

IMPACT OF DONOR *CYP3A5* GENOTYPE ON PHARMACOKINETICS OF TACROLIMUS IN SOUTH AFRICAN PAEDIATRIC LIVER TRANSPLANT PATIENTS

Caitlin Wheeler

Student number: 2490170

Supervisor: Dr Janine Scholefield

Co-supervisors: Dr June Fabian and Prof Jean Botha



**UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG**

A research report (in the format of a “submissible” paper)
submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg,
in partial fulfilment of the requirements for the Degree of Master of Science
in Medicine (Genomic Medicine) by Research and Coursework.

Johannesburg, 2022

NATIONAL HEALTH LABORATORY SERVICE

School of Pathology, University of the Witwatersrand



DIVISION OF HUMAN GENETICS



UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG

Hospital Street, Johannesburg, 2001 | PO Box 1038, Johannesburg, 2000
[T] +27 11 439 9223 | [M] +27 78 080 8841 | [F] +27 11 439 9226 | [E] human.genetics@nhls.ac.za

30 November 2022

Re: Research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, by Ms Caitlin Wheeler in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Genomic Medicine)

Dear examiner,

Thank you for agreeing to examine the research report submitted by Ms Caitlin Wheeler (student number 2490170) entitled "Impact of donor CYP3A5 genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients". This research report is being submitted by the candidate in the format of a "submissible" paper as per the Style Guide for Thesis, Dissertation and Research Reports (updated March 2016) by the University of the Witwatersrand Faculty of Health Sciences. The research report contributes 30% towards the final mark of the MSc (Med) Genomic Medicine degree for which the candidate is enrolled.

The candidate and her supervisors have chosen to submit the paper to the Journal of Translational Medicine. This international journal is a peer-reviewed open-access medical journal published by BioMed Central. The submissible paper attached is therefore written in accordance with the author guidelines of the stipulated journal. These guidelines have been attached for your reference in the appendices section of this document. The only deviation from these guidelines is that the manuscript has been presented as one complete document (including all tables and figures) for ease of marking, rather than being submitted separately.

The research protocol has also been attached as an appendix for the purpose of providing an extended literature review and further contextualise the study. The protocol has already been assessed and approved by the Faculty of Health Sciences Post-Graduate Assessors committee and is therefore not for examination.

Please refer to the Table of Contents for a complete list of the contents provided in this research report.

Yours sincerely,

Caitlin Wheeler (Candidate)

Dr Janine Scholefield (Supervisor)

Dr June Fabian (Co-supervisor)

Prof Jean Botha (Co-supervisor)

This report is intended solely to record the observations and/or opinion of the writer. It does not constitute a medico-legal report

Declaration

I, Caitlin Wheeler, declare that this Research Report (in the format of a “submissible” paper) is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine (Genomic Medicine) by Research and Coursework at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

A handwritten signature in black ink, consisting of a large, stylized 'C' followed by a series of sharp, vertical strokes and a horizontal line.

Caitlin Wheeler

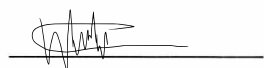
Student number: 2490170

27th day of February 2022 in Johannesburg

Contribution of Candidate

Student's contribution to article(s) and agreement of co-author(s)

I, Caitlin Wheeler, student number 2490170, declare that this Research Report, entitled "Impact of donor *CYP3A5* genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients", is my own work and that I contributed significantly towards the research findings presented in the paper intended for publication below.



Signature of Student

30/11/2022

Date



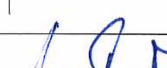
Signature of Primary Supervisor

30th November 2022

Date

Agreement by co-authors:

By signing this declaration, the co-authors listed below agree to the use of the research findings intended for a published article by the student as part of their Research Report.

Authors	Name	Signature	Date
1 st	Ms Caitlin Wheeler		30 November 2022
2 nd	Dr Janine Scholefield		30th November 2022
3 rd	Dr June Fabian		30 November 2022
4 th	Prof Jean Botha		30 November 2022

Dedication

In memory of
all liver transplant heroes

Presentations arising from this research

1. University of the Witwatersrand Faculty of Health Science Postgraduate Biennial Research Day, 15th September 2022 (poster presentation)
2. University of the Witwatersrand Molecular Biosciences Research Thrust (MBRT) Postgraduate Symposium, 1st December 2022 (poster presentation)
3. Young Investigator Forum of the International Congress of Human Genetics 2023, 21st February 2023 (speed talk and poster presentation)
4. International Congress of Human Genetics 2023 Cape Town, 22nd-26th February 2023 (poster presentation)

Abstract

Tacrolimus is characterised by a narrow therapeutic target range and wide interpatient variability. Pharmacogenetic research attributes *CYP3A5* single nucleotide polymorphisms to the variable inter-patient tacrolimus concentration dose ratios (CDR). In this study, we compared the mean tacrolimus CDR in paediatric liver transplant recipients and their living liver donors' *CYP3A5* rs776746 T>C genotypes (**1/*1*, **1/*3* and **3/*3*), accounting for donor and recipient characteristics. The graft-to-recipient weight ratio and the *CYP3A5* donor genotypes were found to be independent factors significantly impacting the mean tacrolimus CDR. Donor *CYP3A5* expressors (**1/*1* and **1/*3*) were shown to have significantly lower recipient tacrolimus CDRs, therefore, higher dosages would be required in comparison to *CYP3A5* non-expressors to reach the same therapeutic target range. The potential implementation of a stratified medicine dosage algorithm, combining living liver donor *CYP3A5* genotyping with the calculation of the graft-to-recipient weight ratio, may predict the optimal tacrolimus dosage schedules for liver transplant recipients.

Acknowledgements

I would like to express my deepest gratitude to my supervisors for their support at every stage of this research project. To Dr Janine Scholefield, I am deeply indebted to you for this incredible opportunity and your support on this project. Your passionate attitude is inspiring and you have opened my eyes to the “beautiful” side of science. To Dr June Fabian, I could not have undertaken this journey without your encouragement and invaluable guidance from the outset. It has been such a privilege to learn from your wealth of experience. To Prof Jean Botha, thank you for your insight and for opening a unique window for me into transplant surgery. My gratitude extends to the National Research Foundation for the funding opportunity to undertake my studies and complete this project.

I am so grateful for my time at the CSIR in the BIGG Research Group. Special thanks to Dr Lara Contu for taking me under your wing and guiding me in my lab work. Thank you to the rest of BIGG for providing such significant support both in the lab and at the lunch table.

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Thank you to my loved ones Krisha, Lia, and Luke, as well as Charles, for your support.

Lastly, I would like to express my deepest appreciation to all the liver donors who answered my calls, met with me and were a part of this project.

Table of Contents

Declaration	i
Contribution of Candidate	ii
Dedication.....	iii
Presentations arising from this research.....	iv
Abstract	v
Acknowledgements.....	vi
Table of Contents	vii
List of Figures	ix
List of Tables	ix
List of Abbreviations	x
Research report in the format of a “submissible” paper	xi
1. Abstract for Journal Submission	xii
2. Background.....	1
3. Methods	5
3.1. Retrospective arm	5
3.2. Prospective arm	6
3.3. Data collection	6
3.4. Donor Genetic Analysis	7
3.5. Recipient tacrolimus concentration dose ratio	10
3.6. Statistical Analysis	10
4. Results	11
4.1. Donor <i>CYP3A5</i> genotype	11
4.2. Donor <i>CYP3A5</i> genotype effect on tacrolimus CDR	14
4.3. Clinical factors associated with tacrolimus concentration dose ratio	16
5. Discussion	18
6. Conclusion	21
7. Declarations.....	22
8. References.....	23

Appendices

Appendix 1: Human Research Ethics Committee (Medical), University of the Witwatersrand (M220351)

Appendix 2: Human Research Ethics Committee (Medical), University of the Witwatersrand (M191006)

Appendix 3: Human Research Ethics Committee (Medical), University of the Witwatersrand (M190749)

Appendix 4: Supplementary Material and Methods

Appendix 5: Information and Consent forms

Appendix 6: Approved Research Protocol

Appendix 7: Turnitin Report and Plagiarism Declaration

Appendix 8: Journal Author Guidelines for Submissible Format

List of Figures

Figure 1. Sequence variation defining <i>CYP3A5</i> *1, *3, *6, and *7 alleles.	3
Figure 2. Estimated frequencies of <i>CYP3A5</i> *1, *3, *6 and *7 alleles in the Sub-Saharan African and European biogeographical groups (adapted from PharmGKB).	4
Figure 3. Schematic and agarose gel result of SNP genotyping methodology for <i>CYP3A5</i> *3 rs776746 single nucleotide polymorphisms	9
Figure 4. Flow diagram of patient recruitment process and exclusion criteria.	11
Figure 5. Phenotype frequencies of <i>CYP3A5</i> expressors and non-expressors in this study cohort in comparison to the African and European biogeographical cohorts adapted from PharmGKB.	13
Figure 6. CDR for each day in the first 15 days of tacrolimus therapy for donor <i>CYP3A5</i> genotype groups *1/*1 (TT), *1/*3 (TC) and *3/*3 (CC)..	14
Figure 7. Mean concentration dose ratio (CDR) over first 15 days of tacrolimus therapy for donor <i>CYP3A5</i> genotype groups *1/*1, *1/*3 and *3/*3.	15

List of Tables

Table 1. <i>CYP3A5</i> single nucleotide polymorphisms, associated clinical implications and allele frequencies. ...	3
Table 2. Demographic information and clinical phenotypes stratified by genotype group for living liver donors and their corresponding liver transplant recipients	12
Table 3. Frequency and distribution of <i>CYP3A5</i> genotypes across living donor groups	12
Table 4. Results of univariable and multivariable regression to determine factors associated with the mean CDR over the first 15 days of immunosuppression post-transplant in paediatric liver recipients	17

List of Abbreviations

African Liver Tissue Biorepository (ALTBio)
Calcineurin-inhibitor (CNI)
Concentration dose ratio (CDR)
Council for Scientific and Industrial Research (CSIR)
Cytochrome P450 3A5 gene (*CYP3A5*)
Cytochrome P450 3A5 protein (CYP3A5)
End-stage liver disease (ESLD)
Ethylenediaminetetra-acetic acid (EDTA)
Expressor (ex)
General Linear Model (GLM)
Genomic DNA (gDNA)
Graft-to-recipient weight ratio (GRWR)
Hardy–Weinberg Equilibrium (HWE)
Interquartile range (IQR)
Living donor liver transplantation (LDLT)
Non-expressor (non-ex)
Polymerase chain reaction (PCR)
Restriction Fragment Length Polymorphism (RFLP)
Sydney Brenner Institute for Molecular Biosciences (SBIMB)
Wits Donald Gordon Medical Centre (WDGMC)

Research report in the format of a “submissible” paper

To be submitted to the Journal of Translation Medicine

Impact of donor *CYP3A5* genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients

Caitlin Wheeler¹, Janine Scholefield², June Fabian³, Jean Botha³

¹Division of Human Genetics, National Health Laboratory Service and School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, JHB, South Africa

²Bioengineering and Integrated Genomics Group, NextGen Health, Council for Scientific and Industrial Research, Pretoria, South Africa

³Wits Donald Gordon Medical Centre, School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, Parktown, JHB, South Africa

Corresponding Author:

Caitlin Wheeler

Division of Human Genetics, Corner Hospital and De Korte Streets, JHB, South Africa, 2001

[T]: 071 070 3091 [E]: cawheeler02@gmail.com/2490170@students.wits.ac.za

1. Abstract for Journal Submission

Background: Tacrolimus is characterised by a narrow therapeutic index and wide inter-patient variability. It is critical to reach optimal therapeutic blood levels to prevent solid organ rejection or overimmunosuppression, especially in paediatric liver transplant recipients where in the South African context poor treatment outcomes are frequently observed in the clinic. Pharmacogenetic research, although scarce in the South African population, suggests that single nucleotide polymorphisms within the cytochrome P450 3A5 (*CYP3A5*) gene contribute to the variability in tacrolimus dosing requirements. The rs776746 T>C polymorphism, *CYP3A5**3, results in a splice defect and a non-functional enzyme. Clinically, to reach the same tacrolimus concentration dose ratio, patients with at least one *1 allele, classified as expressors (*1/*1 and *1/*3), require a higher tacrolimus dose than patients homozygous for the *3 allele (non-expressors). In this study, we compare the pharmacokinetics of tacrolimus for paediatric liver transplant patients to the donors' *CYP3A5* genotypes considering both donor and recipient characteristics.

Methods: Blood samples from 46 living liver donors were collected, gDNA extracted and *CYP3A5* rs776746 T>C genotype established (PCR, RFLP analysis and validated by Sanger sequencing). The relationship of donor and recipient characteristics with the mean concentration dose ratio over the first 15 days of tacrolimus immunosuppression was analysed using a general linear model. Non-confounding significant variables were included in a multiple regression model.

Results: This study showed that all expressor donors genotyped as *CYP3A5**1 homozygotes were of Black African self-reported ethnicity. The graft-to-recipient weight ratio and the *CYP3A5* rs776746 T>C donor genotypes were independent factors that significantly ($p < 0.05$) impacted the mean tacrolimus concentration dose ratio over the first 15 days of immunosuppression. Donor hepatic *CYP3A5* expressors (*CYP3A5**1/*1 and *1/*3) have significantly lower recipient mean tacrolimus concentration dose ratios post-transplant and therefore, higher dosages are required in comparison to *CYP3A5* non-expressors (*3/*3) to reach the same therapeutic target range for tacrolimus.

Conclusions: Including the rs776746 T>C genotyping of living liver donors for *CYP3A5*, the calculation of the graft-to-recipient weight ratio, and other characteristics in a clinical setting may predict the optimal tacrolimus trough levels in the recipient. This genetic information

will contribute towards the development of an Afrocentric tacrolimus precision medicine dosing algorithm that can be utilised to improve the post-transplant drug administering routine.

Keywords: Tacrolimus, Liver Transplant, *CYP3A5*, Pharmacokinetics, Precision Medicine

2. Background

Liver transplantation is the standard of care for children with end-stage liver disease (ESLD). Organs for liver transplantation are sourced from living donors (who donate a portion of their liver, usually a lobe) or from deceased donors [1,2]. The Wits Donald Gordon Medical Centre (WDGMC) hosts the largest paediatric liver transplant unit in South Africa [1,2]. Children with ESLD in South Africa are wait-listed and, when an organ becomes available, those with the most severe disease are prioritised for transplantation [1,3]. In response to deceased donor organ shortages and high wait-list mortalities, a living donor liver transplant (LDLT) programme was initiated at WDGMC in 2012 with ongoing success [2].

To prevent organ rejection, lifelong immunosuppressive drugs are administered starting from the time of surgery. The backbone of immunosuppressive regimens includes a combination of corticosteroids, calcineurin-inhibitors (CNIs), and mycophenolic acid [4]. In the WDGMC transplant unit, children are weaned off corticosteroids within 6 months of their transplant whilst the CNI, tacrolimus, remains the primary immunosuppressive drug with addition of mycophenolic acid as needed [2]. Dosing schedules for tacrolimus aim to achieve the optimal therapeutic blood levels to avoid organ rejection, whilst reducing potential for drug toxicity and adverse drug reactions [4]. Tacrolimus is characterised by a well-established narrow therapeutic index and high inter-patient variability, which makes achieving blood target levels difficult [5,6]. Currently, at WDGMC, there is no personalised dosing schedule for initiating tacrolimus in paediatric liver transplant patients. All children are started on a standard tacrolimus dose within 24 hours of their liver transplant and, thereafter, daily doses are titrated based on trough concentrations [7]. The aim is to achieve blood concentrations of between 12-15 ng/ml in the immediate post-operative period [5,7]. Anecdotally, clinicians at WDGMC have observed that children of African ancestry require much higher doses to achieve therapeutic target drug levels [7].

In the setting of solid organ transplantation, the wide interpatient variability in tacrolimus response has been attributed to cytochrome P450 3A5 (*CYP3A5*) genetic variation, drug-drug interactions (such as fluconazole), as well as patient and graft characteristics [5,8–11]. Specifically relating to liver transplantation, significant factors include graft-to-recipient weight ratio (GRWR), recipient age, and body weight [5,10,12].

Studies in North America, Europe, Asia, and Africa have investigated the association between *CYP3A5* polymorphisms and tacrolimus pharmacokinetics in liver, kidney, lung and heart transplantations [6,13–16]. Paediatric patients tend to be the focus of this research due to the high interpatient pharmacokinetic variability observed at a younger age [5,11,12,17]. Three *CYP3A5* single nucleotide polymorphisms (SNPs) are significantly associated with tacrolimus metabolism: rs776746 C (*CYP3A5**3), rs10264272 T (*CYP3A5**6), and rs41303343 AA (*CYP3A5**7) (Figure 1 and Table 1) [9,18]. These SNPs result in non-functional *CYP3A5* enzyme products, thus reducing the metabolism/clearance of tacrolimus (Table 1) [9,19,20]. *CYP3A5* expressors are individuals with at least one copy of the *CYP3A5**1 allele and this group is further classified into normal (*1/*1) or intermediate metabolisers (*1/other) [9,18,20]. Non-expressors have two non-functional alleles (e.g., *3/*3) and are classified as poor metabolisers [9,18,20]. Tacrolimus metabolism occurs in the liver and small intestine, with the most significant pharmacokinetic contribution from the liver [5,13,17].

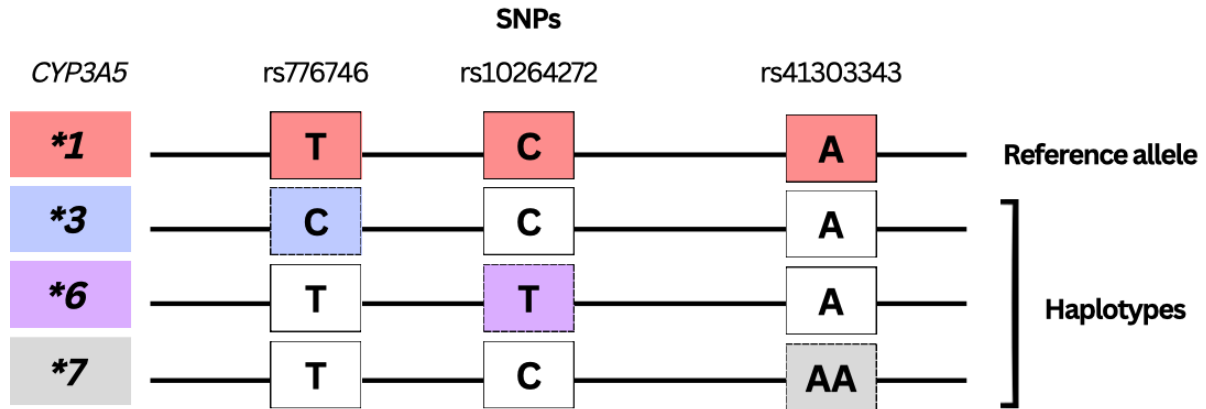


Figure 1. Sequence variation defining *CYP3A51, *3, *6, and *7 alleles.**

Top panel containing *CYP3A5**1 defined as the reference allele sequence. Single nucleotide polymorphisms (SNPs) at rs776746, rs10264272 and rs41303343 shown in the panels below the reference. Variants are orientated in the forward direction (3' to 5') on the minus strand.

Table 1. *CYP3A5* single nucleotide polymorphisms and associated clinical function

Allele name, Variation ¹ and rsID	Variant impact	CPIC allele clinical function ²
<i>CYP3A5</i>*1	Reference allele	Full function →
<i>CYP3A5</i>*3 (99672916T>C) rs776746	splice defect	No function ✗
<i>CYP3A5</i>*6 (99665212C>T) rs10264272	splice defect	No function ✗
<i>CYP3A5</i>*7 (99652770_99652771insA) rs41303343	frameshift (T346fs)	No function ✗

¹Allele variant reported according to genome build GRCh38 (NC_000007.14). ²Clinical Pharmacogenetics Implementation Consortium (CPIC) allele clinical functional status[9,18].

In the *CYP3A5* allele frequency distributions, curated by PharmGKB (Figure 2), the biogeographical group of European cohorts have the highest frequency of *CYP3A5**3 (93%), which contrasts with Sub-Saharan African cohorts where *CYP3A5**1 allele frequency is highest (48%) [18]. Similarly, a study replicated these findings in a Sub-Saharan African cohort where the *CYP3A5**3 allele was observed at higher frequencies in non-African populations in comparison to lower frequencies in African populations[21]. It is noteworthy that *CYP3A5**6 and *7 have been observed in Sub-Saharan African cohorts but have rarely been reported in European cohort studies [18]. Although certain *CYP3A5* alleles, such as *1 and *3, may be more common among African, European and other global biogeographical populations, these populations may have non-negligible frequencies of *CYP3A5* expressors or non-expressors present.

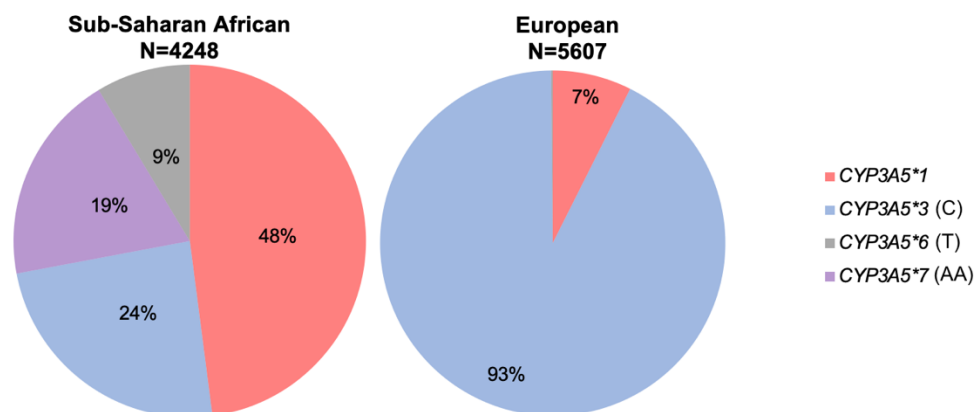


Figure 2. Estimated frequencies of *CYP3A51, *3, *6 and *7 alleles in the Sub-Saharan African and European biogeographical groups (adapted from PharmGKB).**

Clinically, the consequences of the *CYP3A5* polymorphisms are that expressors are predisposed to having low therapeutic drug levels as a result of the rapid metabolism of the drug, which can lead to rejection of the transplanted graft [5,9,17]. By contrast, non-expressors are at risk of overshooting target therapeutic drug levels, predisposing them to various tacrolimus-related toxicities (most commonly neuropsychiatric and renal) as well as adverse drug reactions [4,5]. As illustrated above in Figure 2, since expressors are more prevalent in African populations, this group is likely to require higher tacrolimus doses to

reach the same therapeutic level and may be at higher risk of graft rejection when compared to non-expressors [9,18,22]. Therefore, the implementation of pre-operative living liver donor genotyping analysis for *CYP3A5* could allow transplant physicians, specifically those treating patients of African ancestry, to stratify tacrolimus dosing schedules to achieve therapeutic drug targets more accurately [5,6].

In the setting of paediatric liver transplantation in Southern Africa, no studies have investigated the relationship between *CYP3A5* genotype and the pharmacokinetics of tacrolimus — in part, because access to transplantation is extremely limited [1]. Therefore, research is required to determine *CYP3A5* genotypes of living liver donors, and investigate the association between *CYP3A5* genotype and associated clinical factors with tacrolimus doses in a cohort of children undergoing living liver donor transplantation in a Southern African setting.

The study aimed to (i) determine living liver donor *CYP3A5* genotype frequencies limited to rs776746; (ii) assess the association between *CYP3A5* donor genotype and recipient tacrolimus dose and therapeutic drug levels after transplantation; and (iii) determine the impact of clinical factors such as GRWR, age, weight, sex, and self-reported ethnicity on the mean tacrolimus CDR over the first 15 days post-transplant.

3. Methods

This was a single-centre study of living liver donors and their corresponding paediatric liver transplant recipients from the Transplant Unit at WDGMC. Ethics approval was granted by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (M220351; Appendix 1). The study comprised two components: the first was a retrospective arm and the second a prospective arm.

3.1. Retrospective arm

The retrospective arm was performed using the African Liver Tissue Biorepository (ALTBio), a collaboration between the Council for Scientific and Industrial Research (CSIR), WDGMC, and the Sydney Brenner Institute for Molecular Biosciences (SBIMB), University of the

Witwatersrand. ALTBio was initiated in 2019 with the aim to create a repository of pharmacogenomic profiles from LDLT donors (M191006 Human Research Ethics Committee (Medical), University of the Witwatersrand; Appendix 2). Existing genotype data records from participants already consented to ALTBio study were accessed for this analysis.

3.2. Prospective arm

For the prospective arm, written informed consent was obtained from living liver donors who had undergone transplant procedures at WDGMC prior to the start of ALTBio in 2019 (3.1). Eligibility criteria for recruitment to this study were: (i) any donor that underwent a living donor liver transplant operation at WDGMC since inception of the WDGMC LDLT programme; (ii) the recipient was a first-time liver transplant recipient; and (iii) the recipient was younger than 18 years at time of transplant. Exclusion criteria: (i) simultaneous liver and kidney transplants; (ii) repeat transplantation; and (iii) successful recruitment to ALTBio study.

A six millilitre whole blood sample was collected in ethylenediaminetetra-acetic acid (EDTA) blood collection tubes after receiving written informed consent from each living liver donor. All blood samples were frozen at -20°C until DNA extraction.

3.3. Data collection

For the retrospective and prospective arms (Section 3.1 and 3.2), the study variables were accessed from the WDGMC Paediatric Liver Transplant Research Database (M190749 Wits Human Research Ethics Committee (Medical), University of the Witwatersrand; Appendix 3). Recipient variables included: age, weight, sex, and self-reported ethnicity; Donor variables included: age, weight, sex, self-reported ethnicity, and graft weight. The GRWR was calculated by dividing the graft weight (kg) by the recipient weight (kg). Ethnicity is a multifaceted construct based on common cultural attributes [23]. In this study, self-reported ethnicity was categorised as Black African, Coloured, Indian, Asian or White in accordance with the demographic categories published in the mid-year population estimates for 2019 by Statistics South Africa [24]. In this study, ancestry is defined as a common line of genealogical or genetic descent [23].

3.4. Donor Genetic Analysis

Two methods of genotyping were used to assign *CYP3A5* allelic status:

i) Genotyping of rs776746 by PCR/RFLP (n=27)

Genomic DNA (gDNA) was manually extracted from whole blood samples as per a modified salting out protocol established as the standard operating procedure used at the biobank laboratory at the SBIMB [25,26]. Optimised polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were performed to determine *CYP3A5**3 (rs776746) genotype as illustrated in Figure 3. PCR was performed using a designed forward primer and published reverse primer as provided in Appendix 4 [6]. Phusion Green High-Fidelity DNA polymerase (Thermo Scientific, USA) was used as per manufacturer's instructions with the following cycling conditions: Step 1, 98 °C for 30 s; Step 2, 98 °C for 10 s, 57.9 °C for 15 s, 72 °C for 5 s, repeated for 35 cycles; and Step 3, 72 °C for 10 min. The amplified PCR products were purified using the GeneJET PCR purification kit (Thermo Scientific, USA) according manufacturer's instructions followed by RFLP analysis using SspI-High Fidelity (New England Biolabs, UK). Gel electrophoresis was used to assign genotypes as shown in Figure 2B. Selected samples with respective *1/*1, *1/*3 and *3/*3 genotypes identified through RFLP analysis were selected for Sanger sequencing validation. The designed forward primer was used to sequence the amplicons with the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Scientific, USA) and on an ABI 3500xl genetic analyser (Thermo Scientific, USA). Additional methods are provided as supplementary material (Appendix 4).

ii) Genotyping by GenoPharm SNP array (n=29)

Thirty nine samples had been previously genotyped on a GenoPharm array and retrospective *CYP3A5* genotype data was accessed. This open array chip covers multiple variants including rs776746 (*CYP3A5**3), rs10264272 (*6) and rs41303343 (*7). To increase the sample size of our cohort, the data available for *CYP3A5* rs776746 was included in this analysis as the patients' tacrolimus CDRs had been acquired. However, since this array calls *6 and *7 alleles, which does not extend to the aforementioned PCR-RFLP, samples with *6 and *7 alleles were excluded from this analysis.

Given the timeframe, this preliminary analysis will present findings for the *CYP3A5* rs776746 polymorphism only due to the lack of research in a population representative cohort from Southern Africa considering the definitive knowledge of the impact of this polymorphism on the pharmacokinetics of tacrolimus [9]. In this study we defined individuals with *CYP3A5**1/*1 (TT) or *CYP3A5**1/*3 (TC) genotype at rs776746 as expressors (ex) and individuals with *CYP3A5**3/*3 (CC) genotype as non-expressors (non-ex).

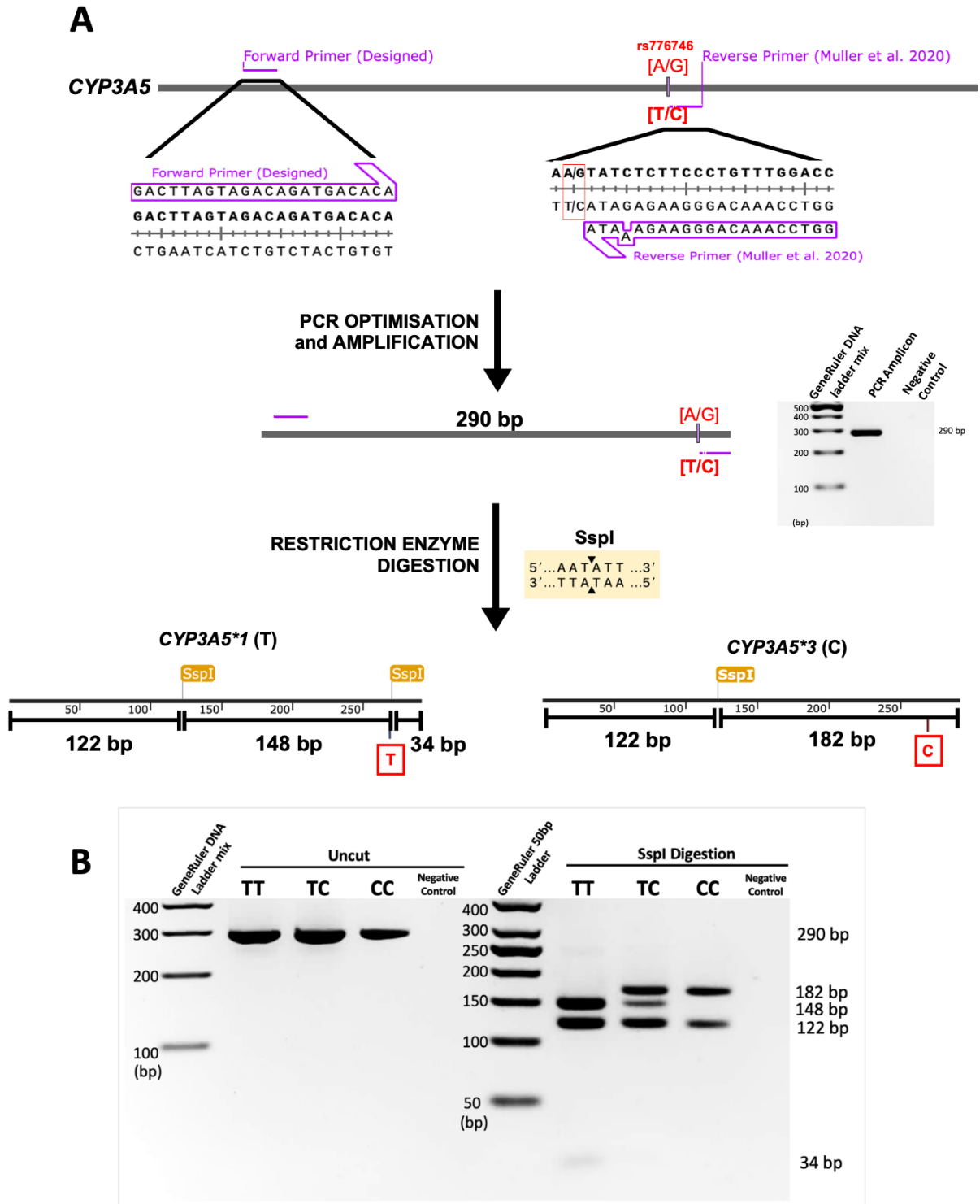


Figure 3. Schematic and agarose gel result of SNP genotyping methodology for *CYP3A53 rs776746 single nucleotide polymorphisms**

A. The rs776746 T>C SNP (*CYP3A5**3 defining variant) is highlighted in red. PCR amplification was performed using a designed forward primer and a published reverse primer [6]. A mismatch in the reverse primer sequence allows for the introduction of a second *SspI* restriction enzyme cut site in the case of *CYP3A5**1 (T). Figure created using SnapGene sequence software. **B.** 3% agarose gel showing the uncut products of *1/*1, *1/*3 and *3/*3 genotype next to the *SspI* digestion results. *SspI* digestion produced the following band sizes are for each genotype: *CYP3A5**1/*1 (148, 122 and 34 bp); *1/*3 (182, 148 and 122 bp); and *3/*3 (182 and 122 bp) allowing for discerning of *CYP3A5* genotypes.

3.5. Recipient tacrolimus concentration dose ratio

In hospital tacrolimus trough levels were monitored in-hospital post-transplant until discharge. The therapeutic drug protocol for tacrolimus requires a trough level to be measured two hours before administration of the next dose. Tacrolimus is measured using a chemiluminescent microparticle immunoassay [27]. Available tacrolimus trough levels and dosages for each recipient were accessed retrospectively from the WDGMCPaediatric Liver Transplant Research Database and from available clinical records. A concentration dose ratio was calculated for each day post-transplant until discharge according to the following equation:

$$CDR = \frac{\text{tacrolimus trough level in blood (ng/ml)}}{\text{total daily tacrolimus dosage (mg)} / \text{recipient weight pretransplant (kg)}}$$

3.6. Statistical Analysis

Possible deviations of the *CYP3A5* genotype frequencies from Hardy–Weinberg Equilibrium (HWE) were investigated through an Exact Test for HWE using the genetics package in R-studio, using a 5% level of significance. Descriptive analysis of all study variables was presented as frequency and percentage tabulation for categorical variables, and mean (with standard deviation) or median (with interquartile range (IQR)) for continuous variables, depending on the distribution of the data. Comparison of categorical study variables between donor genotype groups was performed using Fisher’s exact test. Comparison of continuous study variables between donor genotype groups was performed using one-way ANOVA (or the Kruskal-Wallis test where the data did not meet the assumptions of the one-way ANOVA). The relationship of each study variable with mean CDR over the first 15 days of tacrolimus therapy was analysed using a General Linear Model (GLM). Variables significant at $p < 0.20$ were combined into a multiple regression model; each pair of variables for possible confounding effects. Non-significant variables were removed sequentially until all remaining variables were significant at $p < 0.05$. Confounding between pairs of variables was assessed as follows: For pairs of categorical variables, the chi-squared test (or Fisher’s exact test for 2x2 tables) was used; a value of Cramer’s V (or the phi coefficient for Fisher’s exact test) > 0.50

was regarded as too strong an association to include both variables in the multivariable model. Pairs of continuous variables were assessed similarly, using Spearman's correlation coefficient. Categorical-continuous variable pairs were assessed by one-way ANOVA or the Kruskal-Wallis test, using the corresponding effect size parameter (Cohen's d or the r-value) to assess confounding. Data analysis was carried out using SAS version 9.4 for Windows. A 5% significance level was used.

4. Results

4.1. Donor *CYP3A5* genotype

A total of 46 living liver donors and their corresponding liver transplant recipients were included in this study (Figure 4). Demographic information and clinical phenotype stratified by *CYP3A5* genotype group are summarised in Table 2.

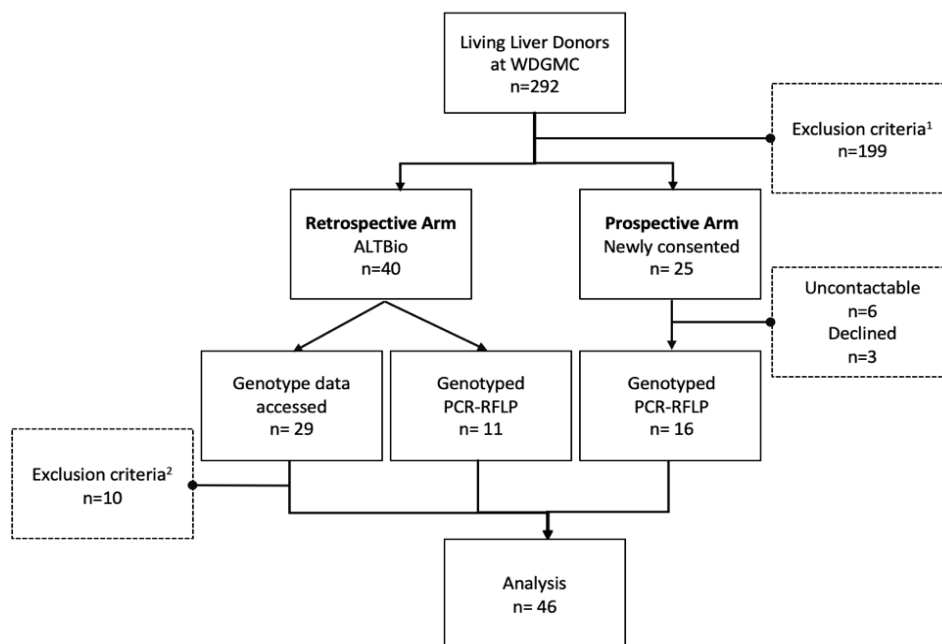


Figure 4. Flow diagram of patient recruitment process and exclusion criteria.

Inclusion criteria: any donor that underwent a living donor liver transplant operation at WDGMC with available tacrolimus trough levels; the recipient was a first-time liver transplant recipient; and the recipient was younger than 18 years at time of transplant. ¹Exclusion criteria: simultaneous liver and kidney transplants; and repeated transplants. ²Exclusion criteria: samples called as *CYP3A5**6 or *7 by GenoPharm array were excluded from this analysis of *CYP3A5* rs776746.

Table 2. Demographic information and clinical phenotypes stratified by genotype group for living liver donors and their corresponding liver transplant recipients (n=46)

Characteristic ¹	Donor <i>CYP3A5</i> genotype			Overall
	*1/*1 (ex)	*1/*3 (ex)	*3/*3 (non-ex)	
n (%)	18 (40)	14 (30)	14 (30)	46 (100)
DONORS				
age (y) median (IQR)	30 (26-41)	33 (29-39)	36 (31-41)	33 (28-40)
sex				
male	11 (61)	5 (36)	2 (14)	18 (39)
female	7 (39)	9 (64)	12 (86)	28 (61)
self-reported ethnicity ²				
Black African	18 (100)	6 (43)	0 (0)	24 (52)
White	0 (0)	1 (7)	10 (71)	11 (24)
Indian	0 (0)	2 (14)	2 (14)	4 (9)
Coloured	0 (0)	5 (36)	2 (14)	7 (15)
weight (kg) median (IQR) ³	71 (63-82)	66 (59-75)	68 (63-73)	68 (62-80)
RECIPIENTS				
age (y) median (IQR)	2.8 (1.5-3.3)	1.9 (0.9-3.1)	3.1 (0.9-8.9)	2.3 (1.4-3.4)
sex				
male	7 (39)	10 (71)	7 (50)	24 (52)
female	11 (61)	4 (29)	7 (50)	22 (48)
self-reported ethnicity ²				
Black African	18 (100)	6 (43)	0 (0)	24 (52)
White	0 (0)	1 (7)	10 (71)	11 (24)
Indian	0 (0)	3 (21)	2 (14)	5 (11)
Coloured	0 (0)	4 (29)	2 (14)	6 (13)
weight (kg) median (IQR) ³	12.2 (10.5-16.0)	11.9 (7.5-15.0)	15.9 (9.2-28.0)	13.3 (9.2-16.9)
GRWR: mean (SD)	2.4 (0.8)	2.4 (1.0)	2.2 (1.4)	2.4 (1.0)

¹Categorical variables were summarised by frequency and percentage tabulation. Continuous variables were summarised by the mean (with standard deviation) or median (with interquartile range), depending on the distribution of the data. IQR= inter-quartile range, SD= standard deviation, GRWR= graft to recipient weight ratio. Missing Data: GRWR was missing for n=2 and donor weight pre-transplant (kg) was missing for n=1. ²Donor self-reported ethnicity as recorded on the WDGMC Paediatric Liver Transplant Research Database in accordance with the demographic categories published in the mid-year population estimates for 2019 by Statistics South Africa [24]. ³Recipient and donor weight (kg) measured pre-transplant.

The distribution of the *CYP3A5* genotype frequencies across donor self-reported ethnicity did not deviate from Hardy Weinberg equilibrium ($p>0.05$) (Table 3).

Table 3. Frequency and distribution of *CYP3A5* genotypes across living donor groups

Donor self-reported ethnicity ¹ n(%)	Donor <i>CYP3A5</i> genotype			Overall (N=46)
	*1/*1 (ex)	*1/*3 (ex)	*3/*3 (non-ex)	
Black African	18 (100)	6 (43)	0 (0)	24 (52)
Coloured	0 (0)	5 (36)	2 (14)	7 (15)
Indian	0 (0)	2 (14)	2 (14)	4 (8)
White	0 (0)	1 (7)	10 (71)	11 (24)

¹As recorded on the WDGMC Paediatric Liver Transplant Research Database in accordance with the demographic categories published in the mid-year population estimates for 2019 by Statistics South Africa [24].

All expressors with *CYP3A5**1/*1 haplotypes were individuals of self-reported Black African ethnicity. None of the individuals in this study who self-reported as Black African ethnicity were *3/*3 non-expressors. To contextualise our results we compared the phenotype frequencies of expressors and non-expressors with those in African and European biogeographical cohorts adapted from PharmGKB (Figure 5) [18].

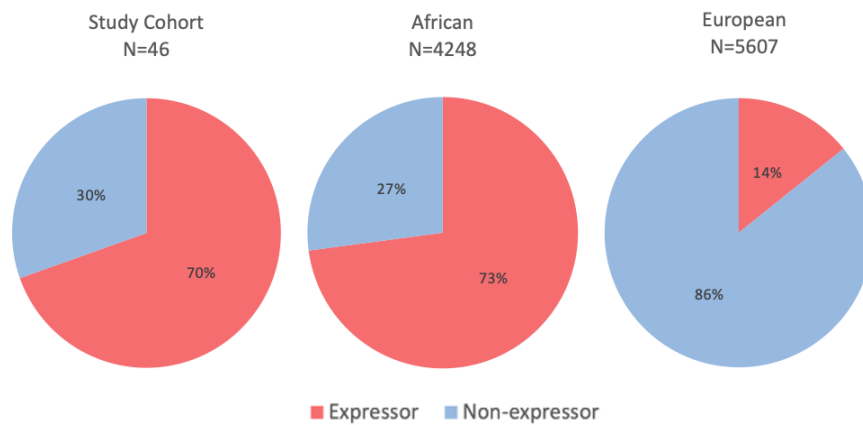


Figure 5. Phenotype frequencies of CYP3A5 expressors and non-expressors in this study cohort in comparison to the African and European biogeographical cohorts adapted from PharmGKB.

For the purpose of this study's cohort, CYP3A5 expressors are classified as donors with *1/*1 or *1/*3 genotype status and non-expressors as donors with CC genotype status. Phenotype frequencies for the African and European biogeographical cohorts were adapted from PharmGKB [18] where expressors included normal and intermediate metabolisers and non-expressors were classified as poor metabolisers. Phenotype frequencies in the PharmGKB database estimated using the equation describing Hardy Weinberg equilibrium based on reported *CYP3A5* allele frequencies from different cohorts in the biogeographical areas [18].

4.2. Donor *CYP3A5* genotype effect on tacrolimus CDR

The tacrolimus CDRs for each of the first 15 days of tacrolimus immunosuppression post-transplant are shown in Figure 6. Significant differences in the median CDR between **1/*1* and **3/*3* donor genotype groups were observed on day 2-9, 11 and 14-15 post-transplant. The **3/*3* donor genotype groups have significantly higher CDRs ($p<0.05$) in comparison to the **1/*1* donor genotype group. There was no significant difference between the **1/*1* expressor and **1/*3* partial expressor donor genotype groups during the first 15 days post-transplant except for day 4 ($p=0.034$). However, the CDRs reported in the **3/*3* non-expressor group were significantly higher than those reported in the **1/*3* partial expressor group on days 3, 9, 11, and 14-15.

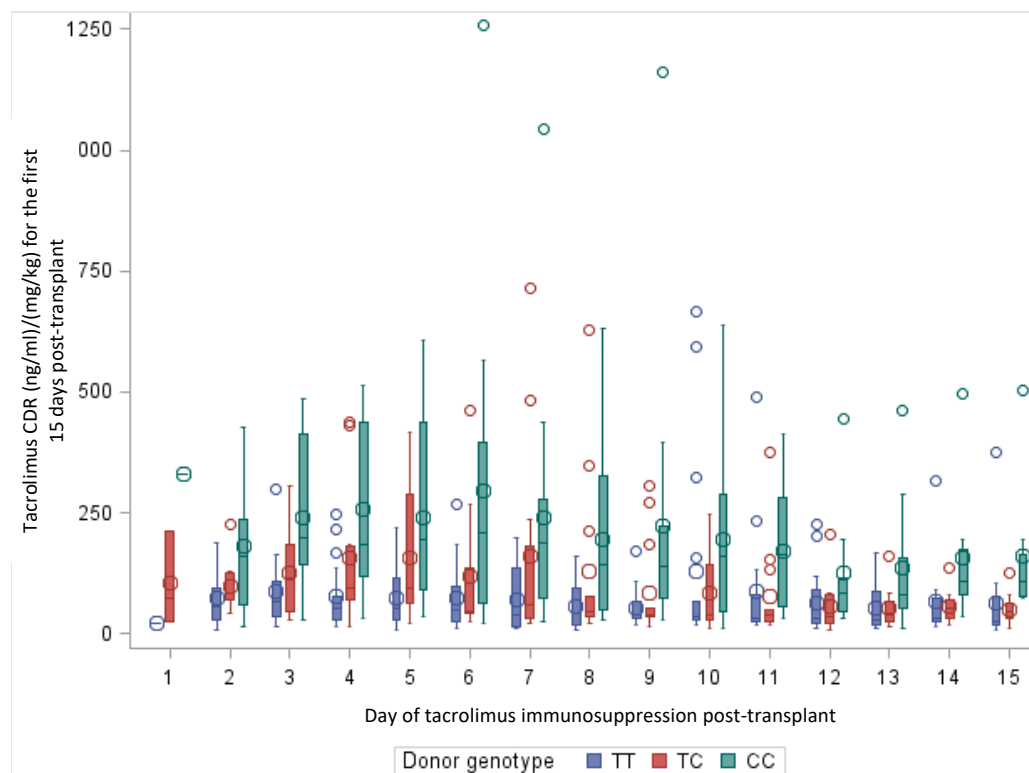


Figure 6. CDR for each day in the first 15 days of tacrolimus therapy for donor *CYP3A5* genotype groups **1/*1* (TT), **1/*3* (TC) and **3/*3* (CC). Box and whisker plots illustrate the interquartile range (IQR), mean and median for tacrolimus CDR. The median value is denoted by horizontal lines inside the boxes. The mean value is denoted by open circles inside the boxes. The whisker lines extend to $\pm 1.5 \times \text{IQR}$. Outlier values ($1.5 \times \text{IQR}$ below the first quartile or $1.5 \times \text{IQR}$ above the third quartile) shown as open circles outside the IQR. Significant differences ($p<0.05$) were observed in the median CDR value between **1/*1* and **3/*3* donor genotype groups on days 2-9, 11, and 14-15. CDR=concentration dose ratio (ng/ml)/(mg/kg).

Figure 7 demonstrates the effect of *CYP3A5* genotype on recipient mean CDRs for tacrolimus over the first 15 days of tacrolimus immunosuppression. The median CDR value was significantly higher for the *CYP3A5**3/*3 non-expressor group (184; IQR 88-247) compared to that of the *1/*3 expressor (73; IQR 40-92; p=0.031) and *1/*1 expressor groups (40; IQR 33-99; p=0.0041).

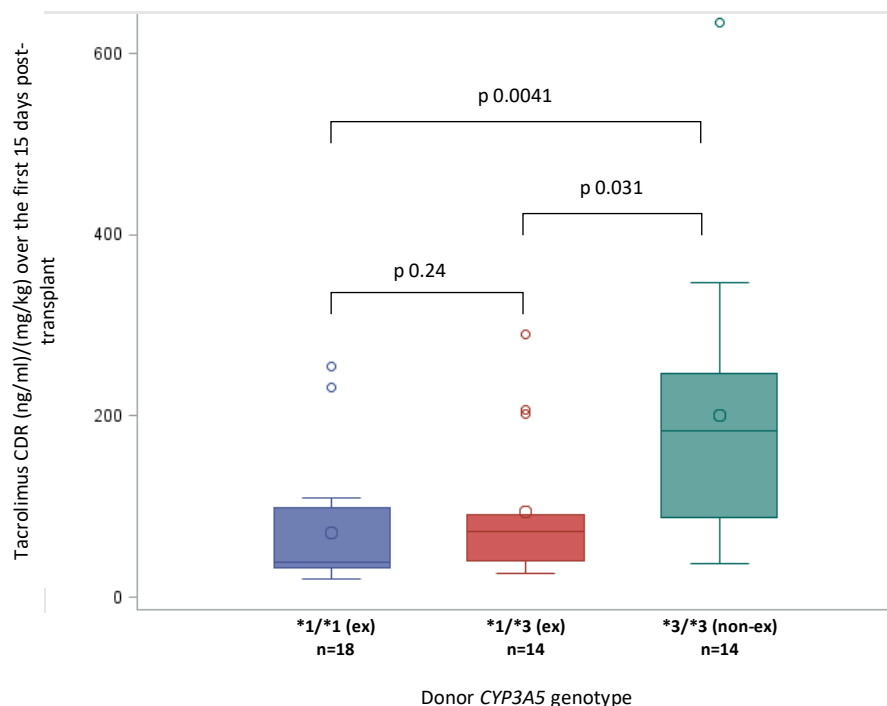


Figure 7. Mean concentration dose ratio (CDR) over first 15 days of tacrolimus therapy for donor *CYP3A5* genotype groups *1/*1, *1/*3 and *3/*3. Box and whisker plots illustrate the interquartile range (IQR), mean and median for CDR (mean CDR over the first 15 days post-transplant). The median value is denoted by horizontal lines inside the boxes. The mean value is denoted by open circles inside the boxes. The whisker lines extend to $\pm 1.5 \times \text{IQR}$. Outlier values ($1.5 \times \text{IQR}$ below the first quartile or $1.5 \times \text{IQR}$ above the third quartile) shown as open circles outside the IQR. The p value is indicated between each donor genotype group. Significant differences ($p < 0.05$) were observed in the median CDR value between the *CYP3A5**3/*3 (non-ex) donor genotype group in comparison to that of the *1/*3 (ex) and *1/*1 (ex) groups. CDR=concentration dose ratio (ng/ml)/(mg/kg).

4.3. Clinical factors associated with tacrolimus concentration dose ratio

In the univariable analysis GRWR, donor genotype, recipient age, recipient weight, recipient ethnicity and donor ethnicity were significantly associated with mean tacrolimus CDR in the first 15 days post-transplant. Donor genotype, donor ethnicity and recipient ethnicity were found to be strongly confounded variables and, therefore, were represented by donor genotype in the multivariable regression. Strong correlations were also identified between recipient age, recipient pre-transplant weight, and GRWR with GRWR chosen as the most meaningful representative variable. The independent variables in the multivariable analysis were GRWR, donor genotype, and donor age. Only GRWR and donor genotype remained significantly associated with mean tacrolimus CDR in the multivariable analysis (Table 4).

Table 4. Results of univariable and multivariable regression to determine factors associated with the mean CDR over the first 15 days of immunosuppression post-transplant in paediatric liver recipients

Characteristic	Overall n=46	Univariable regression model ¹ Estimated coefficient (p-value)	Multivariable regression model ² Estimated coefficient (p-value)
Intercept		varies by model	275.0 (p <0.0001)
GRWR ratio: mean (SD)	2.4 (1.0)	-48.0 (p 0.0002)	-46.2 (p <0.0001)
Donor <i>CYP3A5</i> genotype: n (%)			
*3/*3 (non-ex)	14 (30)	reference	reference
*1/*3 (ex)	14 (30)	-72.8 (p 0.028)	-65.0 (p 0.023)
*1/*1 (ex)	18 (40)	-95.9 (p 0.0028)	-93.0 (p 0.0009)
Recipient age (y): median (IQR)	2.3 (1.4-3.4)	14.8 (p <0.0001)	
Recipient weight pre-transplant (kg): median (IQR)	13.3 (9.2- 16.9)	8.6 (p <0.0001)	
Recipient sex: n (%)			
male	24 (52)	reference	
female	22 (48)	-4.7 (p 0.86)	
Recipient self-reported ethnicity ³ : n (%)			
Black African	24 (52)	reference	
Coloured	6 (13)	46.4 (p 0.23)	
Indian	5 (11)	44.8 (p 0.29)	
White	11 (24)	98.1 (p 0.0035)	
Donor age (y): median (IQR)	33 (28-40)	3.1 (p 0.056)	
Donor weight pre-transplant (kg): median (IQR)	68 (62-80)	-0.9 (p 0.46)	
Donor sex: n (%)			
male	18 (39)	reference	
female	28 (61)	35.5 (p 0.20)	
Donors self-reported ethnicity ³ : n (%)			
Black African	24 (52)	reference	
Coloured	7 (15)	33.9 (p 0.35)	
Indian	4 (9)	66.2 (p 0.15)	
White	11 (24)	98.1 (p 0.0034)	

Statistically significant p values are highlighted in bold. ¹The relationship of each study variable with mean CDR over the first 15 days of tacrolimus therapy was analysed by a General Linear Model (GLM). Variables significant at p<0.20 were combined into a multiple regression model; after examining each pair of variables for possible confounding. ²Non-significant variables were removed sequentially until all remaining variables were significant at p<0.05. ³Donor and recipient self-reported ethnicity as recorded on the WDGMC Paediatric Liver Transplant Research Database in accordance with the demographic categories published in the mid-year population estimates for 2019 by Statistics South Africa [24].

5. Discussion

This study demonstrated that donor *CYP3A5* genotype varied significantly between self-reported ethnic groups. All homozygote **1/*1* expressors and the majority of heterozygote **1/*3* expressors were of Black African self-reported ethnicity. By contrast, the homozygous expressor genotype was not observed in any donors of self-reported White ethnicity. Tacrolimus CDR was found to be significantly lower in the expressor donor *CYP3A5* genotype groups compared to the non-expressor group, and multivariable analysis showed that GRWR was significantly associated with tacrolimus CDR. Although these findings are preliminary, to the best of our knowledge, this is the first study to investigate the associations between living liver donor *CYP3A5* genotype and tacrolimus pharmacokinetics in a liver transplant cohort from Southern Africa.

This study included extensive clinical phenotype data with associated genotype from a well characterised population-representative cohort. However, there are several limitations. Part of the study design was retrospective, the sample size was small, and it was a single-centre study — so findings may not be generalised. While considered less relevant, intestinal metabolism of tacrolimus has not been accounted for in this study [5,17]. Sequence variation of the *CYP3A5***6* and **7* alleles (Figure 1) were not investigated in this study. However, we note that some of the study's cohort that have been genotyped as **1/*1* expressors may harbour **6* and **7* variants. Given the allele frequencies from available data curated by PharmGKB we predict these to be representing less than 10% of our samples [18].

Regarding the *CYP3A5* genotype frequencies (limited to rs776746), our findings are generally consistent with a previous South African study [6] where the expressor genotype was predominant in Black African and Coloured ancestry patients but not present in patients of European ancestry. Similarly, a study in a North American renal transplant cohort showed that over 90% of *CYP3A5* expressors were African American participants compared to 5% who were individuals of White ancestry [28]. These findings (although from kidney transplant cohorts) reinforce the association between high frequencies of the *CYP3A5**1 expressor allele in populations of African ancestry. European studies, conducted in liver transplant patients, have rarely detected homozygous expressor genotypes in individuals of White ethnicity which furthermore supports the association of European ancestry to *CYP3A5* non-expressor alleles such as *3 [5,12,29].

Similar to previous studies in different biogeographical cohorts, the data presented here shows tacrolimus levels are significantly impacted by the *CYP3A5* genotype [5,6,10,17,30]. The only other study in liver transplant patients from Africa, which was conducted in Egypt (n=48), showed that expressor *CYP3A5**1/*1 or *1/*3 genotypes resulted in lower CDR in comparison to *3/*3 non-expressor genotypes [30]. This association between expressor genotype and low recipient tacrolimus CDR post-transplant was furthermore confirmed in recent Egyptian and South African kidney transplant studies [6,31]. A European study on paediatric liver transplant patients, notably lacking homozygous *1/*1 expressor representation, illustrated that the CDR was significantly lower in recipients of heterozygous expressor grafts in comparison to non-expressors [5]. In Chinese cohorts, the homozygous expressor genotype was observed at lower frequencies than this study, yet donor *CYP3A5* genotype significantly impacted recipient CDR post-transplant [10,17].

The stratification of tacrolimus dosing has been previously proposed based on GRWR in addition to donor *CYP3A5* genotype [5,10,32]. Tacrolimus CDR has been shown to be significantly lower in recipients with higher GRWR in European and Chinese cohorts [5,10] which is corroborated by our findings. It should be noted that theoretically, higher graft weights are directly proportional to the liver tissue volume and therefore the amount of *CYP3A5* enzymes present in the graft [5,10].

This study's findings are clinically relevant for physicians transplanting solid organs from individuals of African ancestry. *CYP3A5* hepatic expressors will have low tacrolimus blood concentrations making it difficult to achieve therapeutic levels, which places recipients at significant risk for graft rejection. Thus, doses required are much higher which substantially increases the cost of treatment however this would be significantly outweighed by patients avoiding graft rejection and potential secondary surgeries. In contrast, non-expressors are susceptible to an increased risk of tacrolimus toxicity and associated adverse side effects. Therefore, pre-operative living liver donor genotyping analysis for *CYP3A5* could allow transplant physicians to initiate personalised tacrolimus dosing schedules at the time of transplant to allow for more accurate therapeutic drug monitoring in the immediate post-transplant period. This pre-operative strategy is entirely feasible for a LDLT setting as there is time prior to transplant to conduct genotyping analysis [1]. Financially, in a resource limited setting a pre-operative genotype test would be beneficial considering the high expense associated with the transplant procedure. Recommended dosing would need to be adjusted post-operatively in accordance with the results of the genotyping analysis. However, more investigations need to be conducted into the association between the highly polymorphic *CYP3A5* gene and tacrolimus dosing requirements in order to develop a clinical precision medicine approach to tacrolimus.

We acknowledge the presence of *CYP3A5**6 and *7 alleles in African populations. However, these SNPs have not been investigated in this study. Future studies should consider the analysis of the function obliterating *CYP3A5**6 and *7 alleles due to the higher allele frequency observed in African populations in comparison to the European counterparts as shown in Figure 2 [18]. This would possibly shed further light on the study by Muller et al. 2020 who did not observe *CYP3A5**6 in a South African renal study population which is similar in size and ethnic distribution to this study [6]. The *CYP3A5**8 allele should also be of interest for future work to investigate the impact of *CYP3A5* missense variants in African populations. Therefore, further investigation is needed to assess the impact of these *CYP3A5* SNPs, both individually and as part of a haplotype, in a tacrolimus dosing requirement algorithm. The wide interpatient variability in tacrolimus response has been attributed several other factors which should be further investigated. Future work should consider investigating the impact

cytochrome P450 3A4 isoenzyme which has overlapping specificities with the CYP3A5 enzyme for tacrolimus. In a clinical research setting, the impact of drug-drug interactions between tacrolimus and fluconazole should also be considered during the complex post-transplant management of paediatric patients in transplant units.

6. Conclusion

Whilst aspects of this study have been previously published, this is the first dataset in a liver transplant cohort to have predominant representation of homozygous *CYP3A5* expressors, prevalent in the African population, with tacrolimus CDR data in association with GRWR. The findings from this study, while preliminary, suggest that a stratified tacrolimus precision medicine approach could be developed from future work, which would better inform immunosuppression treatment schedules especially in patients of African ancestry undergoing solid-organ transplantation.

7. Declarations

Ethics approval and consent to participate

Ethics approval was granted by the Human Research Ethics Committee (HREC) Medical, University of the Witwatersrand (Certificate number: M220351). Certificates and consent forms are contained within the Appendix section of this document

Availability of data and materials

The datasets from the WDGMC Paediatric Liver Transplant Research Database used are available from the corresponding author on reasonable request.

Competing Interests

The authors declare they have no competing interests

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Appendices

Appendix 1: Human Research Ethics Committee (Medical), University of the Witwatersrand (M220351)

Appendix 2: Human Research Ethics Committee (Medical), University of the Witwatersrand (M191006)

Appendix 3: Human Research Ethics Committee (Medical), University of the Witwatersrand (M190749)

Appendix 4: Supplementary Material and Methods

Appendix 5: Information and Consent forms

Appendix 6: Approved Research Protocol

Appendix 7: Turnitin Report and Plagiarism Declaration

Appendix 8: Journal Author Guidelines for Submissible Format



R49 Ms C Wheeler

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220351**

NAME:
(Principal Investigator)

Ms C Wheeler

DEPARTMENT:

School of Pathology
Division of Human Genetics
Medical School
University

PROJECT TITLE:

*Impact of donor CYP3A5 genotype on pharmacokinetics
of tacrolimus in South African paediatric liver transplant
patients*

DATE CONSIDERED:

2022/03/25

DECISION:

Approved unconditionally

CONDITIONS:

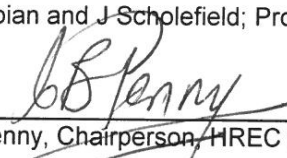
NOTE:

If contact information regarding student study participants is required,
please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>

SUPERVISOR:

Drs J Fabian and J Scholefield; Professor J Botha

APPROVED BY:


Dr CB Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL:

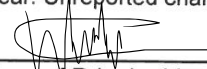
2022/07/06

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report.** When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **March** and therefore reports and re-certification will be due in the month of **March** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).


Signature of Principal Investigator

06/07/2022

Date



R14/49 Prof Collen Masimirembwa and Prof Jean Botha

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M191006

NAME: Prof Collen Masimirembwa and Prof Jean Botha
(Principal Investigator)
DEPARTMENT: Sydney Brenner Institute for Molecular Bio-sciences

PROJECT TITLE: Establishment of a uniquely African Liver Tissue Biorespository and its application in drug metabolism, pharmacokinetics and pharmacogenomics research (ALTBIO)

DATE CONSIDERED: 25/10/2019

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR:

APPROVED BY:


Dr. C Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL: 06/12/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **October** and will therefore be due in the month of **October** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES



R14/49 Professor J Botha

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190749

NAME:
(Principal Investigator)
DEPARTMENT:

Professor J Botha
School of Clinical Medicine
Department of Surgery
Transplant Unit
Wits Donald Gordon Medical Centre

PROJECT TITLE:

WDGMC Paediatric Liver Transplant Research Database

DATE CONSIDERED:

2019/07/26

DECISION:

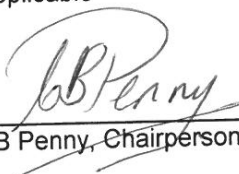
Approved unconditionally

CONDITIONS:

SUPERVISOR:

Not applicable

APPROVED BY:


Dr CB Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL:

2019/12/09

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report.** When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **July** and will therefore reports and re-certification will be due early in the month of **July** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

Donor Genetic Analysis

Frozen samples were thawed at room temperature. The modified salting out protocol was as follows: cell lysis was performed using 35 ml cold sucrose-Triton X lysis buffer consisting of 10 ml 1M Tris-HCl pH 8.0, 5 ml 1M MgCl₂, 10 ml Triton-X 100 and 109.5 g sucrose. After centrifugation at 2 400 rpm at 4°C for 5 minutes, the supernatant was poured off and the pellet was washed by 20 ml cold sucrose-Triton X lysis buffer twice. White blood cells were lysed and protein degraded overnight at 42°C after the addition of 3 ml 20 mM TRIS 5 mM EDTA, 200 µL 10% SDS and 500 µL of Proteinase-K mix to each extraction. Proteinase-K mix for one extraction is made up of 50 µL 10% SDS, 2 µL 0.5M EDTA, 0.35 ml dH₂O and 100 µL 10m g/ml Proteinase-K. Day 2 of the protocol was followed as described in Ali [26].

The concentration of gDNA was quantified using a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). Spectrophotometry was utilised to exclude potential contamination of protein by calculating the OD 260nm/280nm ratios which were required to be between 1.8 and 2.0.

Each PCR reaction was performed using 2.5 ng/µL template gDNA in a reaction mix containing 1x of 5x Phusion Green HF Buffer (Thermo Scientific, USA), 200 µM of 10 mM deoxyribonucleotide triphosphate mix (Thermo Scientific, USA), 0.5 µM of each of the forward and reverse primers (Inqaba Biotechnical Industries, SA) and 0.02 U/µL of Phusion DNA Polymerase (Thermo Scientific, USA) made up to a final volume of 50 µL using Milli-Q H₂O.

Table A. List of forward and reverse primers

Primer	Sequence (5'->3')	Length	Reference
Forward	GACTTAGTAGACAGATGACACA	22	Designed
Reverse	GGTCCAAACAGGGAAGAAATA	21	Muller et al. (2020)

The forward primer sequence published by Muller et al. (2020) contained mismatches with the CYP3A5 reference sequence. The designed primer in this study did not contain mismatches.

To examine the integrity of the amplified PCR product, 5 μ L of the PCR reaction was electrophoresed on a 1% agarose gel at 100 V for 60 minutes. Band sizes of the amplicon were expected to be 290 bp.

PCR products were purified/cleaned up with a final elution volume of 35 μ L. The purified PCR product concentration was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA).

Sspl restriction enzyme digestion was performed on 0.5 μ g of purified PCR product. Each RFLP reaction contained the following reagents: 1X rCutSmart NEBuffer, 10 units Sspl restriction enzyme, 0.5 μ g DNA and a final volume of 75 μ L was made up using Milli-Q water. After the initial incubation step of 45 minutes at 37°C, 10 units Sspl restriction enzyme was added and a second incubation step was performed for 45 minutes at 37°C to avoid undigested product. Sspl-High Fidelity can be used overnight with no star activity. Heat inactivation was performed at 65°C for 20 minutes. To determine the results of the digestion and genotype, 30 μ L of the digested product with 5 μ L purple loading dye was electrophoresed on a 3% agarose gel at 80 V for 60 minutes.

Sanger sequencing was performed as a validation of the genotype results obtained from RFLP analysis. Each cycle sequencing reaction was performed using purified PCR product in a reaction mix containing 2 μ L Bigdye terminator v3.1 Ready Reaction Mix (Applied Biosystems, USA), 5x sequencing buffer (Applied Biosystems, USA), 3.3 μ M forward primer (Inqaba Biotechnical Industries, SA) and made up to a final volume of 10 μ L using Milli-Q H₂O.

Post-cycle sequencing clean-up was performed as follows: 40 μ L injection solution was added to each reaction. The 50 μ L final volume of each reaction was transferred to clean PCR clean-up filter plate and vacuumed until dry. The process was repeated with 25 μ L injection solution twice. After the second addition, the plate was placed on a shaker for seven minutes at a low speed. The contents of each well was transferred to a 96-well plate for sequencing. 5 μ L injection solution was added to blank wells. The plate was heated at 80 °C for 7 min and then placed on ice for 5 min. The plate was placed inside the ABI 3500xl genetic analyser and the

status of all consumables including POP-7 polymer was checked. Sequencing results in .ab1 format were analysed using Snapgene software. Electropherograms were created using Sequencing Analysis Software (Applied Biosystems, USA).

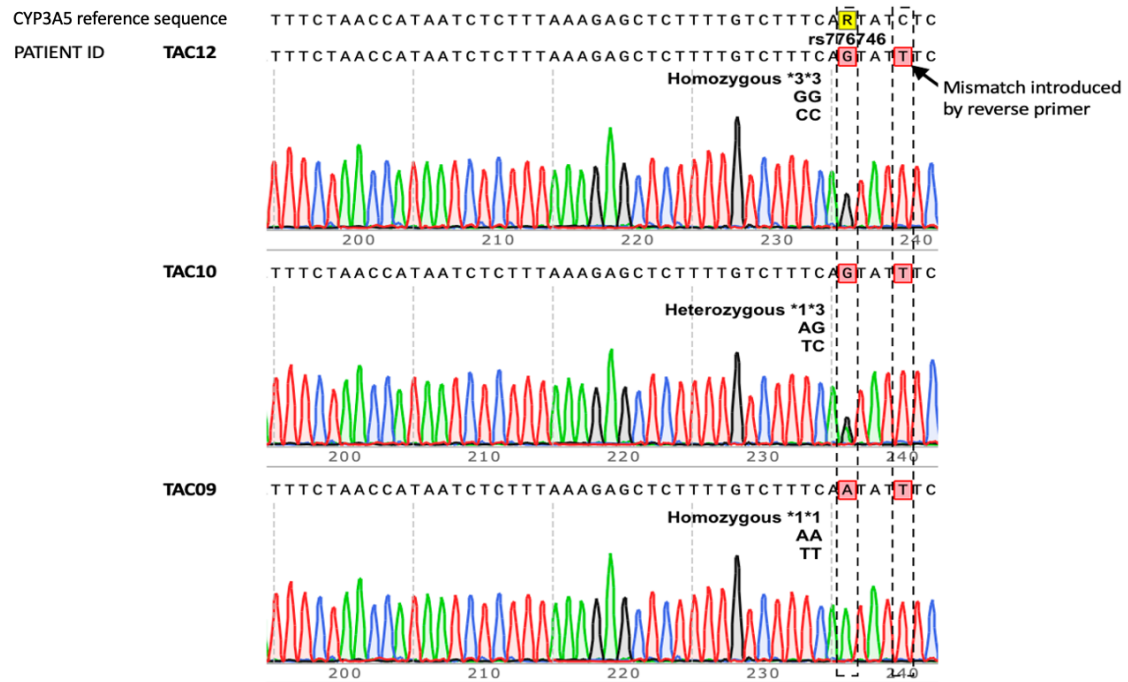


Figure A. Sanger sequencing validation for *CYP3A53 rs776746 genotype**

Panel 1 shows the *CYP3A5* reference sequence for the region of interest. Accessed off Ensembl. Chromosome 7: 99,648,194-99,679,998 reverse strand. GRCh38:CM000669.2 assembly. Panel 2, 3 and 4 shows the region of interest for Patient ID TAC12, TAC10 and TAC09 respectively. At rs775746, there was a homozygous GG peak for TAC12, a heterozygous AG double peak for TAC10 and a homozygous AA peak for TAC09. The results of RFLP genotyping were therefore validated.



STUDY INFORMATION DOCUMENT

Study title: The impact of donor CYP3A5 genotype on the pharmacogenomics of tacrolimus in paediatric liver transplant patients

Hello,

My name is Caitlin, and I am carrying out a Masters study. Together with Dr Scholefield, Dr Fabian and Prof Botha we are doing research on how different people respond to medicines after having a liver transplant. Because you are a living liver donor from Wits Donald Gordon Medical Centre, we would like to invite you to participate in our research. Our research has been approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg ("Committee"). A principal function of this Committee is to safeguard the rights and dignity of all human subjects who agree to participate in a research project and the integrity of the research. To help you make a decision, we will give you a bit more information. If there's anything you aren't sure about, please ask us at any time.

Research is a process used in seeking new knowledge. In this study we want to learn if your (liver donor) particular genes (genetic makeup) can affect how a donated liver responds to medication after transplant.

What is involved in the study?

When we take medicines prescribed by the doctor either as tablets, capsules, solutions or injections, they enter our bodies. To avoid buildup of these chemicals in our bodies, we have organs such as the liver and the kidneys that help remove them from the body. The liver is the main organ involved in this process. It changes the structure of the medicines so that they can easily be removed from the body either through urine or feces. The effectiveness of the liver in this process differs between individuals and between different population groups. In this research we want to learn how people from the different population groups of South Africa handle medicines.

These differences are important in the clinic because we can give the same dose of a drug to people from different population groups, and the treatment outcome may be different. For some it'll work just fine, in others it may fail, and other people may experience side effects that make them feel sick.

Most drugs on the market were developed for people from Europe and North America. The companies that developed these drugs used liver tissue from people from these countries to come up with treatment guidelines. However, these drugs end up being used across the world. Because the drugs are tested in European people, they might not work the same way in African or Indian people. For instance, we have seen that some of these drugs developed for European people are not safe and/or effective in African populations. Because of this, we want to look at how your genetic makeup, as a donor, could have affected the liver recipients' response to the medicine used.

Some of this research involves doing genetic tests on your samples. From these tests, we get important information about how you, as an individual, tolerate drugs differently to other individuals. This is a process that is driven by your genetic make-up.

What are the processes if I agree to take part in this research?

2

If you are willing to participate, we would like you to provide a blood sample of 6 mls (about a teaspoon). We will take your blood sample at the WDGMC in a clinic setting under strict COVID-19 precautionary measures. We will use your blood sample to conduct genetic tests that will tell us about the capacity of your body to handle various medicines. This will be useful for the recipient of your liver, as it may help us work out what the best medicine is for the recipient, and we might be able to use it to guide our decision making. The blood sample will be stored in a “biobank” – explained below.

Because each person is different, we also need access to some demographic information about you. This will help us make sure our results are accurate. For instance, a very big tall man might have different results to a very small short man. Because of this, we would like your permission to access some of your clinical information. This includes your age, body weight, height, medicines you are on, alcohol consumption patterns, and smoking patterns. We can get all this information from your patient file at WDGMC, because it will have been recorded at your appointments before surgery.

What is a biobank and how will we use the biobank in the research?

A biobank is a scientific entity that keeps tissues and blood samples donated for research purposes. Biobanks are very strictly regulated. In order for us to do this research, we have permission from the Biobank Ethics Committee of Wits University under a study called the African Liver Transplant Biorepository (ALTBio). Your samples will be stored in a biobank called the Sydney Brenner Institute for Molecular Biosciences, which is an established biobank in Johannesburg. The left-over blood sample will be stored in the biobank for a maximum of 15 years, or until the sample runs out. At this point, you will no longer be part of the biobank study.

Will any future testing be done on my samples?

In the future, we may run other tests on your samples that will be useful in the drug development process. These may include genetic tests. We cannot do these tests unless they are approved by the Wits Human Research Ethics Committee (HREC[M]).

What is the consent process?

If you agree to take part in this research, we will ask you for consent to participate. Because we are also going to be storing your samples, we will ask you to sign a second consent form for the storage of samples.

Will anyone know my identity if I agree to take part in the study?

In this study your personal information will be treated in the strictest confidence and will only be available to the Principal Investigators (PI). However, the information may be disclosed if required by law and the Human Research Ethics Committees of the University may exceptionally require personal data to respond to a formal complaint, or for a compliance audit. By signing the consent form, you understand that your personal information will be processed in terms of the Protection of Personal Information Act No 4 of 2013 (POPIA).

Because we need to be able to give you your test results, your sample will be stored in the biobank with a unique code. If we need to find your sample, we will go to a list with your name, and we will be able to see what your unique code is. The list with names and codes will only be accessible to the study team and will never be shared with anyone else.

As you know, some of the tests we are doing are genetic tests. No one will be able to tell who you are from the results of these tests.

What are the risks of taking part in this study?

3

The risks of taking part are very small. Drawing 6 mls of blood (about a teaspoon) for the sample will not harm you. You may experience slight dizziness, bruising, brief pain or other temporary side effects. However, we will be prepared for this possibility and will aid you accordingly.

What are the benefits of taking part in the study?

There is a small possibility that being in this study could have direct benefit to you and to the recipient of your liver.

Through testing your blood sample, we may find results that could be useful to you as it will tell your doctor how you are likely to handle medicines you might currently be on or will take in the future. However, this benefit is not guaranteed.

The blood samples that you donate will only have benefit to future patients, because they might help us make better use of drugs in future.

How long will the study process take?

We will ask you to travel to the WDGMC which will require transport and time. However the process of drawing blood will not be long. If you have questions about the study at any point, this may take a bit more time to answer. We are happy to answer your questions at any time.

Do I have to take part in the study?

You are under no pressure to take part in this study, and it is entirely up to you. If you decide not to take part, this will not influence your past or future treatment here at Wits Transplant in any way. You can also withdraw from the study at any time. You don't need to tell us why you have decided to withdraw. If you withdraw from the study, any data collected on you will be destroyed.

Will I be paid for participating and will it cost me anything?

You will not be paid for participating in the study. Participation will not cost you anything. However, since we are asking you to provide a blood sample at WDGMC we will compensate you for an amount of R350 for your travel costs and time.

How will the research be made public?

The outputs of this research will be:

1. A biobank of blood samples
3. Research publications and conference presentations

Should you be interested in these outcomes, we will be able to share them with you.

Who can I contact if I have questions or complaints?

You are welcome to contact us any time if you have questions:

Caitlin Wheeler (cawheeler02@gmail.com) 071 070 3091

Prof. Jean Botha (jeanf.botha@gmail.com) 071 218 7328

If you have any concern over the way the study is being conducted, please contact the Chairperson of this Committee who is Professor Clement Penny, who may be contacted on telephone number 011 717 2301, or by e-mail on Clement.Penny@wits.ac.za. The telephone numbers for the Committee secretariat are 011 717 2700/1234 and the e-mail addresses are Zanele.Ndlovu@wits.ac.za and Rhulani.Mukansi@wits.ac.za

Thank you for reading this Study Information Sheet, we really appreciate your time. Please feel free to ask us any questions you might have. You are in no rush to make a decision about taking part, please take as much time as you need.

If you have any questions concerning this study or consent form beyond those answered by the investigator, including questions about the research, your rights as a research participant or research-related injuries; or if you feel that you have been treated unfairly and would like to talk to someone other than a member of the research team, please feel free to contact the Chairperson of this Committee who is Professor Clement Penny, who may be contacted on telephone number 011 717 2301, or by e-mail on Clement.Penny@wits.ac.za. The telephone numbers for the Committee secretariat are 011 717 2700/1234 and the e-mail addresses are Zanele.Ndlovu@wits.ac.za and Rhulani.Mukansi@wits.ac.za

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Thank you for reading this Study Information Sheet.

Date: March 2022

Authorization:

5

You are making a decision whether or not to participate in this study.

Your signature indicates the following:

1. I have read and understood the information provided above, have had all my questions answered, and have decided to participate.
2. I consent to my personal information being processed in terms of the Protection of Personal Information Act No 4 of 2013.

Name of Research Participant (please print)

Date

Signature of Participant or legally authorized representative

Time

Name of Witness (*if required*)

Signature

Date

Relationship to the Participant (*if required*)

Name of Staff Obtaining Consent

Signature

Date

YOU WILL BE OFFERED A COPY OF THIS CONSENT FORM TO KEEP.



CONSENT FORM – SAMPLE STORAGE AND FUTURE TESTING

Study title:

Establishment of a uniquely African Liver Tissue Biorepository and its Application in Drug Metabolism, Pharmacokinetics and Pharmacogenomics Research

Authorization:

I have been informed about the study entitled: “Establishment of a uniquely African Liver Tissue Biorepository and its Application in Drug Metabolism, Pharmacokinetics and Pharmacogenomics Research”.

As a participant in this study, I have been made aware of the following:

1. My blood sample will be stored in a Biobank. The samples will be used to test how the liver processes different medicines. The samples will be stored till they run out. I agree for my sample to be stored.
2. There may be future testing done on my samples which the researchers have not yet determined. I am aware that if such testing takes place, it will be approved by the Wits Human Research Ethics Committee (Medical). I agree that future tests may be run on my sample under these conditions.
3. I consent to my personal information being processed in terms of the Protection of Personal Information Act No 4 of 2013.

Name of Research Participant (please print)

Date

Signature of Participant or legally authorized representative

Time

Relationship to the Participant (*if required*)

Name of Witness (*if required*)

Signature

Date

Name of Staff Obtaining Consent

Signature

Date

YOU WILL BE OFFERED A COPY OF THIS CONSENT FORM TO KEEP.

If you have any questions concerning this study or consent form beyond those answered by the investigator, including questions about the research, your rights as a research participant or research-related injuries; or if you feel that you have been treated unfairly and would like to talk to someone other than a member of the research team, please feel free to contact the Chairperson of this Committee who is Professor Clement Penny, who may be contacted on telephone number 011 717 2301, or by e-mail on Clement.Penny@wits.ac.za. The telephone numbers for the Committee secretariat are 011 717 2700/1234 and the e-mail addresses are Zanele.Ndlovu@wits.ac.za and Rhulani.Mukansi@wits.ac.za

POPIA Consent Form

Impact of donor CYP3A5 genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients

POPIA Information Sheet and Consent Form

Dear _____,

Thank you so much for considering participation in our liver transplant research programme. In terms of the Protection of Personal Information Act of 2013 (POPIA), we need to give you some very specific details about how your personal information (also referred to as 'data') will be used in this project. This information is required over-and-above the study details contained in the Study Information Sheet, and in terms of POPIA, we would like to invite you to sign this form as an indication that you consent to our using your data in this manner.

Below is a set of questions you may wish to ask and to which we have provided answers so that you have more information before you make your decision.

What is Personal information?

Personal information includes any type of data that can identify a person. For this project, you have been invited to donate liver tissue and blood. Along with these specimens, we collect some personal information about you. This includes your name, age, sex, ethnicity and some clinical details.

What kind of information will be collected about me?

We will collect your basic demographic information: name, age, sex, ethnicity and some clinical details. This information will be requested directly from you and obtained from your transplant file.

Where will my data be stored?

Your data will be stored on an academic research database called REDCap. REDCap is POPIA compliant and the backup server is located in South Africa. REDCap has extensive layers of data security, so your data will be well-protected.

How long will my data be stored?

Your data will be stored indefinitely.

Who will have access to my data?

Only members of the core research teams will have access to your full data set on REDCap. The core teams include collaborators at the Sydney Brenner Institute for Molecular Bioscience (SBIMB), Wits Donald Gordon Medical Centre (WDGMC), the Council for Scientific and Industrial Research (CSIR) and Cradleomics. These teams will be able to see your sex and your medical information, and sometimes your name. They are legally and ethically required to keep this information totally confidential.

Do I have to agree for my data to be collected and stored?

No – whether you are willing for us to collect your data, or not, it totally up to you. If you decide not to have your data collected for research purposes it will not jeopardise the treatment you receive from the Unit in any way. However, it is important to note that we cannot include you in the ALTBio project if you would prefer us not to collect your data – because demographic data is very important for the research and the science associated with it.

Can I agree now, and change my mind later?

Yes, you can agree to us collecting your data for research purposes now, and then change your mind at a later stage. If you change your mind, please contact us at the details towards the end of this form. We will permanently delete all data related to you from the REDCap research database, and we can send you proof that this has been done. However, please be aware that where your data has been anonymised and combined with other data for the purposes of analysis or third-party transfer, we will not be able to delete it, as we will not be able to tell what data belongs to you.

How will my data be utilised?

When we decide to publish a research project using information from our research databases, we first have to approach the Wits Human Research Ethics Committee (Medical) and get permission to analyse the data in order to answer a specific research question. Once we have this permission we will make the data available to a biostatistician, who will analyse the data. We will then write-up the analysis in an article for an academic journal, or include it in conference presentations. No personal, identifying information about you will ever be included in any of these outputs that are published in academic journals or presented at conferences.

What if a third party wants to access my data

Sometimes, we get requests from scientists, students or other doctors to access our research databases so that they can do research projects. These projects will always be related to aspects of ALTBio, but at this point, we do not know exactly what these projects would involve. When we get these requests, we do the following:

1. We require that the third party gets permission to do their research project from the Wits Human Research Ethics Committee (Medical)
2. We review the request for data and approval by our Data Access Committee – which is constituted in terms of the POPI Act
3. If approved at both levels, we will share ANONYMOUS data with the third party.

We will never share your name with anyone else, and we will only share the absolute minimum of data needed for the third party to undertake their research project.

Am I allowed to see the data that you collect about me for research purposes?

Yes. If you would like to see the data we have collected about you on the REDCap database for research purposes at any time, that is totally fine. Please contact us on the details below to arrange for this.

Who should I contact if I have questions related to POPIA?

If you have any questions about our research projects, POPIA and the use of your data, please contact:

The WDGMC Clinical Research Office on 011 356 6395 Please feel free to make contact at any time.

POPIA Consent

In accordance with the provisions of the Protection of Personal Information Act 4 of 2013 (as amended) and the information contained within the document above, I hereby consent:

1. To my personal information being collected, processed, shared and stored in accordance with the research protocol or future research projects as approved by the University of the Witwatersrand Human Research Ethics Committee (Medical)
2. To my data being shared, processed and transferred to third parties and between third parties, and where relevant beyond the jurisdictional borders of South Africa;
3. To all findings and results flowing from my data being broadly shared and published on the conclusion of the research and future research projects using my data.

Name: _____

Signature: _____

Date: _____

Witness (If necessary): _____

Appendix 6: Approved Research Protocol



Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

27 June 2022
Person No: 2490170
PAG

Miss CA Wheeler
16 Kentucky Circle
Saddlebrook Estate
Midrand Ext 7
1682
South Africa

Dear Miss Caitlin Wheeler

Master of Science in Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Impact of donor CYP3A5 genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

A handwritten signature in black ink, appearing to read 'S. Benn', with a horizontal line underneath.

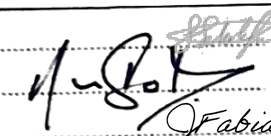
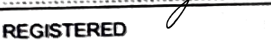
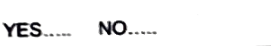
Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG



FACULTY OF
HEALTH SCIENCES

CANDIDATE'S SURNAME: [Please print]	WHEELER	FIRST NAME/S:	CAITLIN	STUDENT NUMBER:	2490170
CURRENT QUALIFICATIONS: BSc, BSc Honours					
TEL: N/A	CELL: 0710703091	E-MAIL: 2490170@students.wits.ac.za	FAX: N/A		
DEGREE FOR WHICH PROTOCOL IS BEING SUBMITTED: MSc Med Genomic Medicine					
PART-TIME OR FULL-TIME: FULL TIME					
FIRST REGISTERED FOR THIS DEGREE:	TERM : 1		YEAR: 2022		
DEPARTMENT: HUMAN GENETICS					
TITLE OF PROPOSED RESEARCH: Impact of donor CYP3A5 genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients					
CANDIDATE'S SIGNATURE:				DATE: 20th MARCH 2022	
SUPERVISOR 1 (NAME & SURNAME): DR JANINE SCHOLEFIELD				% Supervision: 50%	
SUPERVISOR'S QUALIFICATIONS: PhD					
SUPERVISOR'S DEPARTMENT: CSIR Bioengineering and integrated genomics group (BIGG) Group Leader					
SUPERVISOR'S ADDRESS / TEL / E-MAIL: CSIR, NextGen Health, Building 20, Meiring Naude Rd, Pretoria, 0001 JScholefield@csir.co.za 076 963 9272					
SUPERVISOR 2 (NAME & SURNAME): DR JUNE FABIAN				% Supervision: 30%	
SUPERVISOR'S QUALIFICATIONS: MBBCh, PhD					
SUPERVISOR'S ADDRESS / TEL / E-MAIL: Wits Donald Gordon Medical Centre, 21 Eton Road, Parktown, Johannesburg june.fabian@mweb.co.za 082 785 3579					
SUPERVISOR 3 (NAME & SURNAME): PROF JEAN BOTHA				% Supervision: 20%	
SUPERVISOR'S QUALIFICATIONS: MBBCh (Wits)					
SUPERVISOR'S ADDRESS / TEL / E-MAIL: Wits Donald Gordon Medical Centre, 21 Eton Road, Parktown, Johannesburg jeanf.botha@gmail.com 071 218 7328					
<u>SYNOPSIS OF RESEARCH:</u> SEE NEXT PAGE					

WITS ETHICS NOT REQUIRED: ☐ Yes ☐ No WITS ETHICS PENDING: ☒ Yes ☐ No WITS ETHICS APPROVED: ☐ Yes ☐ No (circle appropriate symbol)* CONDITIONALLY APPROVED	IF Y SUPPLY ETHICS CLEARANCE CERTIFICATE AS ATTACHMENT AND INCLUDE ETHICS NUMBER HERE: M220351
*Please note the final human ethics clearance certificate or animal ethics certificate must be available prior to starting research	
As supervisor/s, I/we confirm that I have read the protocol which has been submitted for assessment.	
SIGNATURE OF SUPERVISORS:	 ...Dr Janine Scholefield  ...Prof Jean Botha  ...Dr June Fabian
SIGNATURE PG OFFICE STAFF	REGISTERED YES..... NO..... STAMP

SYNOPSIS OF RESEARCH

Introduction:

Achieving the correct concentration dose ratio (CDR) of the immunosuppressant drug, tacrolimus, is the most important aspect of avoiding acute organ rejection in liver transplant patients. Clinically, there is high variability in tacrolimus dosing requirements, especially in paediatric patients. Pharmacogenetics research attributes CYP3A5 SNPs to the specific inter-patient tacrolimus CDRs observed. There is little to no data available on the relationship between tacrolimus administration and the CYP3A5 genotype in a South African paediatric liver transplant population.

South Africa has a highly diverse ethnic population and the post-transplant drug administering routine would be directly impacted by this correlation. This demonstrates the need for the development of an Afrocentric tacrolimus dose requirement algorithm that considers the influencing characteristics of recipients and donors.

Aim:

The aim of this study is to compare the pharmacokinetics of tacrolimus for paediatric liver transplant patients in relation to the donors' CYP3A5 genotypes in a South African context considering both donor and recipient characteristics

This will be achieved from the following objectives:

1. Determine CYP3A5 genotype of the donor
1. Determine age, graft to recipient weight ratio of the recipient
1. Determine the association between recipient tacrolimus CDR post-transplant and the donor and recipient factors from objectives 1 and 2

Proposed Methodology:

- 1) Blood sample collection from living liver donors at WDGMC
- 2) Accessing of HREC approved (M191006) ALTBio blood samples from liver donors
- 3) Accessing of HREC approved (M190749) WDGMC Paediatric Liver Transplant Research Database for donor and recipient variables
- 4) Laboratory genotyping of blood samples at CSIR
- 5) Establish correlation weighting between genetic and clinical factors

Anticipated Outcome:

This genetic information will contribute towards the development of a tacrolimus dosage requirement algorithm that can be utilised in clinical settings.

11 March 2019/MP

**Impact of donor CYP3A5 genotype on pharmacokinetics of
tacrolimus in South African paediatric liver transplant patients**

Caitlin Wheeler

2490170

MSc (Med) Genomic Medicine

Division of Human Genetics, School of Pathology, University of Witwatersrand

Supervisors:

Dr Janine Scholefield, PhD

Council for Scientific and Industrial Research

Dr June Fabian, PhD, MBBCh

WITS Donald Gordon Medical Centre

Professor Jean Botha, MBBCh

WITS Donald Gordon Medical Centre

Table of Contents

1. Background: Literature analysis and critique.....	1
1.1 Paediatric Liver Transplants	1
1.2 Immunosuppression.....	1
1.3 Tacrolimus immunosuppression.....	3
1.4 CYP3A5: Metabolizing enzyme for tacrolimus	4
1.5 Contribution of Donor CYP3A5 Genotype	6
2. Study Aims and Objectives.....	8
2.1 Aim	8
2.2 Objectives	8
3. Methods	9
3.1 Donor sample collection	9
3.2 Data collection.....	10
3.3 Genotype Identification	11
3.4 Sample size.....	12
3.5 Data analysis	12
4. Ethics	13
5. Timing	13
6. Funding.....	14
7. Potential Problems.....	14
8. References.....	15
9. Appendix.....	I

1. Background: Literature analysis and critique

1.1 Paediatric Liver Transplants

Liver transplantation is the standard medical therapy performed in children in response to end-stage liver failure. The most common reasons for liver transplantation in paediatric patients are Biliary Atresia, viral infections and metabolic conditions (Bruckmann et al., 2020; Fabian et al., 2019). Historically, organs from deceased donors were the only available source for liver transplantation, resulting in many potential recipients dying while on the transplant waiting list (Botha et al., 2019). Thus, alternative organ sources have been explored in response to the shortage of deceased donors. These include deceased donor split grafts, reduced grafts, ABO incompatible grafts and, most predominantly, living donor liver transplants (Bruckmann et al., 2020). The Wits Donald Gordon Medical Centre (WDGMC) pioneered living donor liver transplants in Southern Africa in 2012 (Bruckmann et al., 2020). This transplant program, located in Johannesburg, is one of only two paediatric liver transplant programs in Sub-Saharan Africa and the only one performing living donor liver transplantation (LDLT). The implementation of new surgical techniques, such as living donor liver transplants, has allowed transplant teams to partially overcome the shortages of liver donor availability and perform more liver transplants in children.

1.2 Immunosuppression

Despite the success of liver transplantation, these patients remain at a high risk of post-operative complications, particularly infections and acute cellular rejection (Chen et al., 2014; Muller et al., 2020). These adverse outcomes can be reduced by the correct administration of prophylactic immunosuppressant drugs (Choudhary et al.,

2017). The administration of immunosuppressants needs to be carefully balanced with the risk of infection due to the inhibition of T lymphocyte activation (Spada et al., 2009). Modern immunosuppressive regimens are designed to reduce drug toxicity, while still reaching the therapeutic target levels to avoid allograft rejection. Paediatric liver transplant recipients require life-long immunosuppression. As no standard immunosuppressant treatment schedule exists for liver transplant patients, the regimen that is followed is dependant mostly on the specific transplantation unit. The main postoperative stages of immunosuppression regimens are induction and maintenance. Induction immunosuppression is performed at the time the graft is transplanted into the recipient when the risk of acute rejection is highest. The calcineurin inhibitor, Tacrolimus forms the backbone of maintenance immunosuppression and is continued indefinitely.

The most common drug groups that are involved in the immunosuppression of paediatric liver transplant patients are corticosteroids, calcineurin inhibitors, anti-metabolites, mTOR (mammalian target of rapamycin) inhibitors and IL-2 receptor antibodies (Spada et al., 2009).

The immunosuppression schedule currently used currently at WDGMC is the administration of the corticosteroid methylprednisolone in the induction phase; and corticosteroids (oral prednisone) in combination with Tacrolimus in the maintenance phase. Patients are generally weaned off the corticosteroids during the first 3-6 months post-transplantation. Tacrolimus doses are titrated as per the established protocol in the transplant unit.

Long term use of corticosteroids is associated clinically with a range of adverse drug reactions. The risk of infection, as well as negative metabolic effects, increases dramatically due to over-suppression when administering steroids especially in

paediatric patients (Spada et al., 2009). Therefore, the transplant unit at WDGMC is transitioning to corticosteroid-free regimens. Two doses of basiliximab are administered as induction therapy with the dosage titrated according to body weight. The first dose is administered within 8 hours and the second dose on day 4 post-transplantation. Thereafter, each child patient is administered a corticosteroid-free, single agent tacrolimus-based regimen as maintenance immune suppression.

1.3 Tacrolimus immunosuppression

Tacrolimus, a calcineurin inhibitor, is an important immunosuppressant drug that is heavily involved in the liver transplant postoperative process (Pinon et al., 2021). Mediated through the inhibition of the activation of the signal transcription factor, NF-AT, T lymphocyte activation is decreased (Hemenway & Heitman, 1999; Hoofnagle, 2013).

It is well established that tacrolimus has a narrow therapeutic index, as well as specific interpatient pharmacokinetics, pharmacodynamics, and pharmacogenetics (Muller et al., 2020; Pinon et al., 2021). Achieving the correct concentration dose ratio (CDR) of tacrolimus is the most important factor to avoid organ rejection (Muller et al., 2020; Pinon et al., 2021). CDR is calculated by dividing the drug blood trough concentration (C) to the corresponding daily Tac dose (D). These values are obtained postoperatively before patient discharge. The calculated CDR value for each patient is recorded on the WDGMC established Paediatric Liver Transplant Research Database which will be accessed in this study.

The metabolism of tacrolimus is predisposed to drug-drug interactions, genetic factors, and environmental influences (such as age, sex, and body weight) (Chen et al., 2014; Hoofnagle, 2013). Sub-therapeutic post-operative tacrolimus levels can lead to acute

rejection. Conversely, high levels result in drug toxicity (Chen et al., 2014; Dong et al., 2022). In paediatric patients it is considerably more difficult to determine the initial dose requirements and treatment schedule due to high pharmacokinetic inconsistencies observed in inter- and intra-patient studies (Chen et al., 2014; Pinon et al., 2021).

1.4 CYP3A5: Metabolizing enzyme for tacrolimus

The cytochrome P450 enzyme group CYP3A is involved in the metabolism of tacrolimus and is expressed in the liver and small intestine tissue (Liu et al., 2017). The main isoenzymes are CYP3A4 and CYP3A5, with the former generally being predominant (Dong et al., 2022; Kamdem et al., 2005). However, it is CYP3A5 which is primarily responsible for tacrolimus metabolism in the liver (Muller et al., 2020).

Research relating to tacrolimus pharmacogenetics suggests that *CYP3A5* single nucleotide polymorphisms (SNPs) affect the specific inter-patient tacrolimus dosing requirements (Chen et al., 2014; Helal et al., 2017). Homozygote/heterozygote individuals harbouring the *CYP3A5* *1 allele express high levels of CYP3A5 protein leading to rapid tacrolimus metabolism (Chen et al., 2014; Muller et al., 2020). In comparison to *CYP3A5* *1, homozygotes for the non-functional *CYP3A5* *3 allele do not express the CYP3A5 protein- therefore tacrolimus is not metabolised as efficiently summarised in Table 1 (Muller et al., 2020). The *3 allele T>C polymorphism (rs776746) of *CYP3A5* results in aberrant mRNA splicing and leads to a non-functional truncated CYP3A5 protein (Helal et al., 2017). The subsequent decrease in CYP3A5 protein expression has been shown to impact tacrolimus metabolism (Chen et al., 2014; Dong et al., 2022).

Table 1. Analysis of the CYP3A5 alleles including genetic and clinical correlations of T>C polymorphism.

Allele Name	SNP name	mRNA	Protein	Tacrolimus metabolism	Required CDR
CYP3A5*1 (T allele)	rs776746	Full length	Fully functional	High	Low
CYP3A5*3 (C allele)		Aberrant splicing	Truncated non-functional	Low	High

Clinically, the consequences are that *CYP3A5* *1 patients require a higher tacrolimus dose than *CYP3A5* *3 to reach the same tacrolimus CDR (Chen et al., 2014). The *CYP3A5* *3 genotype (C allele) is the most common in the global European, Asian and American populations (Fig. 1) which consequently influenced the development of baseline tacrolimus dose requirements. However, the T>C allele ratio is inverted in the African population as shown in Figure 1. Therefore, African populations with increased prevalence of *1 alleles require higher tacrolimus doses. This demonstrates the need for the development of an Afrocentric tacrolimus dose requirement algorithm.

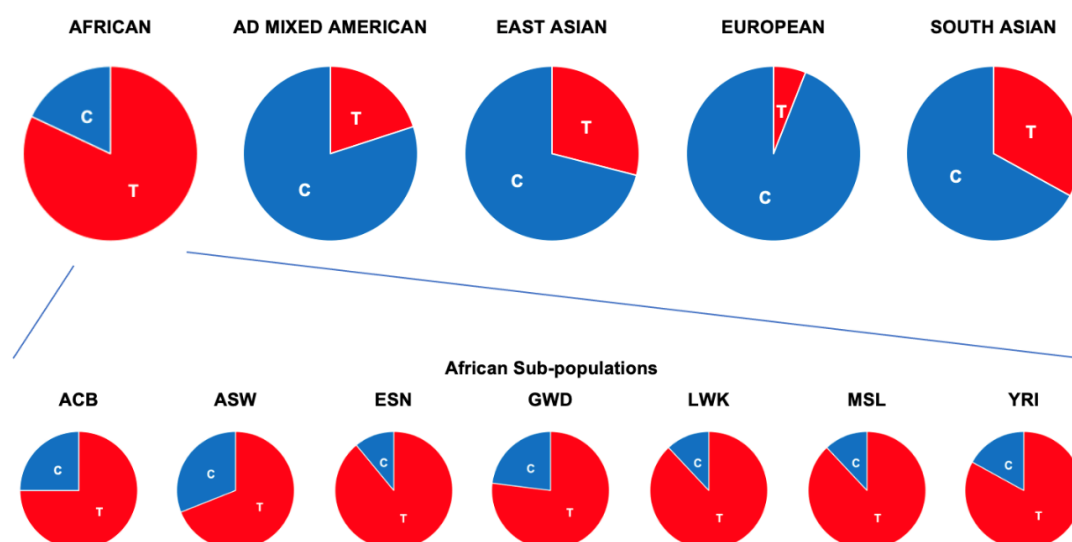


Figure 1. rs776746 1000 Genomes Project Phase 3 allele frequencies adapted from Ensembl showing global populations and the African subpopulation T>C allele frequency for the rs776746 SNP of CYP3A5. The African subpopulation codes are as follows: ACB- African Caribbean in Barbados; ASW- Americans of African Ancestry in SW USA; ESN- Esan in Nigeria; GWD- Gambian Genome Variation Project; LWK- Luhya in Webuye, Kenya; MSL- Mende in Sierra Leone; YRI- Yoruba in Ibadan, Nigeria.

1.5 Contribution of Donor *CYP3A5* Genotype

The *CYP3A5* genotype of the donor is hypothesized to play a role in the required initial dosage of tacrolimus after transplant. Therefore, routine genotyping of the donor could inform a personalised medicine approach to determining the tacrolimus dosing regimen for individual paediatric recipients (Chen et al., 2014). Aside from the *CYP3A5* genotype, the tacrolimus dose needed for each child after a liver transplant is informed by additional factors such as the graft-to-recipient weight ratio and the age of the child (Pinon et al., 2021). The recipients diseased liver is completely removed during surgery and replaced with the donor's graft. Therefore, tacrolimus metabolism by *CYP3A5* post-transplantation occurs in the transplanted donor liver and to a lesser

extent in the recipient intestine (Chen et al., 2014). Studies that have been conducted to determine the relationship between recipient CYP3A5 genotype (intestine) and tacrolimus metabolism have concluded that there is a minor contribution of recipient genotype to tacrolimus dosage variability in comparison to the abovementioned environmental factors (Chen et al., 2014; Dong et al., 2022). These conclusions support the development of a clinical algorithm that could consider both donor and recipient genotype and environmental factors in order to determine the correct tacrolimus dosing regimen (Chen et al., 2014; Muller et al., 2020). More investigations need to be conducted into the association between the highly polymorphic *CYP3A5* gene and tacrolimus dosing requirements with regards to a clinical setting in which genotyping would result in personalised tacrolimus dosing requirements (Chen et al., 2014).

In the context of ethnic diversity, Muller et al., 2020 illustrated that the *CYP3A5**1/*1 genotype was predominant in black African patients and those of mixed ancestry but not present in white individuals.. The opposite was observed for the non-expressor *CYP3A5* *3/*3 genotype which was observed in majority white patients as well as mixed ancestry patients to a lesser extent. This is consistent with the T>C allele ratios shown in Figure 1. It was observed that black African patients needed higher tacrolimus dosage requirements in comparison to white patients (Muller et al., 2020). Therefore, these findings from the renal transplant patient group can be extended to support a correlation between *CYP3A5* expression in hepatic tissue with black African ancestry and *CYP3A5* non-expression with white ancestry (Muller et al., 2020). This suggests there will be high interpatient variability for tacrolimus dose requirements dependant on ancestry of the patient. South Africa has a highly diverse ethnic

population and the post-transplant drug administering routine would be directly impacted by this correlation.

Although research focusing on the relationship between tacrolimus administration and the CYP3A5 genotype has been conducted, little to no data are available about this relationship, specifically the donors' CYP3A5 genotype, in a South African paediatric liver transplant population. Therefore, the aim of this study is to compare the pharmacokinetics of tacrolimus for paediatric liver transplant patients in relation to the donors' CYP3A5 genotypes in a South African context, considering both donor and recipient characteristics.

2. Study Aims and Objectives

2.1 Aim

To compare the relationship between liver donor CYP3A5 genotypes and mean concentration dose ratio of the immunosuppressant drug, tacrolimus, administered to South African paediatric liver recipients, taking donor and recipient characteristics into account.

2.2 Objectives

1. Determine CYP3A5 genotype of the donor
2. Determine Recipient age, Recipient sex, Recipient weight, Recipient self-reported ethnicity, Graft weight, Donor age, Donor sex, Donor self-reported ethnicity from WDPMC Liver Transplant Research Database.
3. Determine the association between the mean of recipient tacrolimus CDR over the first 15 days post-transplant and the donor and recipient factors from objectives 1 and 2.

3. Methods

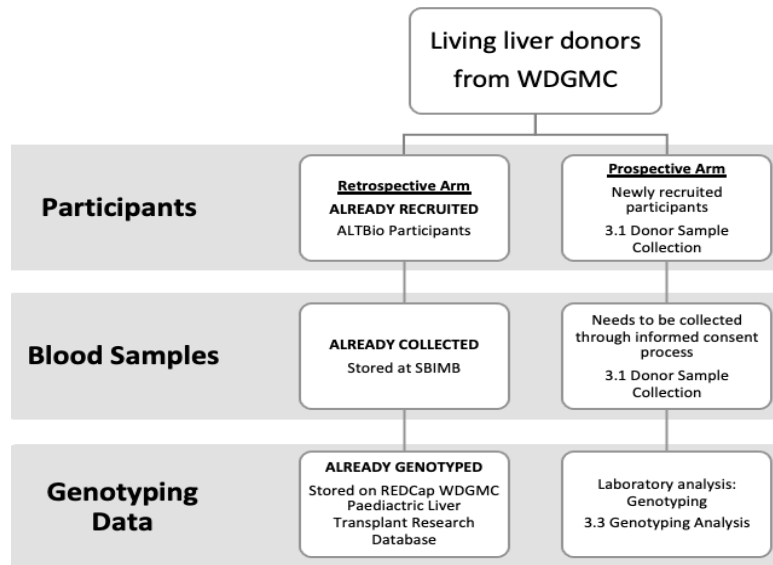


Figure 2. The retrospective and prospective arms of the project detailed in terms of participants, blood samples and genotyping data.

3.1 Donor sample collection

For some background relevant to the sample collection process for this study, the paediatric living donor liver transplant programme at WDGMC was started in 2012 (Bruckmann et al., 2020). In 2019, WDGMC began a research collaboration with the Sydney Brenner Institute for Molecular Biology (SBIMB) and the Council for Scientific and Industrial Research (CSIR) to establish an African Liver Tissue Biorepository (ALTBio). ALTBio aims to create a repository of pharmacogenomic profiles from donor DNA that will assist future research, such as this project (Fig. 2). For ALTBio, living liver donors are invited to participate, and for those who agree, written informed consent is obtained and a sample of blood collected for genotyping (M191006 Wits Human Research Ethics Committee (Medical)). In this study, the existing genotype data from participants in the ALT-BIO study will be accessed. Due to the ongoing

nature of the ALTBio project, the numbers consented may change. Currently there is already genotype data for 40 samples stored in the WDGMC Paediatric Liver Transplant Research Database which will be used in data analysis.

As part of standard practice in the WDGMC Transplant Unit, all donors are asked to sign a consent form to participate in research. On this basis, for the paediatric living liver donor transplant procedures that occurred prior to the start of the ALT-BIO study, living donors will be contacted primarily by the transplant unit and asked to for their details to be given to the primary researcher of this study be used. The donors that agree to be contacted by the researcher will be asked to participate in this study. If they agree to participate, donors will be asked to come to the WDGMC Transplant Clinic where they will be informed about the study, sign a written informed consent form and a 6ml EDTA sample of blood will be collected.

The inclusion criteria is any donor that underwent a living donor liver transplant operation at WDGMC where the recipient paediatric patient was a first time liver transplant recipient and is currently still alive. Recipients involved in transplants procedures involving simultaneous liver and kidney transplants were excluded.

The proportions of self-reported ethnicity groupings, of the 40 participants already recruited in the ALTBio study, are approximately 60% black African and 40% white.

3.2 Data collection

The WDGMC Paediatric Liver Transplant Research Database (M190749 Wits Human Research Ethics Committee (Medical)) will be accessed for the following retrospective variables at the time of transplant: Recipient age, Recipient sex, Recipient weight, Recipient self-reported ethnicity, Graft to Recipient weight ratio, Donor age, Donor sex, Donor self-reported ethnicity and Tacrolimus CDR.

3.3 Genotype Identification

- a) Perform DNA extraction (SBIMB):
 - i) Manual extraction of genomic DNA (gDNA) by Salting Out from blood samples. DNA extraction Standard Operating Procedure developed by Busisiwe Mthembu of the SBIMB Biobank.
 - ii) gDNA quantification: NanoDrop® (Inqaba Biotechnical Industries Ltd, South Africa).
- b) PCR amplification of each sample (CSIR)
 - i) Identify from literature or alternatively design primers for the correct amplification of the CYP3A5 gene
 - ii) Optimise PCR with varying conditions including a temperature gradient
- c) Genotype analysis (CSIR)
 - i) Amplified PCR product will be cleaned with the PCR QIAGEN QIAquick clean-up kit
 - ii) Perform RFLP on the amplicon as per Muller et al. and assign genotypes on known samples to establish relevant homozygote controls.
 - iii) Perform sanger sequencing on a subset of samples to confirm genotypes. The subset of 3 samples will include *1 and * homozygous and heterozygous genotypes, which were determined by RFLP analysis. Sanger sequencing will be performed at the WITS Division of Human Genetics using the BigDye™ Terminator v3.1 Cycle Sequencing Kit and the BigDye XTerminator™ Purification Kit supplied by ThermoFisher
 - iv) Perform RFLP on the remaining samples and assign genotype status.

3.4 Sample size

Sample size estimation is based on the key research question, in this case the determination of factors affecting the mean CDR level. This requires a multiple regression analysis. Considering the list of variables which could possibly be included in a multivariable model, donor and recipient ethnicities are likely to be confounded with each other and with donor genotype and so would be omitted from the model. This leaves the estimation of at most 7 parameters (2 for genotype, 2 for donor and recipient sex, 1 for Graft to Recipient weight ratio, 2 for donor and recipient age). At least 5-10, preferably 20, cases per parameter to be estimated are required (Hair et al., 2013). This translates to a minimum sample size of 70 (10 cases per parameter).

3.5 Data analysis

Hardy-Weinberg equilibrium calculations for the quality control of genotyping data will be performed using the BeadStudio 3.2 software package.

Descriptive analysis of all study variables will be carried out as follows: Categorical variables will be summarised by frequency and percentage tabulation. Continuous variables will be summarised by the mean (with standard deviation) or median (with interquartile range), depending on the distribution of the data.

The relationship of each study variable with the outcome (average CDR over the first 15 days post-transplant) will be analysed by simple regression. Categorical variables will be coded as dummy variables using an appropriate reference category.

Variable significant at $p < 0.20$ will be combined into a multiple regression model; multicollinearity will be assessed using the Variance Inflation Factor (VIF). Non-significant variables will be removed sequentially until all remaining variables are

significant at $p < 0.05$. If the sample size allows, interaction effects between key variables will be tested. Data analysis will be carried out in R and R-studio.

4. Ethics

Human Research Ethics Committee (HREC) Medical application provisionally approved. Protocol No. M220351. (Memorandum of conditional approval attached).

However, research will not begin until final approval is received from the Assessors Committee and the Human Research Ethics Committee (Medical).

5. Timing

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec
Literature review												
Protocol preparation												
Protocol review												
Ethics application												
Data collection												
Data analysis												
Write up- thesis												
Write up- paper												

6. Funding

This study is funded through the National Research Foundation (NRF) under the research project number 133135, “Development of a Genome Engineered Stem Cell Drug Toxicity Model for Developing Countries” (Confirmation of funding certificate attached)

Activity	Details	Estimated Cost
General consumables	Gloves, Tips, Glass ware, Plastic ware	R7 500
Data Collection	Blood collection tubes with EDTA	R300 per 100
for 42 donors	Blood collection needle	R110 each= R4 620
	Database access	
	Travel remuneration +-42 donors (R350)	R14 700
Analysis	GenePure Kit	Quote required
PCR	PCR Buffer	R685
	Taq DNA polymerase	R814
	Primers for <i>CYP3A5</i>	R400
	dNTPs	R1 674 for 100 µL
Visualization	Loading Dye	R502
	TAE Buffer	R858 for 4L
	EtBr	R820
	Agarose gel: SeaKem LE Agarose	R2 770
	DNA Molecular marker/ladder	R1 028
RE Digestion	Restriction enzymes	R1 500
Sequencing	BigDye terminator v3.1. sequencing kit	R200 per sample
	BigDye Xterminator purification kit	= R600 total
Total		R 36 000

7. Potential Problems

- Availability of research participants who are willing to travel to WDGMC to donate blood sample for storage in the SBIMB Biobank. We will be providing monetary remuneration for travel and appointment costs.
- Since tacrolimus is not only metabolised by CYP3A5 but also by CYP3A4 to a lesser extent, confounding effects need to be considered.

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9. Appendix of Protocol

9.1.1. Confirmation of Funding Letter



CSIR NextGen Health

PO Box 395 Pretoria 0001 South Africa
Tel: +27 12 841 2911
Email: Enquiries@csir.co.za

25 April 2022

RE: Confirmation of Project Funding

Project Title: Impact of donor CYP3A5 genotype on pharmacokinetics of tacrolimus in South African paediatric liver transplant patients

To whom it may concern,

This letter is to confirm funding for the above project has been granted by the NRF under the research project number 133135, "Development of a Genome Engineered Stem Cell Drug Toxicity Model for Developing Countries". This project is further covering her studentship stipend.

The project is being conducted by Caitlin Wheeler who is registered for MSc (Med) Genomic Medicine at WITS University with Dr Janine Scholefield as the primary supervisor.

A handwritten signature in black ink, appearing to read 'Janine Scholefield'.

Janine Scholefield, PhD

Research Group Leader
Bioengineering and Integrated Genomics Group,
NextGen Health, CSIR, Building 20, Meiring Naude Rd, Pretoria, 0001, South Africa
email: jscholefield@csir.co.za
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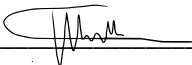
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The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the [CONSORT](#) extension for abstracts. The abstract must include the following separate sections:

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- **Methods:** how the study was performed and statistical tests used
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- **Conclusions:** brief summary and potential implications
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Keywords

Three to ten keywords representing the main content of the article.

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The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate
- Consent for publication

- Availability of data and materials
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