following kidney transplantation in the context of TAC TDM.

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Conflicts of interest

There are no conflicts of interest.

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Chapter 4: Relationship between tacrolimus trough whole blood concentration and acute kidney rejection

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Contribution to the Paper	Study design, collected clinical data, performed statistical analysis, interpreted the results, wrote the first manuscript draft and acted as the corresponding author		
Overall percentage (%)	70%		
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By signing the Statement of Authorship, each author certifies that:

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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SHORT COMMUNICATION

Is There a Temporal Relationship Between Trough Whole Blood Tacrolimus Concentration and Acute Rejection in the First 14 Days After Kidney Transplantation?

Rong Hu, MSc, Daniel T. Barratt, PhD,* Janet K. Coller, PhD,* Benedetta C. Sallustio, PhD,*† and Andrew A. Somogyi, PhD*‡*

Background: There are inconsistent findings regarding the relationship between trough whole blood tacrolimus concentration (TAC C_0) and acute kidney rejection in recipients undergoing TAC therapeutic drug monitoring (TDM). However, studies have not always assessed TAC C_0 at the time of rejection or accounted for variability in hematocrit. Therefore, this study aimed to investigate the temporal relationship between TAC C_0 and acute rejection, including when accounting for variation in hematocrit.

Methods: For 38 recipients who developed biopsy-proven acute rejection (BPAR) in the first 14 days after kidney transplantation, daily TAC C_0 from TDM and hematocrit was collected from case notes. Differences in $\log_{10}$-transformed TAC C_0 between the day of BPAR (log C_r), 1 day before BPAR (log C_{r-1}), and 2 days before BPAR (log C_{r-2}) and the combined median concentrations for the days preceding these (log C_{prior}) were examined by repeated-measures analysis of variance with Dunnett post hoc testing. Generalized linear mixed-effects regression (glmer) examined the ability of TAC C_0 to predict acute rejection episodes with and without controlling for hematocrit.

Results: Log C_{r-1} [mean difference (95% confidence interval) = −0.13 (−0.21 to −0.048), post hoc $P = 0.002$] and log C_r [−0.13 (−0.24 to −0.025), post hoc $P = 0.013$] were significantly lower than log C_{prior}. TAC C_0 was a significant ($P = 0.0078$) predictor of rejection episodes (area under the receiver operating characteristic curve = 0.79) only in glmer models accounting for variability in hematocrit.

Conclusions: In recipients who developed BPAR, there was a significant temporal relationship between TAC C_0 and BPAR incidence under TAC TDM that may not be detected in cross-sectional studies, especially if variability in hematocrit is not addressed. This supports a TAC C_0–rejection relationship, which differs between recipients, and may explain why some recipients do or do not experience rejection within or below the TDM range, respectively. However, studies with larger sample sizes are needed to confirm this finding.

Key Words: kidney transplantation, acute rejection, trough whole blood tacrolimus concentration, therapeutic drug monitoring

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INTRODUCTION

Tacrolimus (TAC) is the mainstay of immunosuppressive therapy after kidney transplantation. However, TAC has large intraindividual and interindividual variability in apparent whole blood clearance (coefficients of variation of 40%–71% and 30%–42%, respectively).[1] TAC therapeutic drug monitoring (TDM) has been recommended to reduce both acute rejection and toxicity.[2] However, the relationship between trough whole blood TAC concentration (TAC C_0) and acute rejection has not been adequately defined.[2]

Inconsistent findings exist regarding a TAC C_0–rejection relationship under TAC TDM.[3–5] Borobia et al[3] reported that a TAC C_0 higher than 9.3 ng/mL on day 5 after transplantation was significantly associated with lower biopsy-proven acute rejection (BPAR) incidence in the first 3 months after transplantation. However, Bouamar et al[4] and our previous work[5] showed that in the first year or the first 2 weeks after transplantation, respectively, there was no significant difference in BPAR risk between TAC C_0 above and below 5, 8, or 10 ng/mL, respectively. Notably, for all 3 studies, TAC C_0 used to categorize recipients into rejection and nonrejection groups was not always concurrent with BPAR. Indeed, to the best of our knowledge, the temporal relationship between TAC C_0 and rejection has not been assessed.

In addition, TAC is highly erythrocyte bound,[6] and variability in hematocrit affects interpretation of TAC C_0.[7] Therefore, accounting for hematocrit variability might assist in identifying TAC C_0–rejection relationships but has not been used in any TAC C_0–rejection study.

We hypothesize that there is a temporal relationship between TAC C_0 and rejection in recipients who develop BPAR. As such, TAC C_0 on the days immediately preceding BPAR and on the day of BPAR would be lower compared with other days, especially when variability in hematocrit is

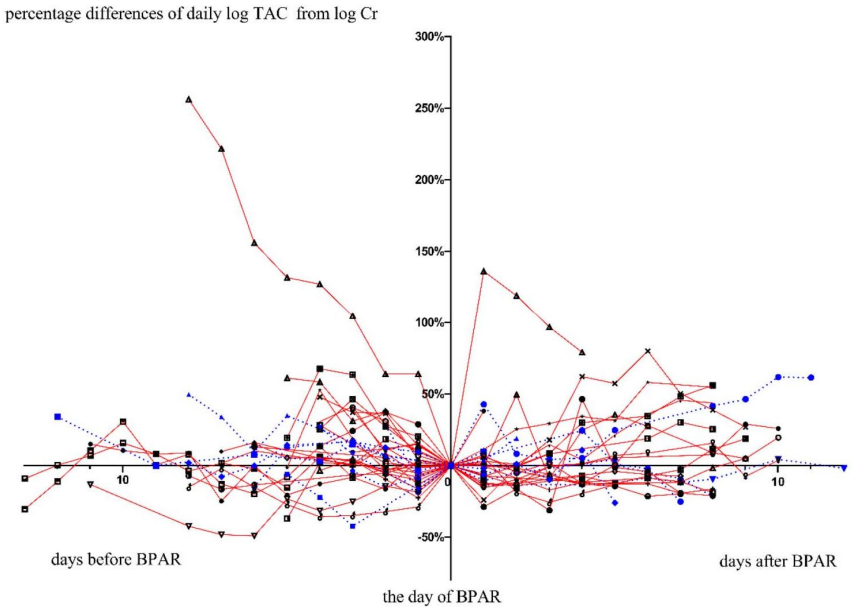

FIGURE 1. Daily log10-transformed trough whole blood tacrolimus concentration (log TAC) as a percentage of BPAR day concentrations (log_{10}-transformed; log C_r) in recipients with BPAR (n = 38) in the first 14 days after transplantation. Blue symbols and dashed lines stand for recipients with BPAR who had missing values on the day of (log C_r), 1 day (log C_{r-1}) or 2 days (log C_{r-2}) before BPAR, or the preceding days (log C_{prior}), in the first 14 days after transplantation.

taken into account. Therefore, in the same cohort of kidney transplant recipients who developed BPAR in our previous study,[5] we aimed to examine whether there is a temporal relationship between TAC C_0 and rejection, using repeated measures of TAC C_0, hematocrit, and rejection status within individuals over the first 14 days after transplantation, when the majority of BPAR episodes occurred in our study.[5]

MATERIALS AND METHODS

Study Participants and Data Collection

This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). All procedures followed the Declaration of Helsinki and/or institutional research committee ethical instructions.

Acute rejection was confirmed by either routine protocol biopsy performed within the first 14 days post-transplantation or for-cause biopsy performed due to a clinical suspicion of rejection. Borderline rejection was not included in this study. In total, 165 kidney transplant recipients were enrolled in this study and 38 had BPAR within the first 14 days post-transplantation. All recipients gave informed consent before participation. Participant inclusion and exclusion criteria, demographics, anti–CD-25 induction therapy, triple immunosuppressive therapy comprising TAC, mycophenolate mofetil and prednisolone, and BPAR data have been described previously.[5,8] Only the first episode of BPAR in each recipient was investigated.

TAC C_0 was targeted to 8–15 ng/mL under TDM as described previously.[5,8] Daily TAC C_0 and hematocrit data over the first 14 days after transplantation were collected from recipients' case notes. In recipients with BPAR, the day of biopsy was assigned as the rejection day. TAC C_0 and hematocrit on the day of BPAR, 1 day before BPAR, and 2 days before BPAR were defined as C_r, C_{r-1}, and C_{r-2}, and H_r, H_{r-1}, and H_{r-2}, respectively. TAC C_0 and hematocrit on the days preceding C_{r-2} and H_{r-2} were defined as C_{prior} and H_{prior}, respectively.

Statistical Analysis

Statistical analyses were conducted in R version 3.5.0[9] unless stated otherwise. Normality of TAC C_0 and hematocrit was assessed using histograms and quantile–quantile plots (R package::function; graphics[9]::hist, stats[9]::qqnorm and qqline). Hematocrit was normally distributed, whereas TAC C_0 was log_{10}-transformed (log C_0) to a normal distribution.

In recipients with BPAR (n = 38), differences in log-transformed C_r, C_{r-1}, and C_{r-2} (log C_r, log C_{r-1}, and log C_{r-2}, respectively) versus C_{prior} (log C_{prior}; recipient median), and H_r, H_{r-1}, and H_{r-2} versus H_{prior} (recipient median), were examined by repeated-measures analysis of variance (ANOVA) with Dunnett post hoc testing, in GraphPad Prism v8 (GraphPad Software, San Diego, CA). Because repeated-measures ANOVA is incompatible with missing values, TAC C_0 data from 12, and hematocrit data from 3, recipients with BPAR, respectively, were excluded from this analysis because of partial missing data.

In recipients with BPAR (n = 38), generalized linear (binomial) mixed-effects regression (glmer; lme4[10]::glmer) was used to assess the ability of TAC C_0 to predict acute rejection episodes with and without controlling for hematocrit. In glmer analyses, based on repeated-measures ANOVA results, the outcome on the day of BPAR and 1 day before BPAR was marked as "rejection," with all preceding days (including 2 days before BPAR) marked as "no-rejection," for each recipient. Days after BPAR were excluded. All

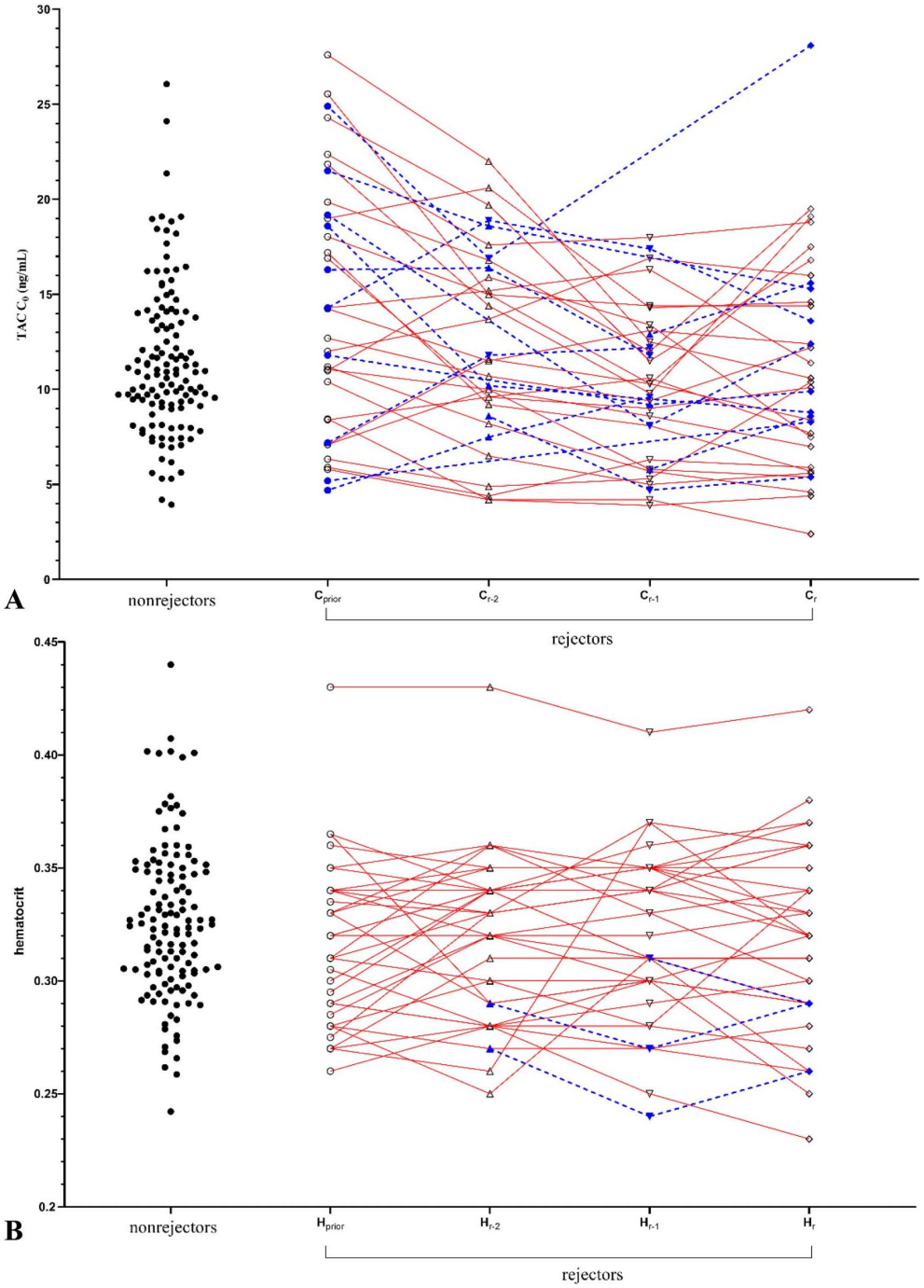

FIGURE 2. A and B. Trough whole blood tacrolimus concentration (TAC C_0) and hematocrit, respectively, for recipients without BPAR (combined median) in the first 14 days after transplantation. And TAC C_0 and hematocrit, respectively, for recipients with BPAR on the day of BPAR (C_r and H_r), 1 day before BPAR (C_{r-1} and H_{r-1}), 2 days before BPAR (C_{r-2} and H_{r-2}), and preceding days (C_{prior} and H_{prior}; combined median). Blue symbols and dashed lines in (A, B) stand for recipients with BPAR who had missing values of C_r, C_{r-1}, C_{r-2}, or C_{prior} (n = 12), and H_r, H_{r-1}, H_{r-2}, or H_{prior} (n = 3), respectively, in the first 14 days after transplantation.

TABLE 1. Comparison of Log C_r, Log $C_{r\text{-}1}$, and Log $C_{r\text{-}2}$ to Log C_{prior} by Repeated-Measures ANOVA (Dunnett Post hoc Testing) in the First 14 Days After transplantation

Comparisons	Mean Difference	95% CI	*P*
Log C_r versus log C_{prior}	−0.13	−0.24 to −0.025	0.013
Log $C_{r\text{-}1}$ versus log C_{prior}	−0.13	−0.21 to −0.048	0.0020
Log $C_{r\text{-}2}$ versus log C_{prior}	−0.069	−0.14 to 0.005	0.08

Because repeated-measures ANOVA is incompatible with missing values, only 26 recipients with BPAR were included in this analysis and 12 recipients were excluded because of 1 or more instances of missing data.

Log C_r, $\log_{10}$-transformed trough whole blood tacrolimus concentration (log TAC) on the day of BPAR; log $C_{r\text{-}1}$, log TAC 1 day before BPAR; log $C_{r\text{-}2}$, log TAC 2 days before BPAR; log C_{prior}, log TAC (recipient median) on preceding days; P, Dunnett post hoc *P* value.

recipients with BPAR (n = 38) were included in glmer analyses because it can handle missing values. Three models were compared, all with random effects on intercept and slope for each subject: (1) log C_0 as a fixed effect; (2) log C_0 and hematocrit (Z-score standardized to enable model convergence) as fixed effects; and (3) $\log_{10}$-transformed predicted trough plasma TAC concentration (log C_p) based on $C_{wb} = C_p + [C_p \times B_{max} \times f_{HCT}/K_D]$ (where C_{wb} = TAC C_0; B_{max} = 418 mcg/L erythrocytes; f_{HCT} = hematocrit as a fraction and K_D = 3.8 mcg/L plasma)[6,11] as a fixed effect. Models were assessed by type-II ANOVA (χ^2 test, car[12]::ANOVA) and calculation of odds ratios, 95% confidence intervals (CIs). The sensitivity and specificity of models were evaluated by area under the receiver operating characteristic curve (ROC AUC; ROCR[13]::performance and AUC).

The percentage of TAC C_0 values in the first 14 days after transplantation (1) within the TAC TDM range (8–15 ng/mL) and (2) below the lower limit of 8 ng/mL was both calculated for all recipients (n = 165). Differences between recipients with and without BPAR were compared by Mann–Whitney tests (stats[9]::wilcox.test).

RESULTS

In total, 15 recipients had BPAR between days 2–5, 13 recipients between days 6–9, and 10 recipients between days 10–14 after transplantation. Figure 1 shows daily $\log_{10}$-transformed TAC C_0 as a percentage difference from log C_r in the first 14 days after transplantation. Figures 2A, B show TAC C_0 (ng/mL) and hematocrit data, respectively, for 127 recipients without BPAR (combined median for the first 14 days after transplantation) and for 38 recipients on the day of BPAR, 1 and 2 days before BPAR, and preceding days (combined median).

There was a significant overall difference between log C_{prior}, log $C_{r\text{-}2}$, log $C_{r\text{-}1}$, and log C_r (ANOVA $P = 0.0008$). Table 1 summarizes Dunnett post hoc testing results. Log $C_{r\text{-}1}$ was significantly lower than log C_{prior} [mean difference (95% CI) = −0.13 (−0.21 to −0.048), post hoc $P = 0.002$], equivalent to 26% (10%–38%) lower geometric mean TAC C_0. In addition, log C_r was also significantly lower than log C_{prior} [−0.13 (−0.24 to −0.025), post hoc $P = 0.013$], equivalent to 26% (6%–42%) lower geometric mean TAC C_0. However, there was no significant difference between log $C_{r\text{-}2}$ and log C_{prior} (post hoc $P = 0.08$). There was no significant difference between H_r, $H_{r\text{-}1}$, $H_{r\text{-}2}$, and H_{prior} (ANOVA $P = 0.39$).

Glmer results are summarized in Table 2. TAC C_0 alone did not significantly predict rejection ($P = 0.077$) but was a significant ($P = 0.0078$) predictor of rejection when hematocrit was included in the model (ROC AUC = 0.79). Calculated TAC C_p was also a significant ($P = 0.048$) predictor for rejection.

There was no significant difference in the percentage of TAC C_0 values within 8–15 ng/mL [median (range): 46% (8%–93%) versus 50% (0%–92%), point-wise $P > 0.3$] or below 8 ng/mL [21% (0%–92%) versus 17% (0%–100%), point-wise $P > 0.7$] between recipients with and without BPAR in the first 14 days after transplantation.

DISCUSSION

The rejection rate (23%) in this kidney transplant cohort is consistent with the rate (~20%) reported by the Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry.[14]

Although there are conflicting findings regarding the TAC C_0–rejection relationship under TAC TDM,[3–5] the TAC C_0 was not always concurrent with BPAR, which may be due to limited TAC C_0 data available for analysis in some studies. We are the first to report a temporal TAC C_0–rejection relationship, with lower TAC C_0 on the day of, and 1 day before, BPAR linked to acute rejection risk in kidney transplant recipients. Decreased TAC absorption (eg, food–drug interaction[15]) or increased TAC metabolism (eg, induction of cytochrome P450 enzyme and P-glycoprotein by high-dose corticosteroid[16]) might lower TAC C_0 within a recipient. However, declining TAC C_0 alone is not necessarily a trigger for rejection. For example, similar proportions of recipients with and without BPAR had a decline in log (TAC C_0) ≥0.13 at least once in the first 14 days after transplantation (data not shown). It is likely that the (whole blood) TAC C_0–rejection relationship varies between recipients because of a combination of factors such as, where on the concentration–response curve (of each patient) rejection occurs, variable hematocrit, TAC plasma protein binding, intracellular distribution and pharmacodynamics, or variable mycophenolate (MPA) concentrations.

Our findings also showed the importance of accounting for hematocrit. Hematocrit should not be ignored when interpreting the TAC C_0–rejection relationships because it can influence interpretation of, and contribute to interindividual and intraindividual variability in, TAC C_0.[7] Hematocrit is generally low because of kidney dysfunction and can usually recover in the weeks after transplantation; nonetheless, post-transplantation anemia and erythrocytosis may still contribute to hematocrit variability.[17] In this study, there was no significant difference between H_r, $H_{r\text{-}1}$, $H_{r\text{-}2}$, and H_{prior}, suggesting that the decline of TAC C_0 1 day before BPAR and on the day of BPAR was unlikely to be caused by changes in hematocrit. Nonetheless, accounting for (random) variability in hematocrit helped identify the relationship

TABLE 2. The Impact of TAC C_0 on Daily Risk of BPAR in the First 14 Days After transplantation, With and Without Controlling for Hematocrit

Glmer Models	Estimate	SE	*P*	OR	95% CI	ROC AUC
Intercept	1.01	1.10		—	—	0.72
Log C_0	−1.75	0.99	0.077	0.17	0.025–1.21	
Intercept	2.14	1.14		—	—	0.79
Log C_0	−2.78	1.04	0.0078	0.062	0.0080–0.48	
Hematocrit*	0.54	0.26	0.041	1.72	1.02–2.88	
Intercept	−1.85	0.50		—	—	0.70
Log C_p	−1.97	1.00	0.048	0.14	0.020–0.98	

Because generalized linear mixed-effects regression is compatible with missing values, all recipients with BPAR (n = 38) were included in this analysis.

hematocrit*, Z-score standardized hematocrit; log C_0, $\log_{10}$-transformed trough whole blood tacrolimus concentration; log C_p, $\log_{10}$-transformed predicted trough plasma tacrolimus concentration; *P*, type II ANOVA *P* value.

between TAC C_0 and rejection and provided better prediction of BPAR (ROC AUC = 0.79). Although the difference between H_r, $H_{r\text{-}1}$, $H_{r\text{-}2}$, and H_{prior} was not statistically significant in the first 2 weeks after transplantation, our glmer results show that accounting for variability in hematocrit can help identify the TAC C_0–rejection relationship and provide better prediction of BPAR (ROC AUC = 0.79). For recipient(s) with the same TAC C_0 under TAC TDM, hematocrit variability may help to explain the difference of rejection incidence within and/or between recipients. We are unaware of any other TAC C_0–rejection studies incorporating hematocrit.

Replication studies are needed to confirm our findings, and some limitations should also be noted when interpreting our results. First, although all recipients were previously genotyped for *ABCB1* and *NR1I2* polymorphisms,[5] we could not test their effect on the temporal TAC C_0–rejection relationship because of the limited sample size. Second, not all TAC C_0 data were available for all recipients with BPAR. Finally, MPA monitoring was not conducted in our transplant unit during the time of this retrospective study. Therefore, we are unable to investigate the impact of MPA concentrations on BPAR incidence.

CONCLUSIONS

In recipients who developed BPAR, there was a significant temporal relationship between TAC C_0 and BPAR incidence under TAC TDM that may not be detected in cross-sectional studies, especially if variability in hematocrit is not addressed. This supports a TAC C_0–rejection relationship, which differs between recipients, and may explain why some recipients do or do not experience rejection within or below the TDM range, respectively. However, studies with larger sample sizes are needed to confirm this finding.

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Chapter 5: Relationship between innate immunogenetics and acute kidney rejection

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Principal Author

Name of Principal Author (Candidate)	Rong Hu		
Contribution to the Paper	Study design, collected clinical data, performed statistical analysis, interpreted the results, wrote the first manuscript draft and acted as the corresponding author		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14th May 2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Daniel T Barratt		
Contribution to the Paper	Assisted with results interpretation and manuscript critical review		
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Contribution to the Paper	Study design, collected all genotyping data, assisted with results interpretation and manuscript critical review		
Signature		Date	15/5/19
Name of Co-Author	Benedetta C Sallustio		
Contribution to the Paper	Study design, assisted with clinical data collection, results interpretation and manuscript critical review		
Signature		Date	15-5-19
Name of Co-Author	Andrew A Somogyi		
Contribution to the Paper	Study design, assisted with results interpretation and manuscript critical review		
Signature		Date	17/05/2019

Effect of innate immune genetics on acute kidney rejection in the first 2 weeks post-transplantation

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Abstract

Background Innate immunity contributes to acute rejection after kidney transplantation. *IL2* -330T>G, *IL10* -1082G>A, -819C>T and -592C>A, and *TNF* -308G>A are not associated with acute rejection risk in European kidney transplant recipients. However, for other important innate immune genetic markers in recipients and donors, associations with rejection have not been studied or results have been inconclusive. **Objective** To investigate the effect of recipient and donor *CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *LY96*, *MYD88*, *TGFB*, *TLR2*, *TLR4* and *TNF* genetics on acute kidney rejection in the first 2 weeks post-transplant. **Methods** This study included 165 kidney transplant recipients and 129 donors. Recipient and donor genotype/diplotype differences in acute rejection incidence within the first 2 weeks post-transplantation were assessed by logistic regression, adjusted for induction therapy, human leukocyte antigen mismatches, kidney transplant number, living donor and peak panel-reactive antibody scores. **Results** Although recipients with *IL6* -6331C/C genotype had a higher incidence of acute rejection compared to T/T genotype (Odds Ratio [95% confidence interval] = 6.6 [1.7 to 25.8], likelihood-ratio test P = 0.02), no genetic factors were associated with rejection after correction for multiple comparisons (P-value threshold = 0.0036). **Conclusions** Recipient and donor innate immune genetics investigated in this study did not significantly impact on acute kidney rejection incidence in the first 2 weeks post-transplantation.

Key words kidney transplantation, acute rejection, recipient, donor, innate immune genetics, genotype, diplotype

1. Introduction

Acute rejection remains the biggest short-term challenge following kidney transplantation and it affects long-term graft survival [1]. Although induction therapy, human leukocyte antigens (HLA) mismatches, number of kidney transplants, living donor and peak panel-reactive antibodies (PRA) have been studied as rejection predictors [2, 3], acute rejection still occurs.

The T-cell driven adaptive immune system plays a major role in acute rejection. However, innate immunity is also essential. Damage-associated molecular patterns (DAMPs) from transplantation surgery and ischemia/reperfusion injury can activate nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) via the myeloid differentiation primary response 88 (MyD88)-dependent Toll-like receptor (TLR) signaling pathway in leukocytes, which leads to pro-inflammatory cytokine (e.g. interleukin (IL)-1β, IL-2, etc.) secretion [4]. These cytokines assist T-cell proliferation, differentiation, and intensify kidney tissue damage [4]. In contrast, anti-inflammatory cytokines (e.g. IL-10) can decrease pro-inflammatory cytokine release [5] and therefore may have the potential to attenuate rejection risk.

Meta-analyses show that recipient and/or donor *IL2* -330T>G (rs2069762) [6], *IL10* -1082G>A (rs1800896), -819C>T (rs1800871) and -592C>A (rs1800872) [7], and *TNF* -308G>A (rs1800629) [8] are not significantly associated with acute rejection incidence in European kidney transplant recipients. However, as recently reviewed [9], the impact of some important innate immune genetic variations (e.g. *IL1B* 3954C>T (rs1143634) and *TLR4* 896A>G (rs4986790)) on acute rejection remains inconclusive. This is likely due to different criteria for acute rejection (biopsy-proven acute rejection (BPAR) versus clinical evidence, e.g. serum creatinine change), recipient ethnicities and time of rejection post-transplantation between cross-sectional studies. In addition, adjustment for multiple statistical comparisons was not always conducted. Finally,

other important innate immune genetic loci, including *CASP1*, *CRP*, *IL6R*, *LY96*, *MYD88* and *TLR2* have not been examined for their impact on acute rejection in kidney transplant recipients.

Therefore, this study aimed to explore the impact of innate immune genetics on acute rejection risk in a cohort of predominantly European kidney transplant recipients and donors [3]. An innate immune gene panel, including SNPs of *CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *LY96*, *MYD88*, *TGFB*, *TLR2*, *TLR4* and *TNF*, has been designed and reported by our group [10-12]. We hypothesized that these recipient and donor innate immune genetics would affect acute rejection incidence in kidney transplant recipients in the first 2 weeks post-transplantation.

2. Methods

2.1. Study participants and data collection

This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). All procedures complied with the Declaration of Helsinki and/or institutional research committee ethical requirements. As described previously, 165 kidney transplant recipients and 129 donors were recruited [3, 13]. All recipients and living donors gave informed consent before participation. For deceased donors, their respective recipients gave informed consent to use excess donor tissue blood vessels for genotyping. Recipient inclusion and exclusion criteria, demographics, anti-CD-25 induction therapy, immunosuppressant regimen, the number of HLA mismatches (HLA-A, -B and -DR antigens) between recipients and donors, number of kidney transplants, donor type (living or deceased), peak PRA and BPAR have all been described [3, 13].

2.2. Genotyping

Genomic DNA was extracted from blood and kidney tissue as described previously [3, 13]. A panel of 19 SNPs in 13 innate immune genes were assayed by Agena Bioscience (previously Sequenom) MassARRAY at the Australian Genome Research Facility (Brisbane, Australia) [10-12]: MyD88-dependent TLR signaling pathway – *TLR2* 1350T>C (rs3804100), *TLR4* 896A>G (rs4986790) and 1196C>T (rs4986791), *LY96* (rs11466004), and *MYD88* (rs6853); and pro- and anti-inflammatory mediators – *CASP1* 5352G>A (rs580253) and 10643G>C (rs554344), *CRP* -717T>C (rs2794521), *IL1B* -511C>T (rs16944), -31T>C (rs1143627) and 3954C>T (rs1143634), *IL2* -330T>G (rs2069762), *IL6* -6331T>C (rs10499563), *IL6R* (rs8192284), *IL10* -1082G>A (rs1800896) and -819C>T (rs1800871), *TGFB* -1287G>A (rs11466314) and -509C>T (rs1800469), and *TNF* -308G>A (rs1800629). The panel also included SNPs in *BDNF* (rs6265) and *OPRM1* (rs1799971) but these were not included in the analysis as they were considered outside the scope of this study. Notably, only 154 recipients and 81 donors (3 donors each provided kidneys for 2 recipients) had sufficient DNA for genotyping. Donors were counted only once in Hardy-Weinberg equilibrium (HWE) tests but were treated independently for logistic regression analyses. For some SNPs, 1 to 4 recipients and/or donors had missing genotypes due to genotyping failure.

2.3. Statistical analysis

Hardy-Weinberg equilibrium (HWE) tests for all genotypes, linkage disequilibrium (LD) between SNPs within the same gene, haplotype inference (*CASP1*, *IL1B*, *IL10* and *TLR4*), and logistic regression analyses were as described previously [3, 11-13]. The associations between SNPs, diplotypes and BPAR incidence were analyzed separately by logistic regression, adjusted for induction therapy (yes/no (Y/N)), living donor (Y/N), HLA mismatches (< 3 or ≥ 3), kidney transplant number (1 or ≥ 2) and

peak PRA (≤ 10% or > 10%). The genetic impact on BPAR incidence was assessed by the likelihood-ratio test, Odds ratios (OR) with 95% confidence intervals (CI) as previously described [3]. In addition, genotype differences in BPAR without adjusting for non-genetic variables were tested by Fisher or Chi-square tests and OR with 95% CI. Multiple testing-adjusted P-value threshold for significance was corrected by Bonferroni-adjustment ($\alpha = 0.05/N$, where N is the number of genotype/diplotype tests carried out in the recipient or donor cohort, respectively).

3. Results

In total, 23% (n = 38) of patients developed BPAR in the first 2 weeks post-transplantation. The impact of induction therapy, HLA mismatches, kidney transplant number, living donor and peak PRA scores on BPAR has been reported [3]; none were statistically significant (likelihood-ratio test P-value > 0.1).

3.1. Genetic variability in kidney transplant recipients and donors

No variant allele was found for *TGFB* rs11466314. *LY96* rs11466004 was excluded from further analysis due to its low variant allele frequency in both recipients and donors (3% and 1%, respectively). All other recipient and donor genotypes were in HWE ($P \geq 0.2$). Where required, rare homozygous genotypes ($n < 5$) were combined with heterozygous genotypes for further analyses: recipient *MYD88* rs6853 A/A genotype versus G allele carriers (A/G + G/G); donor *IL6* -6331T/T genotype versus C allele carriers (T/C + C/C); recipient and donor *TLR2* 1350T/T genotype versus C allele carriers (T/C + C/C); recipient and donor *TNF* -308G/G genotype versus A allele carriers (G/A + A/A).

Recipient and donor *CASP1* 10643G and 5352G, *IL1B* -511C and -31T, *IL10* -1082G and -819C, and *TLR4* 896A and 1196C were in strong LD ($D' > 0.99$, $r^2 \geq 0.3$). Recipient and donor genotype, allele, haplotype and diplotype frequencies are

summarized in Table 1. The following rare *CASP1*, *IL1B*, *IL10* and *TGFB* diplotypes (n < 5) were not included in further analyses: recipient and donor *CASP1* (5352G>A & 10643G>C) A-C/A-C, recipient and donor *IL1B* (-511C>T & -31T>C) C-C/C-C, donor *IL10* (-1082G>A & -819C>T) A-T/A-T, and recipient and donor *TLR4* (896A>G & 1196C>T) A-C/A-T and G-T/G-T. Consequently, a multiple testing-adjusted P-value threshold for significance was determined at 0.0036 (α = 0.05/14, 10 individual SNP and 4 gene diplotype tests in recipients and donors).

3.2. Genetic effect on BPAR incidence

Table 2 summarizes associations between recipient and donor genotype/diplotype and BPAR incidence in the first 2 weeks post-transplant, adjusting for induction therapy, HLA mismatches, kidney transplant number, living donor and peak PRA scores. Although recipients with *IL6* -6331C/C genotype had a higher incidence of BPAR compared to T/T genotype recipients (OR [95% CI] = 6.6 [1.7 - 25.8], likelihood-ratio test P-value = 0.02), no genetic factors were significantly associated with rejection after correction for multiple comparisons (P-value threshold = 0.0036). Fisher and Chi-square tests also showed similar results (point-wise P-value > 0.05; data not shown).

4. Discussion

To our knowledge, this is the first innate immunogenetic study investigating both recipient and donor *CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *LY96*, *MYD88*, *TGFB*, *TLR2*, *TLR4* and *TNF* genotypes/diplotypes for their association with BPAR incidence in kidney transplant recipients receiving TAC as the only calcineurin inhibitor.

We are the first to investigate the impact of *IL6* -6331T>C on BPAR incidence in kidney transplant recipients. Our results indicate the C/C genotype might be associated with 6.6-fold higher OR of BPAR, but the effect was not statistically significant after adjusting for multiple comparisons. The physiological and/or pathological impact of

IL6 -6331T>C on inflammation remains uncertain. *IL6* -6331T>C was not significantly associated with plasma IL-6 concentrations in healthy volunteers (n = 421) [14]. In 321 surgical patients at 6 hours after coronary artery bypass grafting, T/T carriers had 73% higher plasma IL-6 concentrations than C/C carriers, but this difference was non-significant at 24 hours post-surgery [14]. However, in 173 patients receiving intensive periodontal therapy, T/T carriers had 4- to 6-fold higher plasma IL-6 concentrations than C/C carriers at 24 hours and 7 days post-therapy, respectively [14]. This inconsistency is likely due to the study participants having different diseases, different time post-surgery and sample size limitations. Moreover, the relationship between -6331T>C and plasma IL-6 concentration has not been explored in kidney transplant recipients. Therefore, more studies might still be needed to elucidate if -6331T>C affects BPAR incidence.

Three meta-analyses reported that *IL2*, *IL10* and *TNF* genotypes/haplotypes were not significant predictors of acute rejection in European kidney transplant patients [6-8]; our results are in accordance with these findings. In addition, *IL1B* and *TLR4* genetics have been associated with rejection risk in some studies, however, results were not always reproducible as reviewed previously [9]. We also did not find any significant impact of *IL1B* and *TLR4* genetics on BPAR incidence. The inconsistency might be due to varied definitions of acute rejection, time post-transplantation, sample size between studies, statistical interpretations (e.g. with or without multiple comparison adjustment) and genetic differences between ethnicities. Overall, these inconsistent results suggest that the *IL1B* and *TLR4* genotypes/diplotypes investigated might not be major factors affecting BPAR incidence in kidney transplant recipients.

The impact of recipient and donor *CASP1*, *CRP*, *IL6R*, *MYD88* and *TLR2* genetics on BPAR incidence in kidney transplant patients has not been reported previously. TLRs

and the MyD88 adaptor are important in innate immune response to DAMPs, which can lead to tissue damage [4]. Caspase-1 (encoded by *CASP1*) activates pro-IL-1β into its active IL-1β form [4], and IL-6/soluble IL-6R enhances the expansion and activation of T- and B-cells and induces several acute phase reactants, e.g. C-reactive protein [15]. Therefore, they are expected to be important for any innate immune contribution to acute rejection. However, common variations in these genes had no significant impact on BPAR incidence in our study.

Our study has several limitations to consider when interpreting these results. Firstly, we had a relatively limited sample size and not all genotypes/diplotypes were available for every recipient and donor. This necessitated combination of some rare homozygous genotypes and exclusion of some rare diplotypes for statistical purposes, therefore, the effect of certain rare diplotypes or homozygous genotypes are unknown. Secondly, a few key SNPs, e.g. *IL6* -174G>C and *IL10* -592C>A, were not included because of incompatibility with the genotyping array, and there was insufficient DNA available to carry out separate genotyping of these SNPs. Thirdly, some important innate immune genes, e.g. *IFNG* (encoding for IFN-γ) [9] and *NFKB1* (encoding for the NF-κB1 subunit) [9], were not included in the gene panel design and are worthwhile exploring in the future.

In conclusion, this study found no statistically significant impact of recipient or donor innate immune genetics on BPAR incidence in kidney transplant recipients in the first two weeks post-transplantation.

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Table 1. Recipient and donor genotype, allele, haplotype and diplotype frequencies.

Genes & SNPs	Recipients[#] (n = 124 - 154)			Donors* (n = 77 - 81)		
	Genotypes/Diplotypes (n, %)	Alleles/Haplotypes (n, %)	HWE P	Genotypes/Haplotypes (n, %)	Allele/Haplotypes (n, %)	HWE P
CASP1	G/G (107, 69)	G (258, 84)	0.8	G/G (58, 72)	G (137, 85)	1
5352G>A	G/A (44, 29)	A (50, 16)		G/A (21, 26)	A (25, 15)	
	A/A (3, 2)			A/A (2, 2)		
	G/G (107, 69)	G (258, 84)	0.8	G/G (58, 72)	G (137, 85)	1
10643G>C	G/C (44, 29)	C (50, 16)		G/C (21, 26)	C (25, 15)	
	C/C (3, 2)			C/C (2, 2)		
	G-G/G-G (107, 69)	G-G (258, 84)		G-G/G-G (58, 72)	G-G (137, 85)	
5352G>A & 10643G>C	G-G/A-C (44, 29)	A-C (50, 16)		G-G/A-C (21, 26)	A-C (25, 15)	
	A-C/A-C (3, 2)			A-C/A-C (2, 2)		
CRP	T/T (77, 50)	T (215, 70)	0.4	T/T (33, 41)	T (103, 64)	1
-717T>C	T/C (61, 40)	C (93, 30)		T/C (37, 46)	C (57, 36)	
	C/C (16, 10)			C/C (10, 13)		
IL1B	C/C (76, 49)	C (215, 70)	0.7	C/C (41, 51)	C (114, 70)	0.8
-511C>T	C/T (63, 41)	T (93, 30)		C/T (32, 40)	T (48, 30)	
	T/T (15, 10)			T/T (8, 10)		

	-31T>C	T/T (74, 48) T/C (63, 41) C/C (16, 10)	T (211, 69) C (95, 31)	0.7	T/T (41, 51) T/C (32, 40) C/C (8, 10)	T (114, 70) C (48, 30)	0.8
	-511C>T & -31T>C	C-T/C-T (75, 49) C-T/T-C (63, 41) T-C/T-C (15, 10) C-C/C-C (1, 1)	C-T (213, 70) T-C (93, 30) C-C (2, 1)		C-T/C-T (41, 51) C-T/T-C (32, 40) T-C/T-C (8, 10)	C-T (114, 70) T-C (48, 30)	
	3954C>T	C/C (84, 55) C/T (61, 40) T/T (9, 6)	C (229, 74) T (79, 26)	0.5	C/C (52, 64) C/T (24, 30) T/T (5, 6)	C (128, 79) T (34, 21)	0.5
IL2	-330T>G	T/T (70, 45) T/G (63, 41) G/G (21, 14)	T (203, 66) G (105, 34)	0.3	T/T (39, 48) T/G (36, 44) G/G (6, 7)	T (114, 70) G (48, 30)	0.6
IL6	-6331T>C	T/T (80, 52) T/C (61, 40) C/C (13, 8)	T (221, 72) C (87, 28)	0.8	T/T (50, 62) T/C (28, 35) C/C (3, 4)	T (128, 79) C (34, 21)	1
IL6R	Asp358Ala A>C	A/A (50, 33) A/C (78, 51) C/C (25, 16)	A (178, 58) C (128, 42)	0.6	A/A (27, 34) A/C (39, 49) C/C (14, 18)	A (93, 58) C (67, 42)	1

IL10		G/G (31, 20)	G (141, 46)	0.6	G/G (16, 20)	G (68, 42)	0.5
	-1082G>A	G/A (79, 52)	A (165, 54)		G/A (36, 44)	A (94, 58)	
		A/A (43, 28)			A/A (29, 36)		
		C/C (88, 58)	C (230, 75)	0.5	C/C (42, 52)	C (119, 73)	0.4
	-819C>T	C/T (54, 35)	T (76, 25)		C/T (35, 43)	T (43, 27)	
		T/T (11, 7)			T/T (4, 5)		
		G-C/G-C (31, 20)	G-C (141, 46)		G-C/G-C (16, 20)	G-C (68, 42)	
		G-C/G-T (43, 28)	G-T (89, 29)		G-C/G-T (14, 17)	G-T (51, 31)	
	-1082G>A & -819C>T	G-C/A-T (36, 24)	A-T (76, 25)		G-C/A-T (22, 27)	A-T (43, 27)	
		G-T/G-T (14, 9)			G-T/G-T (12, 15)		
		G-T/A-T (18, 12)			G-T/-A-T (13, 16)		
		A-T/A-T (11, 7)			A-T/A-T (4, 5)		
LY96		C/C (146, 95)	C (300, 97)	1	C/C (76, 99)	C (153, 99)	1
	Ser157Pro C>T	C/T (8, 5)	T (8, 3)		C/T (1, 1)	T (1, 1)	
		T/T (0, 0)			T/T (0, 0)		
MYD88		A/A (123, 80)	A (275, 89)	0.7	A/A (64, 79)	A (145, 90)	0.6
	rs6853 A>G	A/G (29, 19)	G (33, 11)		A/G (17, 21)	G (17, 10)	
		G/G (2, 1)			G/G (0, 0)		
TGFB	-1287G>A	G/G (154, 100)	G (308, 100)		G/G (81, 100)	G (162, 100)	

	-509C>T	C/C (81, 53) C/T (60, 39) T/T (13, 8)	C (222, 72) T (86, 28)	0.8	C/C (45, 56) C/T (29, 36) T/T (7, 9)	C (119, 73) T (43, 27)	0.6
TLR2	1350T>C	T/T (133, 86) T/C (19, 12) C/C (2, 1)	T (285, 93) C (23, 7)	0.2	T/T (74, 91) T/C (6, 7) C/C (1, 1)	T (154, 95) C (8, 5)	0.2
TLR4	896A>G	A/A (137, 89) A/G (16, 10) G/G (1, 1)	A (290, 94) G (18, 6)	0.4	A/A (71, 88) A/G (10, 12) G/G (0, 0)	A (152, 94) G (10, 6)	1
	1196C>T	C/C (136, 88) C/T (17, 11) T/T (1, 1)	C (289, 94) T (19, 6)	0.4	C/C (70, 88) C/T (10, 13) T/T (0, 0)	C (150, 94) T (10, 6)	1
	896A>G & 1196C>T	A-C/A-C (136, 88) A-C/G-T (16, 10) A-C/A-T (1, 1) G-T/G-T (1, 1)	A-C (289, 94) G-T (18, 6) A-T (1, 1)		A-C/A-C (71, 88) A-C/G-T (10, 12)	A-C (152, 94) G-T (10, 6)	
TNF	-308G>A	G/G (113, 73) G/A (35, 23) A/A (6, 4)	G (261, 85) A (47, 15)	0.2	G/G (50, 62) G/A (30, 37) A/A (1, 1)	G (130, 80) A (32, 20)	0.2

Ala: Alanine; Asp: Aspartic acid; Donors*: donor numbers may differ from those in Table 2, as 3 donors were for 2 recipients, respectively, and they were not counted twice in HWE. In addition, donor numbers may differ within Table 1 due to genotyping failure or if the predicted haplotype probability was < 0.8; HWE P: HWE P-value; Pro: Proline; Recipients[#]: recipient numbers may differ within Table 1 due to genotyping failure or if the predicted haplotype probability was < 0.8; SNP: single nucleotide polymorphism. Percentages may not sum up to 100% due to rounding.

Table 2. Recipient and donor innate immune genotype/diplotype differences in BPAR incidence in the first 2 weeks post-transplantation, adjusting for HLA mismatches, induction therapy, kidney transplant number, living donor and peak PRA scores.

Genes & SNPs	Recipients[#] (n = 124 - 154)				Donors* (n = 73 - 84)			
	Genotypes/Diplotypes (n, %)	BPAR (n, %)	OR [95% CI]	P	Genotypes/Diplotypes (n, %)	BPAR (n, %)	OR [95% CI]	P
CASP1	G-G/G-G (107, 71)	19, 18	Ref	0.07	G-G/G-G (60, 73)	16, 27	Ref	0.9
5352G>A & 10643G>C	G-G/A-C (44, 29)	15, 34	2.2 [0.9 - 5.2]		G-G/A-C (22, 27)	6, 29	1.0 [0.3 - 2.9]	
CRP	T/T (77, 50)	12, 16	Ref	0.05	T/T (34, 41)	6, 18	Ref	0.1
-717T>C	T/C (61, 40)	18, 30	3.0 [1.2 - 7.6]		T/C (39, 47)	15, 38	3.1 [1.0 - 10.5]	
	C/C (16, 10)	5, 31	2.1 [0.5 - 7.8]		C/C (10, 12)	2, 20	1.3 [0.2 - 7.5]	
IL1B	C-T/C-T (75, 49)	18, 24	Ref	0.9	C-T/C-T (41, 49)	13, 32	Ref	0.5
-511C>T & -31T>C	C-T/T-C (63, 41)	13, 21	0.8 [0.3 - 1.9]		C-T/T-C (34, 40)	9, 26	0.7 [0.2 - 2.2]	
	T-C/T-C (15, 10)	4, 27	0.9 [0.2 - 3.6]		T-C/T-C (9, 11)	1, 11	0.3 [0.01 - 1.9]	
	C/C (84, 55)	16, 19	Ref	0.2	C/C (54, 64)	13, 24	Ref	0.07
3954C>T	C/T (61, 40)	18, 30	2.0 [0.9 - 4.6]		C/T (25, 30)	10, 40	2.3 [0.8 - 6.6]	
	T/T (9, 6)	1, 11	0.6 [0.03 - 4.1]		T/T (5, 6)	0, 0	NA	
IL2	T/T (70, 45)	12, 17	Ref	0.3	T/T (41, 49)	10, 24	Ref	0.09
-330T>G	T/G (63, 41)	16, 25	1.5 [0.6 - 3.6]		T/G (37, 44)	9, 24	1.1 [0.4 - 3.2]	
	G/G (21, 14)	7, 33	2.4 [0.7 - 7.2]		G/G (6, 7)	4, 67	8.1 [1.2 - 78.5]	

IL6	T/T (80, 52)	14, 18	Ref	0.02	T/T (52, 62)	11, 21	Ref	0.09
-6331T>C	T/C (61, 40)	15, 25	1.6 [0.7 - 4.0]		T/C+ C/C (32, 38)	12, 38	2.4 [0.9 - 6.9]	
	C/C (13, 8)	6, 46	6.6 [1.7 - 25.8]					
IL6R	A/A (50, 33)	12, 24	Ref	0.9	A/A (29, 35)	4, 14	Ref	0.09
Asp358Ala A>C	A/C (78, 51)	16, 21	0.8 [0.3 - 2.1]		A/C (39, 47)	11, 28	2.3 [0.6 - 10.1]	
	C/C (25, 16)	6, 24	0.9 [0.3 - 3.2]		C/C (15, 18)	7, 47	5.4 [1.2 - 27.5]	
IL10	G-C/G-C (31, 20)	8, 26	Ref	0.7	G/C-G/C (18, 23)	2, 11	Ref	0.2
	G-C/G-T (43, 28)	12, 28	1.3 [0.4 - 4.1]		G-C/G-T (14, 18)	4, 29	2.1 [0.4 - 13.3]	
-1082G>A & -819C>T	G-C/A-T (36, 24)	7, 19	0.7 [0.2 - 2.5]		G-C/A-T (23, 29)	7, 30	2.6 [0.6 - 14.5]	
	G-T/G-T (14, 9)	2, 14	0.6 [0.08 - 3.2]		G-T/G-T (12, 15)	2, 17	1.2 [0.1 - 8.7]	
	G-T/A-T (18, 12)	3, 17	0.4 [0.05 - 2.0]		G-T/A-T (13, 16)	7, 54	6.7 [1.3 - 43.4]	
	A-T/A-T (11, 7)	3, 27	1.3 [0.2 - 6.1]					
MYD88	A/A (123, 80)	28, 23	Ref	0.6	A/A (66, 79)	17, 26	Ref	0.5
rs6853 A>G	A/G +G/G (31, 20)	7, 23	0.7 [0.2 - 2.0]		A/G (18, 21)	6, 33	1.5 [0.4 - 4.7]	
TGFB	C/C (81, 53)	18, 22	Ref	0.7	C/C (47, 56)	14, 30	Ref	0.5
-509C>T	C/T (60, 39)	13, 22	1.0 [0.4 - 2.3]		C/T (29, 35)	6, 21	0.5 [0.2 - 1.7]	
	T/T (13, 8)	4, 31	1.7 [0.4 - 6.1]		T/T (8, 10)	3, 38	1.3 [0.2 - 6.2]	
TLR2	T/T (133, 86)	33, 25	Ref	0.07	T/T (77, 92)	22, 29	Ref	0.5
1350T>C	T/C +C/C (21, 14)	2, 10	0.3 [0.04 - 1.1]		T/C+C/C (7, 8)	1, 14	0.5 [0.02 - 3.4]	

TLR4	A-C/A-C (136, 89)	30, 22	Ref	0.5	A-C/A-C (74, 88)	20, 27	Ref	0.9
896A>G & 1196C>T	A-C/G-T (16, 11)	4, 25	1.5 [0.4 -5.0]		A-C/G-T (10, 12)	3, 30	0.9 [0.2 - 3.8]	
TNF	G/G (113, 73)	21, 19	Ref	0.04	G/G (53, 63)	13, 25	Ref	0.5
-308G>A	G/A+A/A (41, 27)	14, 34	2.4 [1.0 - 5.7]		G/A+A/A (31, 37)	10, 32	1.4 [0.5 - 3.8]	

Ala: Alanine; Asp: Aspartic acid; BPAR: biopsy-proven acute rejection; Donors*: donor numbers may differ from those in Table 1, as 3 donors were for 2 recipients, respectively, and they were treated independently when associated with BPAR of the individual recipients. In addition, donor numbers may differ within Table 2 due to genotyping failure or due to exclusion of rare diplotypes (n < 5) so the total number of diplotypes in each SNP may be different; human leukocyte antigens (HLA-A, -B and -DR) mismatches; NA: not available; OR: Odds ratio; P: likelihood-ratio P-value; peak PRA: peak panel-reactive antibodies scores assessed by serum lymphocytotoxicity assay; Recipients[#]: recipient numbers may differ within Table 1 due to genotyping failure or due to exclusion of rare diplotypes (n < 5) so the total number of diplotypes in each SNP may be different; Ref: reference group; 95% CI: 95% confidence interval.

Chapter 6: Conclusion

TAC is the backbone of maintenance immunosuppressive therapy post-transplantation. However, challenges exist in its clinical use even under TDM, mainly due to acute rejection and kidney dysfunction. In addition, the relationship between TAC C_0 and acute kidney rejection has not been adequately shown under TDM. Therefore, the main aim of this study was to assess the impact of TAC dispositional genetics on TAC C_0/D, BPAR incidence and eGFR in a retrospective cohort of 165 Australian kidney transplant recipients and their respective donors in the first 3 months post-transplantation. In addition, the innate immunogenetic factors were examined as potential predictors of BPAR. A secondary aim was to explore if there could be a TAC C_0-rejection relationship under TDM in kidney transplant recipients in the first 14 days post-transplantation. Due to the initial study design, only short- but not long-term kidney dysfunction was investigated in this study.

6.1 Significance of the study

- ***ABCB1* 61A>G as a novel SNP contributing to TAC C_0/D variability**

As introduced in sections 1.2 and 1.5.1 in Chapter 1, recipient CYP3A4/5 are the main hepatic/intestinal metabolising enzymes of TAC and P-gp is responsible for TAC efflux. In addition, *NR1I2* and *POR* regulate CYP3A4/5 and/or P-gp expression or activity. Therefore, in Chapter 2, I examined the known key SNPs in TAC dispositional genes and their impact on TAC C_0/D and identified a novel although minor impact of recipient *ABCB1* 61A>G on TAC PK variability (explaining 3-4% of $\log_{10}$ transformed TAC C_0/D) for the first time in kidney transplant recipients in the first 3 months post-transplantation. I also confirmed the major impact of recipient *CYP3A5*3*, accounting for 25-30% of $\log_{10}$ transformed TAC C_0/D variability in the same cohort. Altogether, recipient *CYP3A5*3*, *ABCB1* 61A>G and non-genetic factors (age, sex and haematocrit)

accounted for ~35% of TAC C_0/D variability (log_{10} transformed) in the first 3 months post-transplantation. The residual unexplained TAC C_0/D variability may be due to other genetic (e.g. *CYP3A4* phenotypes) and/or non-genetic factors (e.g. co-medication), both of which could not be addressed in this study due to the initial study design.

- **TDM may attenuate the genetic influence on kidney transplant outcomes**

As introduced in section 1.5.1 in Chapter 1, recipient *ABCB1* and donor *CYP3A5* and *ABCB1* genetics are likely to affect TAC intracellular concentrations in T-cells and kidney cells, respectively. Therefore, TAC dispositional genes may also affect kidney transplant outcomes (e.g. BPAR and decreased eGFR post-transplantation).

In Chapter 3, I examined recipient and donor *CYP3A4/5*, *POR*, *ABCB1* and *NR1I2* genotype/haplotypes for their impact on BPAR incidence in the first 14 days post-transplantation and eGFR in the first 3 months post-transplantation. However, none of these genetic factors can predict these kidney transplant outcomes in this cohort. TDM attempts to limit TAC C_0 within a narrow range (e.g. 8-15 ng/mL in this study), therefore, it may have substantially counteracted the effect of recipient *CYP3A5*3* and *ABCB1* 61A>G on TAC PK and consequently reduced the risk of sub- and supra-exposure of TAC. This helped to explain why TAC dispositional genes affected TAC C_0/D (see Chapter 2), but did not impact on BPAR or eGFR under TDM conditions.

- **A temporal TAC C_0-rejection relationship in kidney transplant recipients**

As discussed in section 1.3 in Chapter 1, there is no agreement if a TAC C_0-rejection relationship exists under TDM. However, this study showed for the first time that there was a temporal relationship between TAC C_0 and BPAR in kidney transplant recipients. In Chapter 3, I found there was no difference in BPAR incidence between groups with TAC C_0 < and ≥ 8 ng/mL or TAC C_0 < and ≥ 5 ng/mL (both P-value = 0.7), this

observation also indicated that the relationship between TAC C_0 and BPAR may be variable between kidney transplant recipients. This variability is likely due to the inter-individual differences in haematocrit, plasma protein binding, TAC intracellular distribution or recipients' immunological risk of BPAR (e.g. immunogenetic differences between recipients and the immunosuppressive effect from MMF and corticosteroid). Therefore, each recipient may have their own optimal TAC C_0 TDM range, which assists to explain why some recipients experience BPAR even within or above current TAC TDM range. Furthermore, in Chapter 4, I reported for the first time that TAC C_0 on the day of, and 1 day prior to, BPAR were lower than on preceding days. In addition, adjusting for haematocrit variability assisted in identifying this temporal TAC C_0-rejection relationship. In conclusion, there was a temporal concentration-response relationship within kidney transplant recipients under TAC TDM.

- **Innate immunogenetic impact on BPAR incidence — not conclusive yet**

As introduced in section 1.5.2.1 in Chapter 1, the innate immune system is essential in rejection pathophysiology. In addition, recipients may have different immunological risks of BPAR due to immunogenetic variabilities. Therefore, the genetics of pro- and anti-inflammatory mediators, and the MyD88-dependent TLR signalling pathway, which mediates pro-inflammatory cytokine secretion, have the theoretical potential to affect BPAR. Most importantly, innate immunogenetics have never been examined in a transplant cohort treated with TAC as the only CNI.

In chapter 5, however, I did not find the recipient and donor innate immunogenetics (*CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *LY96*, *MYD88*, *TGFB*, *TLR2*, *TLR4* and *TNF*) affected BPAR incidence in TAC-treated kidney transplant recipients in the first 14 days post-transplantation. The negative finding may be due to potent

immunosuppression by TAC in both adaptive (T-cell proliferation) and innate (IL-2 secretion) immune system as described in section 1.1.2 in Chapter 1 and the use of anti-CD25 induction therapy, in over 90% of recipients in this Australian cohort, which may have inhibited IL-2/IL-2 receptor signalling. Notably, SNPs in *CASP1*, *CRP*, *IL6R*, *LY96*, *MYD88* and *TLR2* were studied for the first time for BPAR incidence in a relatively limited number of kidney transplant recipients and donors (n = 165 and 129, respectively), with missing genotypes/haplotypes in some individuals. In addition, due to the low rare allele frequency in some of these SNPs (see Table 5 in Chapter 1), it is still not conclusive whether the innate immune genetic factors investigated in this study affect BPAR in TAC-treated kidney transplant recipients.

6.2 Limitations and strengths of the study

Study limitations were summarised in the discussion in Chapters 2 to 5; specifically the limited sample size, lack of information on co-medications (especially MMF) and long-term kidney function. As a retrospective study, the limited sample size (recipient n = 165, donor = 129) may be insufficient to support the negative findings (no major PGx or immunogenetic impact on BPAR incidence and/or kidney function) in Chapters 3 and 5. For example, in Chapter 3, detection of OR > 6.1 for BPAR for *CYP3A5* genotype which the study had 80% power to detect is not biologically plausible. However, the data presented in this thesis, along with other PGx and innate immunogenetic studies may together provide valuable information for meta-analysis in the future.

Two strengths of this study should be highlighted. Firstly, this study employed linear and generalised linear (binomial) mixed effects modelling to account for repeated measurements and confounding factors, which enhanced the chance to identify the minor contribution of recipient *ABCB1* 61A>G to TAC C_0/D variability and the TAC

C_0-rejection relationship (see Chapters 2 and 4). In addition, multiple comparison adjustment was applied throughout the whole study, which can attenuate the chances of interpreting "false positive" results. Secondly, this was the first study to compare TAC C_0 on the days prior to and on the day of BPAR within and between recipients, as they can develop BPAR on different days post-transplantation. Also, it was the first study to account for haematocrit in TAC C_0-rejection relationship as haematocrit contributes to TAC C_0 intra- and inter-individual variability (see section 1.2.2.2 in Chapter 1). Most importantly, it was the first study to allow for the inter-individual variabilities in the relationship between TAC C_0 and BPAR via incorporating random effects (TAC C_0 and haematocrit both on random slope and intercept) in the generalised linear (binomial) mixed effects modelling.

6.3 Future perspectives

- **Progress of TAC PK monitoring: from whole blood to cells**

Current TAC TDM is based on TAC C_0 monitoring, however, TAC C_0-rejection relationship is variable between recipients. In addition, the whole blood TAC concentration is not therapeutically active, as only the plasma unbound TAC can enter the T-cells (the immunosuppressive site of action) to suppress the immune system. It has also been reported that there is a lack of a significant correlation between TAC C_0 and intracellular TAC concentrations in PBMCs (70-85% of which are T-cells) [168], indicating whole blood TAC concentration is unlikely to accurately reflect the immunosuppressive effect of TAC *in vivo*.

Liquid chromatography–mass spectrometry quantification (LC-MS/MS) methods have been established in recent years for plasma unbound and PBMC TAC quantification [169-171], although they are much more laborious than TAC C_0 monitoring. Several studies have already reported that PBMCs but not trough whole blood CNI (TAC/CsA)

concentrations were associated with BPAR incidence following liver or kidney transplantation [172-174]. However, PBMC TAC concentration was not correlated with BPAR in kidney transplant recipients, likely due to the small sample size and low BPAR incidence (n = 6 out of 96 recipients) [112]. Notably, similar to the limitations in those cross-sectional studies of TAC C_0-rejection relationship, PBMC TAC concentrations used to categorize recipients into rejection and non-rejection groups were not always concurrent with BPAR.

Therefore, future studies should investigate what genetic and non-genetic determinants are causing the variable relationship between TAC C_0 and BPAR. Also, it is worthwhile exploring if plasma unbound and/or PBMC TAC concentrations can predict BPAR incidence in kidney transplant recipients.

- **TAC PD monitoring: beyond drug concentrations**

Although TAC PK monitoring is essential to correct inter- and intra-recipient viabilities in TAC disposition, it does not reflect recipients' immunosuppressive response. Therefore, TAC PD biomarker monitoring may play an additional TDM role to TAC PK biomarker monitoring to guide TAC clinical use in the future. As reviewed recently [175], calcineurin phosphatase activity has been quantified to assess the degree of immunosuppression. However, calcineurin phosphatase activity is still not used as a TAC PD biomarker due to low reproducibility, expensive costs and time-consuming laboratory practice. Other PD biomarkers, for example, NFAT-regulated gene expression and NF-κB and p38 mitogen-activated protein kinases phosphorylation, have less specificity than calcineurin phosphatase activity to predict the degree of immunosuppression. However, they may better reflect the biological efficacy of TAC than monitoring concentration alone. More validation studies are still needed for these potential PD biomarkers before clinical implementation.

- **Prospect in biomarkers: early and non-invasive genetic and non-genetic predictors of BPAR incidence**

Current biomarkers widely used for BPAR are graft histology (e.g. needle biopsy) and serum creatinine/eGFR. However, biopsy is invasive and can increase the chance of infection post-transplantation. Most importantly, biopsy is only diagnostic but not predictive of BPAR. In addition, serum creatinine/eGFR is non-specific to BPAR.

Genetic factors in immune system (e.g. NFAT and NF-κB) and TAC PD pathway (e.g. immunophilin and calcineurin-calmodulin complex) may predict BPAR as they are theoretically able to affect individuals' immunological risk of rejection and recipients' response to immunosuppressive therapy. Although no genetic factors have been accepted as predictors of BPAR [176], it might be due to the limited sample size in cross-sectional studies and the selectively designed gene panels. Therefore, genome-wide association studies in large kidney transplant cohorts (likely with multi-centre collaboration) may enhance the chance to identify the genetic predictors of BPAR.

Other biochemical factors, e.g. donor-reactive T-cell response and epitope mismatch load, have been studied as novel biochemical predictors of BPAR but still require validation before clinical implementation [177].

6.4 Conclusion

In conclusion, *ABCB1* 61A>G and *CYP3A5*3* significantly affected TAC C_0/D variability in kidney transplant recipients in the first 3 months post-transplantation. Also, there was a temporal TAC C_0-rejection relationship under TDM in kidney transplant recipients in the first 14 days post-transplantation. However, validations of these findings are still needed in the future due to the limited sample size in this study. Future work can focus on plasma unbound or PBMCs TAC concentration monitoring,

TAC PD monitoring and the novel genetic and non-genetic predictors of BPAR to maximise TAC immunosuppression and minimise BPAR incidence in kidney transplant recipients.

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