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Tacrolimus Pharmacogenomics in Abdominal Solid Organ Transplantation

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Submitted for the Degree of *Doctor of Medicine*

University of Edinburgh

May 2018

PREFACE

I, Stuart John Falconer, declare that this thesis which I submit to the University of Edinburgh for the award of the degree of Doctor of Medicine has been composed of my own work. Where assistance in experimental work has been provided this has been acknowledged in the text. This work has not been submitted for any other degree or professional qualification.

The funding for this research was provided by research grants from NHS Lothian liver transplant endowment funds and renal transplant endowment funds. My salary was paid by an unrestricted educational grant from Astellas® Pharma through the University of Edinburgh as a clinical research fellow in the Transplant Unit.

Stuart John Falconer

May 2018

Acknowledgements

This work would not have been possible were it not for the help and support of colleagues and friends. In particular Dr Claire Cryer and Dr Dave Turner who helped with the genotyping; Dr John Park, Dr Alex Loh, Dr Wen-Ling Choong and Dr Ross Peagam who all helped with data collection. I would like to thank Kirsty Martin, Janice Davidson and Klavs Zarins who assisted me in accessing electronic clinical records and very warm and heartfelt thanks to Ina, in hospital records, who retrieved the case notes of hundreds of transplant patients for me. I would also like to thank everyone at the Wellcome Trust Clinical Research Facility who helped with genotyping. I would like to thank Prof. Steve Wigmore for being one of my supervisors and his ongoing advice and support.

Most importantly I should like to thank my primary supervisor Mr Gabriel Oniscu who has helped and supported me relentlessly over the entirety of this project, who always has a friendly ear to bend when needed, and without whom this project would not have been possible. Gabi, thank you for everything.

Finally, I would like to thank all my family and friends who I have undoubtedly neglected at various times throughout this project but who have always been there for me whenever I have needed them.

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Abstract

Background: Abdominal solid organ transplantation has evolved from an experimental procedure to a well-established therapy within a few decades. This success is largely due to the introduction of calcineurin inhibitor immunosuppression. Tacrolimus is the most widely used calcineurin inhibitor but has a narrow therapeutic range which requires close drug monitoring to prevent both toxicity and inadequate immunosuppression. Previous studies in renal transplantation have shown that genetic polymorphisms, CYP3A5, CYP3A4*22 and ABCB1 can influence the bioavailability and pharmacokinetics of tacrolimus. These polymorphisms are closely linked to ethnicity and have never been studied in a Scottish population before. Additionally, increasing evidence suggests that high variability of tacrolimus is linked to increased graft loss in kidney transplant patients.

Methods: 5889 subjects were genotyped for the genetic polymorphisms CYP3A5 A>G allele transition, CYP3A4*22 C>T and ABCB1 C>T transition. This included 4899 healthy individuals from Generation Scotland bio-resource and 990 patients who underwent renal, liver, or simultaneous pancreas kidney transplants or were organ donors. Tacrolimus dose, trough level and renal function were measured at 11 time points from date of

transplant up to and including 12 months post-transplant. Clinical data including episodes of acute rejection, graft and patient survival were compared between the different genotypes. Separate analyses were undertaken for kidney, SPK transplants, as well as liver transplants, the latter looking at recipient and liver donor genotype. A separate cohort of 103 renal transplant patients converted from twice-daily to once-daily tacrolimus had their tacrolimus variability calculated and compared with graft survival.

Results: The distribution of the 3 different genotypes of CYP3A5, CYP3A4*22 and ABCB1 were comparable with other Caucasian populations studied previously. In renal transplant recipient expression of the A allele (GA/AA) led to significantly increased dose requirements of tacrolimus and initially lower tacrolimus trough levels. The different genotypes of ABCB1 had no effect. Expression of a CYP3A4*22 T allele trended towards a lower tacrolimus dose requirement but this was not significant. There was no difference in renal function, graft survival or patient survival with any of the polymorphisms. SPK patients had comparable results. In the liver transplant patients, the donor genotype had a greater influence than the recipient one. The donors with CYP3A5 A allele expression had significantly higher tacrolimus dose requirements and lower initial tacrolimus levels. This was apparent to a lesser extent with the recipient expression of CYP3A5

and did not reach statistical significance at all time points. There was no significant difference in tacrolimus dose requirements or level with either donor or recipient expression of ABCB1 or CYP3A4*22. There was a significantly higher incidence of acute rejection in donor CYP3A5 A allele expressers of liver transplant patients in univariate and multivariate analysis. There was no significant different in acute rejection with ABCB1 or CYP3A4*22 genotype. No differences in graft or patient survival with either donor or recipient genotype of any of the 3 polymorphisms were noted. Conversion from twice-daily to once-daily tacrolimus in the first 12 months post-transplant reduced tacrolimus variability. Patients with high tacrolimus variability pre and post conversion had significantly greater graft loss than patients with low tacrolimus variability.

Conclusion: CYP3A5 expression results in increased tacrolimus requirements to achieve adequate immunosuppression in renal transplant and SPK patients. Donor rather than recipient CYP3A5 expression is relevant for liver transplantation and dose requirements. There may be an association with donor CYP3A5 expression in liver transplant patients and acute rejection which needs further evaluation. ABCB1 and CYP3A4*22 do not appear to have a significant impact in any of the organ transplants. High tacrolimus variability is associated increased graft loss in renal transplant patients.

Lay Summary

Organ transplantation has become a well-established treatment which saves many lives each year in the United Kingdom. For transplants to be successful, patients must take medication to suppress the immune system and prevent the body from rejecting the transplanted organ.

One of the main drugs used today to reduce the activity of the immune cells in the body and prevent rejection of the transplanted organ is called tacrolimus. This drug is very effective but works within a narrow range – too little drug is ineffective whilst too much becomes toxic and can damage the kidneys and the nervous system. A blood test measures how much of the drug is in the patient's system. This is checked regularly after transplant to allow changes of the doses to achieve a steady level in the blood.

The way the body metabolises the drug is influenced by certain genes that affect how the liver breaks down the drug and how it is absorbed in the intestine. One gene in particular, called CYP3A5, makes a liver protein that very rapidly breaks down tacrolimus. This means that if a patient has a particular format of that gene, they need much higher doses to achieve a sufficient level in the blood to effectively suppress the immune system.

Not everyone has an active CYP3A5 gene. This varies in different populations and ethnic groups. We sampled approximately 5000 Scottish people and found that around 20% had the active gene. This is similar to other studied Caucasian populations. We looked at two other genes called ABCB1 and CYP3A4*22 and found the different variants of these genes were also found in similar numbers to previously studied Caucasian populations.

By testing our kidney, liver and combined kidney/pancreas patients as well as deceased liver donors, for these 3 genes we were able to determine that the ABCB1 and CYP3A4*22 genes did not have much influence on tacrolimus doses and levels. However, we found that those with CYP3A5 activity required approximately two times higher doses of tacrolimus and took a longer to reach an effective level in their blood. In liver transplant patients, a much bigger effect was seen if the donor liver had the active CYP3A5 gene, rather than the recipient. This was associated with an increased rejection rate, but this was only seen if we did not account for other factors that might increase rejection and therefore requires further analysis. There was no increased rejection in the kidney or combined kidney/pancreas patients. The expression of CYP3A5 gene did not affect how long the transplants lasted or how long the patients lived.

The last part of this thesis examined whether the variability of the tacrolimus levels had any impact on the function of the transplanted organ. We looked at patients where there has been a conversion from the usual twice-daily formulation of tacrolimus to a slow release formulation that is taken only once-daily. We found that high variability of tacrolimus led to significantly more patients losing their transplant through a form of low level chronic rejection. Increased variability did not affect how long a patient lived. We found that changing patients from the standard tacrolimus to the slow-release tacrolimus reduced this variability but only if the change happened within the first year after the kidney transplant. The three genes we examined in this thesis had no impact on tacrolimus variability. However, as seen with the other transplants in this thesis, those patients who had active CYP3A5 required two-times higher dose of the once-daily drug.

In summary, this thesis found that active CYP3A5 in kidney, combined kidney/pancreas and liver transplant patients (particularly the liver donor) led to increased tacrolimus dose requirements. Whilst no significant impact on transplant outcomes was noted, patients may have an increased risk of rejection, especially in liver transplantation. We found CYP3A4*22 and ABCB1 genes had little influence on tacrolimus or clinical outcome and

observed that high tacrolimus variability was associated with increased loss of transplanted kidneys but was not dependant on the gene expression.

Chapter I

Introduction

1 INTRODUCTION

Organ transplantation is a triumph of the modern medical age. Transplantation of solid organs such as kidney, liver, heart, lung and pancreas are nowadays routine and advances in surgical techniques, peri-operative care, immunosuppression and organ preservation techniques enable us to push the boundaries of human transplantation allowing ever more complex procedures to be undertaken. The relentless desire to replace defective or damaged body parts has led to some of the most complex surgery undertaken in the world today including composite tissue transplantation of whole limbs and even the human face.

1.1 A very brief history of transplantation

Pioneering work by the French surgeon Alexis Carrel at the turn of the 20th century in vascular anastomotic techniques laid the foundations for grafting transplanted organs into recipients. By 1918 the enthusiasm for organ transplantation was growing almost as quickly as the transplanted organs were failing and Carrel was one of the first to encounter the biggest hurdle to successful organ transplantation, rejection.

In 1933 the Ukranian surgeon Yuri Voronoy attempted the first deceased donor kidney transplant but the graft failed due to rejection [1, 2] .

The first cadaveric kidney transplant in the USA took place in 1950. The transplant recipient was a 44 year old woman with polycystic kidney disease who lost the graft to rejection 10 months later. In 1954 a kidney transplant between identical twins was performed in Boston and without the need for immunosuppression the recipient lived a further 8 years. In the United Kingdom, the first renal transplant was performed in 1960 in Edinburgh, again between identical twins, and the recipient lived for another 6 years before dying of an unrelated illness.

1.1.1 Discovering immunity and the implications for transplantation

The German surgeon Georg Schöne was one of the first to describe the concept of transplant immunity. However, despite various strategies to match the donor and recipient, as well as the attempt to use different agents for immunosuppression, the results remained extremely poor such that by

the end of the first world war, clinical organ transplantation had all but been abandoned.

Towards the end of the 1940s, Peter Medawar began to make significant progress in understanding the mechanism of rejection and his seminal paper along with R. E. Billingham in the *J. Exp. Biol* in 1951 described a variety of skin graft experiments which laid the foundations for further work in describing T-cell mediated rejection of foreign tissue. Through their work Medawar and Billingham were not only able to describe the mechanisms by which transplanted tissue was rejected but also discovered immune tolerance, whereby the immune system is not pre-programmed to distinguish between self and non-self, but rather that the immune system is shaped to do so as a result of exposure to self-antigens in early development [3]. One of the most significant discoveries by Medawar and Billingham was that corticosteroid hormones from the adrenal gland delayed skin graft rejection in rabbits showing that this insurmountable barrier to transplantation could be overcome [4].

1.2 Immunosuppression

1.2.1 Cortisone

Cortisone, the glucocorticoid steroid hormone made by the adrenal gland, was discovered in the 1940s by Edward Calvin Kendall while he was a researcher at the Mayo Clinic and by the early 1950s it was being manufactured commercially although it was not until some time later that is was used in transplant patients in the form of prednisolone [5].

The use of steroid alone would require high doses of Prednisolone in order to achieve adequate immunosuppression, resulting in serious side effects including insulin resistance and diabetes, osteoporosis, increased susceptibility to infection, hypertension and increased risk of malignancy. While it seemed plausible that the immune system could be suppressed in order to facilitate successful transplantation, it was clear that other therapeutic agents would be required in order to reduce the dose of steroid and the associated side-effects.

1.2.2 Azathioprine

In 1957 George Herbert Hitchings and Gertrude Elion synthesized Azathioprine. Originally designed to be a chemotherapy agent, Azathioprine is a prodrug, a precursor to 6-mercaptopurine (6-MP). Azathioprine is converted to 6-MP by non-enzymatic reductive cleavage of the thioether (-S-) mediated by glutathione. Through various methylation and hydroxylation steps the active metabolite of Azathioprine, methyl-thioinosine monophosphate (MeTIMP), is formed. This inhibits purine synthesis and thus significantly reduces the activity of both B and T lymphocytes, the prime facilitators of rejection in transplanted organs. In 1959 Shwartz and Damashcek found that 6-MP inhibits antibody production in rabbits and this, along with Medawar's previous work, led Sir Roy Calne to consider 6-MP as an anti-rejection drug [6, 7]. Calne's experimental renal transplants in dogs confirmed that Azathioprine, rather than 6-MP, provided more consistent results and therefore subsequent kidney transplant human trials used Azathioprine along with corticosteroids[8][9].

Azathioprine allowed a reduction in the dose of corticosteroid and for a time a combination of the two drugs formed the cornerstone of immunosuppression in kidney transplantation. Although progress was made, results remained relatively poor. *Murray et al* reported in the New England

Journal of Medicine in 1963 the first accounts of prolonged survival in kidney transplant recipients, but this was measured in months rather than years. The difficulty lay between the requirement of adequate immunosuppression to prevent allograft rejection and the severe side effects of Azathioprine at the doses required to achieve this. These side-effects included bone marrow suppression leading to anaemia and leukopenia, hepatotoxicity, increased risk of malignancy (particularly skin cancer and lymphoma), diarrhoea, fatigue, dizziness and skin rashes.

1.2.3 Ciclosporin

In 1972 Sandoz (now Novartis) discovered that a drug called Ciclosporin A (CsA), which had been isolated a few years earlier by Hans Peter Frey from a soil sample in Norway, had powerful immunosuppressive effects. In a case series of seven patients who underwent renal transplantation and had CsA as the sole immunosuppressant *Calne et al* demonstrated that CsA could indeed be used to provide effective immunosuppression although nephrotoxic and hepatotoxic effects of the drug were noted in some of the patients [10]. Nevertheless, this new drug provided a longer graft survival compared to what was achieved so far. Calne started using CsA in all types of transplants and reported good results with the use of CsA in 32 renal transplants, 2

pancreas transplants and 2 liver transplants [11]. In 1981 Thomas Starzl published a report describing a series of 14 liver transplant patients who were immunosuppressed with ciclosporin and prednisolone alone, with 83% survival at 12 months follow up despite the fact that liver biopsy showed signs of acute rejection in six patients who required adjustments in steroid dose [12].

The discovery of ciclosporin was one of the milestones in transplantation as it provided, for the first time, adequate graft survival allowing transplantation to become accepted as the treatment of choice for end stage diseases of the kidney, liver, lung and heart.

Ciclosporin is still used today, but even back in the 1980s it was recognised that it had significant side effects, predominantly nephrotoxicity and the development of new onset diabetes after transplantation (NODAT). Given the encouraging results of cyclosporin, but mindful of the serious adverse effects, pharmaceutical companies tried to develop alternative immunosuppressive drugs with greater efficacy and fewer side effects.

1.2.4 Tacrolimus

In 1987, Toru Kino described a novel immunosuppressive agent (FK-506) which had been isolated from the fungus *Streptomyces* [13]. He reported a series of mouse experiments where FK-506 was able to suppress the *in vitro* proliferative response of human lymphocytes to alloantigen stimulation when added at the initiation of cell cultures [14].

Tacrolimus has the chemical formula $C_{44}H_{69}NO_{12}$ and a molecular weight of 804.018 g/mol. Tacrolimus is lipid soluble and most commonly administered in an oral preparation although intravenous, rectal and sublingual preparations are available for systemic administration. Topical tacrolimus is used to treat a variety of dermatological conditions. The structure of the tacrolimus molecule is detailed below.

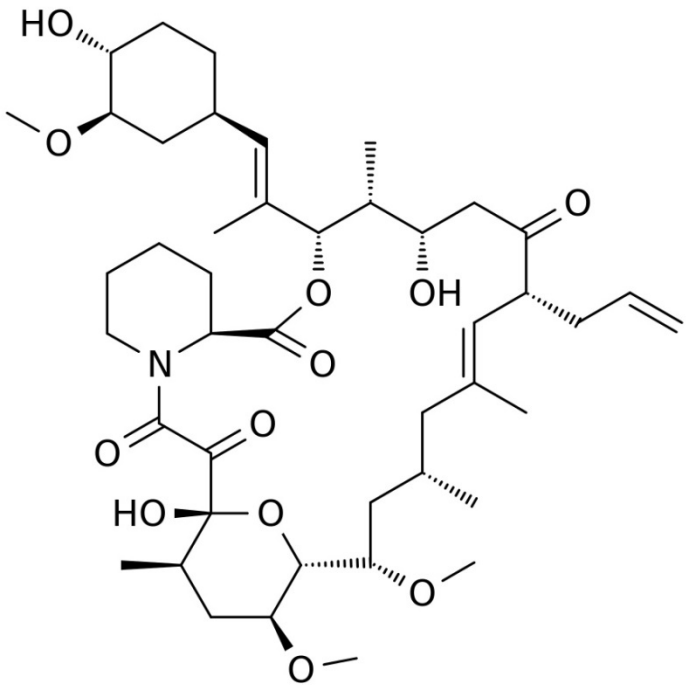

Figure 1.1 Chemical structure of tacrolimus

(Reproduced from the NIH PubChem database)

These early studies showed that FK-506 was considerably more potent than CsA and required concentrations 100 times lower to inhibit the generation of cytotoxic T cells. While FK-506 had a quite distinct molecular structure compared with CsA, its mechanism of action appeared to be similar. During the 1990s the mechanism of action of FK-506 became better understood [15, 16] and FK-506 was considered as a promising new immunosuppressive agent for transplant patients [17]. Following the initial experience using the drug as a rescue therapy for liver transplant patients suffering from rejection with CsA based immunosuppression [18] the Pittsburgh group reported on the use of FK-506 as the primary immunosuppressive agent with promising

results [19, 20]. Shortly after other studies reported an improvement in patient and graft survival at 1 and 2 years with FK-506 when compared with ciclosporin [21]. Similar findings were reported in renal transplant studies [22, 23] where a triple combination of FK-506, Azathiorpine and steroids led to a reduction in rejection rates [24].

In 1994 FK-506 became licensed for use under the unbranded name Tacrolimus (sold as Prograf® by Astellas Pharma) and replaced ciclosporin as the primary immunosuppressant drug used in solid organ transplantation. Similar to ciclosporin, tacrolimus has a narrow therapeutic index with neurotoxicity and nephrotoxicity as two of the main side effects. The introduction of routine blood concentrations assays allowed a better definition of the therapeutic ranges and a clearer understanding of the side effects [25]. The measurement of the tacrolimus trough concentration, which is the tacrolimus whole blood concentration taken 12 hours after the dose is administered, revealed considerable variability between individuals who received the same dose suggesting that maintaining individual patients within the therapeutic window would not be achieved by a 'standard dosing regimen' but would rather require a tailored dose guided by the tacrolimus trough concentrations. The detailed aspects of therapeutic monitoring of tacrolimus are discussed later in this chapter. The narrow therapeutic index

as well as the significant side effects of the calcineurin inhibitors led to development of other immunosuppressant agents.

1.3 A brief summary of transplant immunology

Manipulation of the recipient immune system to prevent organ rejection is essential for maintaining long term graft function. Allograft rejection occurs when the recipient immune system recognises the transplanted organ as 'non-self' and therefore activates the adaptive immune system which would normally respond to foreign antigens. The normal immune response is rapid, antigen specific and protective against invading pathogens although this is, of course, an undesirable effect in the case of a transplanted allograft.

1.3.1 Initial Inflammatory Response

The entire process of allograft rejection is driven by an inflammatory response which is multi-faceted but we now know begins before the transplant has taken place and, in fact, before the organs have even been retrieved in a deceased donor. The neuroendocrine disturbances often called the 'catecholamine cascade' in DBD donors, alongside the resultant haemodynamic disturbances can initiate the inflammatory process which is

further added to by a brief period of warm ischaemia during the retrieval operation and then static cold storage while the organs are transported [26]. Once the initiation of this inflammatory process underway, pro-inflammatory molecules and cellular pathways lead to the development of an ischaemia-reperfusion injury, which will manifest itself following the implantation and reperfusion of the allograft.

1.3.2 Ischaemia-Reperfusion Injury

An ischaemia-reperfusion injury to some degree is inevitable after every transplant and is closely related to the cold ischaemic time, with a longer cold ischaemic time leading to a more significant ischaemia-reperfusion injury [27]. The direct immune response against an allograft is initiated when damaged or dying cells release endogenous proteins or molecules, which are known as damage-associated molecular patterns (DAMPs) and by the release of pro-inflammatory cytokines. This cellular damage occurs due to ischaemia, switching the cell to anaerobic metabolism resulting in reduced ATP. Subsequently the Na^+/K^+ pumps start to fail and the cell becomes oedematous and acidotic causing an influx of calcium ions and mitochondrial injury. The cells become apoptotic and release inflammatory cytokines and DAMPs which, in turn leads to increased MHC class II

expression, furthering the immune response. Inflammatory DAMPs such as heat-shock proteins (HSP), DNA fragments and high-mobility group box 1 (HMGB-1) are recognised by specific cellular receptors such at toll-like receptors (TLRs) [2]. This process occurs very readily in solid organ allografts as the apoptotic cells are not so readily cleared following ischaemia-reperfusion injury and as the membrane integrity of the cells fails large amounts of pro-inflammatory cytokines are released which activate dendritic cells which will migrate from the allograft to lymphoid tissue and stimulate T cell production [28]. The severity of ischaemia-reperfusion injury has been shown to correlate with an increased incidence of acute rejection and a study in 2011 by *Barba et al* reported that a cold ischaemic time of > 18 hours increased the incidence of acute rejection in kidney transplant patients [29]. Other studies have shown that ischaemia-reperfusion injury contributes to delayed graft function however the relationship between ischaemia-reperfusion injury, delayed graft function and acute rejection remains unclear, with some studies suggesting a longer CIT leads to increased acute rejection but no overall negative effects [30]. While ischaemia-reperfusion injury and the long term impact on graft function and survival remains a topic for debate, there is evidence to support that a more significant ischaemia-reperfusion primes the allograft for a more severe immunological response which manifests itself with increased acute rejection.

1.3.3 Immune Response Following Transplantation

The main components of the adaptive immune systems are the cellular response (T cells) and the humoral response (B cells/antibodies) and both play a role in graft rejection. T cells are formed in the thymus derived from stem cells which originate in the bone marrow [31]. T cells differentiate into different subtypes while in the thymus and T cells designated CD4+ and CD8+ are of particular relevance in cellular immunity. T cells can recognise antigens presented by the major histocompatibility complex (MHC), which is also known as the human leukocyte antigen, (HLA) through T cell receptors (TCRs) expressed on their cell surface. B cells express surface immunoglobulins which act as their antigen receptor and are the precursor to plasma cells which, when activated, will secrete soluble immunoglobulin in the form of antibody. In addition to the adaptive system of immunity, there is the innate immune system which comprises of a molecular component (cytokines, Toll-like receptors and complement) and a cellular component (macrophages, dendritic cells, natural killer cells and neutrophils). There are important interactions between the adaptive and innate immune systems when mounting an immune response. The primary targets of the alloimmune response are HLA antigens (MHC). Class I MHC molecules (HLA A, B, and C) are expressed on all cells and are recognised by CD8+ T cells. Class II MHC molecules (HLA DR, DQ, and DP) are recognised

by CD4+ T cells and are found on a few specialised cell types known as antigen presenting cells (APCs) which include dendritic cells, macrophages and B cells.

Allograft rejection is primarily mediated by T cells and many immunosuppressive agents target T cell signalling pathways to prevent T cell activation. In order for T lymphocytes to achieve optimal activation and expansion, they must receive two coordinated signals [32]. The first signal is provided by the T cell receptor (TCR) as it recognises the MHC-antigen complex expressed on the surface of the APC and the second signal comes from costimulatory molecules on the T cells which interact with ligands on the APC [33].

Costimulatory molecules (receptor-ligand pairs) can result in both activation of T cell activity or cause T cell activity to wane depending on which costimulatory molecules are involved in TCR signal transduction. In general terms, binding of the T cell based CD28 molecule results in T cell activation and binding of the CD152 molecule, also known as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) results in reduced T cell activity. The ligands for CD28 and CTLA-4 are the B7 molecules, CD80 and CD86, both of which can bind to either CD28 or CTLA-4, although CTLA-4 has a much higher affinity [34, 35]. This process describes a two-signal model for positive and negative co-stimulation of T cells. In order for a T cell to become activated

it must first receive signal one from MHC-antigen complex interacting with the TCR and the second positive co-stimulatory molecule interacting with their respective ligand on the APC. Once this occurs, T cells will proliferate, secrete cytokines and differentiate into effector cells [32]. The cytokines IL-2 and IFN-γ from T cells and IL-12 from APCs produce an environment which favours the recruitment of T cells and clonal expansion [36]. Interleukin-2 works as an autocrine agent, actively upregulating IL-2 from the cell which is secreting it, as well as upregulating CD25, the high-affinity receptor for IL-2. IFN-γ will induce the expression of class II MHC molecules on non-lymphoid graft tissue and upregulate class I MHC molecules, increasing the amount of alloantigen present [37].

The rejection of the transplanted allograft is brought about by the cytotoxic effect of CD8+ cells as they bind to MHC class I. Cell death occurs by a Ca^{2+} dependent mechanism whereby Ca^{2+} influx results in the exocytosis of cytolytic granules which contain a lytic protein called perforin which perforates the cell membrane and serine proteases called granzymes which then lyse and destroy the cell [38]. In the absence of Ca^{2+}, CD8+ cells can initiate cellular apoptosis by upregulating *fas* ligand on it cell surface. Crosslinking of *fas* on target cells with the *fas* ligand on the CD8+ cells will then induce programmed cell death of the target cell. If this process is

allowed to go on unchecked after solid organ transplantation, the inevitable outcome is acute cellular rejection and the loss of the transplanted allograft.

In order to prevent this and maintain function of the transplanted organ, immunosuppressive drugs are required to inhibit T cell activity and the associated pathways.

Therefore, immunosuppression is usually provided by a combination of different drugs which include a calcineurin inhibitor in most cases. Currently, tacrolimus is the most commonly used calcineurin inhibitor for all types of organ transplantation.

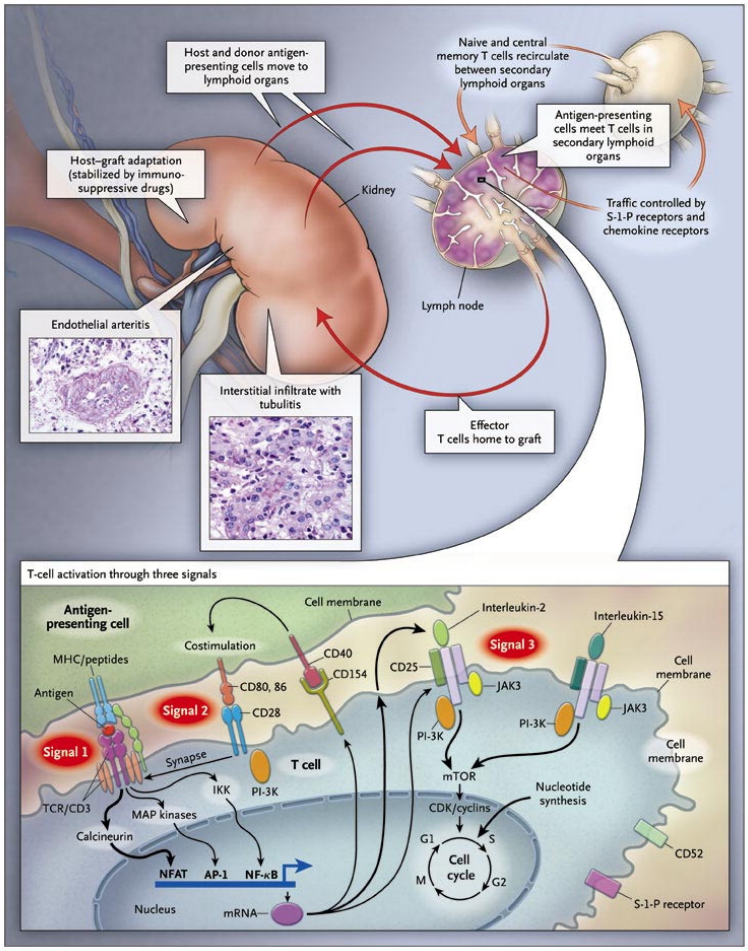

Figure 1.2 Key steps in T-cell mediated acute cellular rejection outlining interaction with the T cell receptor, different signalling pathways leading to the transcription factors that upregulate interleukins and other cytokines which act as co-stimulatory molecules in the activation and proliferation of T cells.

Immunosuppressive Drugs for Kidney Transplantation, New England Journal of Medicine [39].

1.3.4 Tacrolimus Mechanism of Action

In order to understand the mechanism of action of tacrolimus it is necessary to first look closely at what happens when a T cell encounters an alloantigen

and, in particular, its interaction with the intracellular molecule calcineurin, which plays a critical role in the process of IL-2 production and therefore T cell activation and proliferation. The TCR encountering alloantigen leads to an increase of intracellular calcium within the T cell. Calcineurin is formed from 2 subunits: calcineurin A (CnA) which is responsible for the phosphatase activity of calcineurin and acts as a catalyst and calcineurin B (CnB) which regulates the activity of CnA. CnB is particularly responsive to the increase in intracellular calcium which occurs upon TCR interacting with alloantigens. The activation of CnB initiates the phosphatase activity of CnA which then dephosphorylates cytoplasmic Nuclear Factor of Activated T-cells (NFATc), a gene transcription factor [40]. NFATc, along with activated calcineurin, translocates to the nucleus of the T cell where it facilitates the transcription and upregulation of various cytokines and growth factors including IL-2, IL-4, IFN-γ and TNF-α, [41] .

Calcineurin inhibitors block the action of calcineurin within the T cell and thus prevent the dephosphorylation of NFATc, preventing gene transcription of IL-2 and considerably reducing T cell activity. Ciclosporin and tacrolimus achieve this in slightly different ways. Both bind to intracellular proteins known as immunophilins but whilst Ciclosporin A binds to the immunophilin called cyclophilin, tacrolimus (FK506) binds to the FK-binding protein (FKPB). This FK506-FK PB complex will then bind to

calcineurin phosphatase, inhibiting its activity and preventing the dephosphorylation of NFATc. In addition to blocking IL-2 transcription, tacrolimus also inhibits other calcium dependent events such as nitric oxide synthase activation, cell degranulation and apoptosis, markedly reducing T cell activity [42].

1.3.5 Tacrolimus bioavailability and pharmacokinetics

Currently, there are several forms of Tacrolimus, either as immediate release preparation administered twice daily (Tac-BD) or a modified release administered once daily (TAC-QD). The once-daily modified release version of tacrolimus has different pharmacokinetics to the original twice daily preparation.

Tac-BD is absorbed throughout the gastrointestinal tract but absorption is incomplete and is highly variable between individual patients. The absolute bioavailability of tacrolimus is 18±5% in adult healthy volunteers, 17±10% in adult renal transplant patients and 22±6% in adult liver transplant patients (National Center for Biotechnology Information. PubChem Compound Database; CID=445643). There have been some studies published that report bioavailability, ranging as widely as 4 -93% [43, 44]. Numerous factors are thought to affect the bioavailability of Tac-BD including the time between

administration and last ingestion of food, corticosteroid dose, haematocrit level, and variations in cytochrome P450 activity.

Tac-BD binds extensively to erythrocytes, albumin and α_1-acid glycoprotein, is metabolised by cytochrome P450 enzymes in the liver and is counter-transported by the drug efflux pump p-glycoprotein in the enterocyte [45]. The drug is almost entirely excreted through the biliary tract with only 1% excreted in the urine [46].

The half-life (the time taken for half of the drug to be cleared) is variable depending on whether the drug is administered to a healthy volunteer, a renal transplant patient or a liver transplant patient, paediatric or adult patients. Studies in renal transplant patients suggest a median half-life of approximately 19 hours with the mean in the region of 48 hours (range: 4.6 – 939 hours) [43]. The drug has different pharmacokinetics in the liver transplant population, particularly in the early post-transplant period, given the fact that the drug is metabolised in the liver [47].

1.4 Tacrolimus formulations and Pharmacokinetics

1.4.1 Once-daily Modified Release Tacrolimus (Tac-QD)/Advagraf®

A modified release preparation of tacrolimus which could be taken once-daily has been available since 2004. The once-daily preparation, developed

as a means of reducing the pill burden and improving adherence among patients, does have some key differences to its standard-release predecessor. It is absorbed more distally in the gut than the standard twice-daily preparation (Tac-BD) which may make polymorphisms of ABCB1 and P-gp expression more relevant as P-gp expression in the proximal and distal gut is different.

Early studies in healthy volunteers and also stable renal and liver transplant patients reported that conversion on a mg:mg basis from twice daily tacrolimus (Tac-BD, Prograf®) to once-daily modified release tacrolimus (Tac-QD, Advagraf®) was both safe and effective [48]. It was hoped that Tac-QD would improve patient adherence by reducing the pill burden and that the modified release formulation would reduce the within-patient variability of tacrolimus which has been shown to be detrimental to long term graft survival [49]. Further studies of both renal and liver transplant recipients concluded that patients converted on a mg:mg basis had stable tacrolimus levels with no difference in the acute cellular rejection or graft loss compared with patients on the standard twice-daily Tac-BD formulation [50, 51].

As further studies started to emerge, it became apparent that Tac-BD and Tac-QD were not, in fact, bioequivalent and there was a lower tacrolimus trough concentration (C_0) following conversion [52, 53]. By 2011 there had

been 15 published studies comparing Tac-BD and Tac-QD, 4 of which were industry designed phase-II pharmacokinetic studies in adult kidney, adult liver, adult heart and paediatric liver transplants. *Barraclough et al* published a comprehensive review examining these studies and concluded that while the Tac-BD and Tac-QD formulations were bioequivalent by Food and Drug Administration (FDA) and European Medicines Agency (EMA) standards, there was a reduction of total tacrolimus exposure of between 5-15% following conversion to Tac-QD necessitating numerous dose changes in several cases [54]. Furthermore *de novo* transplant recipients exhibited reduced AUC_{24} requiring higher initial doses whilst data from some phase III studies suggested reduced efficacy of Tac-QD with higher rates of acute rejection in both liver and kidney transplant cohorts.

More recently, the OSAKA trial (a randomized controlled trial comparing *de-novo* Tac-BD and Tac-QD without basiliximab induction in kidney transplantation) reported that there was no significant difference in clinical outcomes between patients prescribed 0.2 mg/kg/day of Tac-BD compared with 0.2 mg/kg/day Tac-QD [55].

Tac-QD is increasingly used in clinical transplantation. Given the obvious differences in the pharmacokinetics between the standard and the modified release preparation it cannot be assumed that the SNPs will impact in the

same way. Gut expression of CYP3A5 is more abundant in the proximal gut, such as the jejunum, whereas there is increased P-gp expression more distally in the gut in the colon [56]. It is therefore conceivable that the ABCB1 polymorphisms may have a greater impact on Tac-QD than Tac-BD whilst the gut expression of CYP3A5 could impact less on the pharmacokinetics of Tac-QD. *Niioka et al* reported that the ABCB1 polymorphism had no impact on the bioavailability of either Tac-BD or Tac-QD, although an overall reduced bioavailability of Tac-QD was noted. However, CYP3A5 expressers had significantly reduced bioavailability for both preparations meaning that CYP3A5 expressers who were prescribed Tac-QD had the lowest bioavailability [57]. *Glowacki et al* reported a reduced tacrolimus exposure (AUC_{24}) when renal transplant patients were converted from Tac-BD to Tac-QD. This reduction in exposure was most pronounced in those individuals who were carriers of the CYP3A5*1 allele and therefore CYP3A5 expressers [58]. These studies support the view that tacrolimus exposure reduces when patients are converted from Tac-BD to Tac-QD and highlight the possibility that CYP3A5 expressers may be particularly at risk of achieving sub-therapeutic tacrolimus levels when converted and require careful monitoring post conversion.

In addition to the variability of tacrolimus C_0/D levels seen between different patients, there is now increasing evidence that there can be significant

intra-patient variability. Studies investigating this phenomenon are still relatively few but recent data suggests that high levels of intra-patient variability result in poorer patient outcomes. In a cohort of 297 renal transplant patients treated with Tac-BD, *Borra et al* found that, patients who were deemed to have 'high within-patient variability' had significantly higher rates of graft loss compared with the patients in the low variability group [49]. *Wu et al* demonstrated that conversion from Tac-BD to Tac-QD resulted in a significant drop in C_0 immediately after conversion but also reported a significant reduction in the intra-patient variability [59]. These two studies used different methodologies. *Borra et al* based their estimations on an equation which calculated a percentage by which each patient deviated from their own mean dose corrected tacrolimus levels. In contrast, *Wu et al* used the coefficient of variation to calculate the variability and used C_0 levels that were not corrected for the dose.

There have been very few studies examining the relationship between the genetic polymorphisms and tacrolimus intra-patient variability, as well as whether this is impacted by a change to Tac-QD. In a study of 29 healthy Korean volunteers who took either branded immediate release tacrolimus (Prograf®) or a generic formulation of tacrolimus, *Yong et al* performed AUC_{24} and genotype analysis and suggested that individuals who carried the CYP3A5*1 allele had reduced intra-individual variability compared with

those of the CYP3A5*3/*3 genotype [60]. Another study by *Pashaee et al,* however, examined 208 renal transplant patients taking tacrolimus found no significant link between CYP3A5 genotype and the intra-patient variability of tacrolimus, although the preparation of tacrolimus is not specified in this study [61].

Most recently, *Stifft et al* published a prospective study of kidney transplant patients and using AUC_{24} and the coefficient of variation found that Tac-QD had significantly reduced variability compared with Tac-BD. They suggested that this reduction was more pronounced in patients who carried the CYP3A5*1 allele and concluded that as there was a very small number of subjects who carried this allele, this warrants further study [62]. This study was robust in that it looked at AUC_{24} profiles and the dose of tacrolimus remained constant throughout. What has not yet been established by any study to date is whether the reduction in variability persists long term after conversion and whether thus leads to an improvement in graft survival.

1.4.2 Envarsus® XR

Recently another modified release preparation of tacrolimus that is taken once-daily has come on the market with a different pharmacokinetic profile to both Tac-BD (Prograf®) and Tac-QD (Advagraf®). The 2 main studies to

date by *Gaber et al* which reported on kidney transplant patients and *Alloway et al* which studied liver transplant patients suggest that approximately a 30% lower dose is required to achieve comparable tacrolimus exposure as the twice-daily capsules [63, 64]. Envarsus® XR uses a different type of modified release technology to release the drug called MeltDose® which offers increased solubility and bioavailability compared with other Prograf® [65]. It appears to have a flatter pharmacokinetic profile and reduced peak/tough fluctuations as well as a lower peak concentration (C_{max}) and a longer time to reach that peak (T_{max}) [66]. The pharmacokinetic profile and AUC_{24} for Prograf®, Advagraf® and Envarsus® XR is shown in Figure 1.3 below.

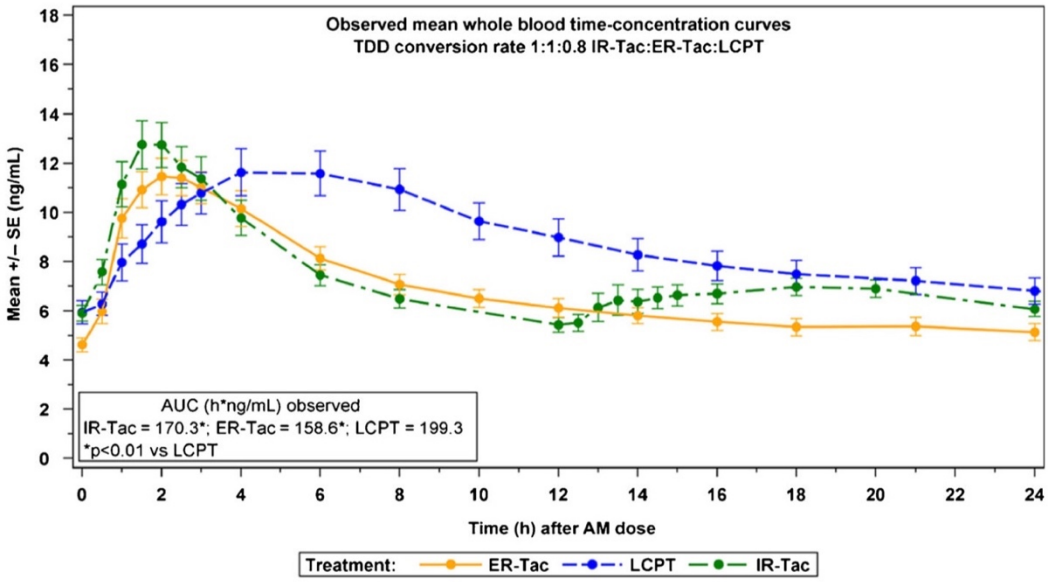

Figure 1.3 Pharmacokinetic whole blood time-concentration curves of Prograf® (IR-Tac), Advagraf® (ER-Tac) and Envarsus® XR (LCPT).

Observed steady-state pharmacokinetic profiles of the 3 tacrolimus formulations. AM morning, AUC area under the curve, ER-Tac tacrolimus extended release, IR-Tac tacrolimus immediate release, LCPT life cycle pharma tacrolimus. Reproduced from Tremblay S, Nigro V, Weinberg, J, Woodle ES, Alloway RR. A Steady-State Head-to-Head Pharmacokinetic Comparison of All FK-506 (Tacrolimus) Formulations (ASTCOFF): an open-label, prospective, randomized, two-arm, three-period crossover study. Am J Transplant. 2017;17(2):432-42. [67]

1.4.3 Sirolimus

Sirolimus was isolated in 1975 from the fungus *Streptomyces hygroscopicus* found in a soil sample collected from Rapa Nui (Easter Island). It inhibits the action of mammalian Target of Rapamycin (mTOR), blocking several signal transduction pathways that results in the arrest of the cell cycle in the G1 phase, preventing the up-regulation of IL-2 mediated by CD28 [68] thus inhibiting T-cell activity. Sirolimus is not used as widely as tacrolimus

although studies have shown that it can be used effectively as an immunosuppressive drug [69, 70].

1.4.4 Mycophenolate Mofetil

Mycophenolate mofetil (MMF) inhibits purine synthesis and selectively inhibits the proliferation of B and T cells while maintaining haematopoiesis and neutrophil activity [71]. Early clinical trials of MMF in renal transplantation showed promising results with a reduction in the rate of acute rejection by 30 to 50% when compared with Azathioprine [72, 73]. This resulted in MMF being widely adopted by the transplant community as a more favourable agent to Azathioprine for renal transplantation and thereafter for liver, lung and heart transplant patients. The Symphony study explored different immunosuppression regimens and showed that MMF in conjunction with low dose tacrolimus results in lower rates of acute rejection in renal transplant recipients, improved graft survival and better renal function [74, 75]. As a result of this study, MMF has largely replaced Azathioprine as part of the primary immunosuppressive regimen across renal transplantation, due to better outcomes in terms of acute rejection and graft survival as well as allowing a reduction in calcineurin inhibitor exposure (primarily tacrolimus) with an associated reduction in side-effects.

1.4.5 Belatacept and Biological Agents

The current 'triple therapy' immunosuppression regimen of a calcineurin inhibitor, an antiproliferative agent and steroids has allowed successful transplantation of kidneys and other organs and undoubtedly saved and improved many lives. The burden of immunosuppression, however, is not without its drawbacks such as the nephrotoxicity and diabetogenic effects of tacrolimus. The variability of tacrolimus can also be problematic and thus interest in other types of immunosuppression has developed in a bid to circumvent these problems. Belatacept, a fusion protein composed of the Fc fragment of IgG1 linked to the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), selectively inhibits T-cell activation through co-stimulation blockade [76]. The BENEFIT was a randomised trial that compared belatacept based immunosuppression (an intense and less intense regimen) against ciclosporin and reported significant reduction of 43% risk of death or graft loss for both the intensive and less intensive or belatacept compared with ciclosporin. Belatacept is associated, however, with increased rates of acute rejection. A large multicentre trial comparing belatacept and tacrolimus is still needed however a smaller randomised study of 40 kidney transplant by *de Graav et al* compared tacrolimus with belatacept *de novo* following kidney transplantation and found higher rates of acute rejection at 1 year [77]. The

role for belatacept is still under evaluation and with other biological agents coming on the market, this expanding class of drugs may help to reduce the burden of calcineurin inhibitors in transplant patients.

1.5 Antibody induction

A different group of immunosuppressive agents used routinely in modern solid organ transplantation are antibody induction agents. These agents were introduced as early as the 1960s however towards the end of the 1980s and early 1990 their use in renal transplantation became much more widely established, primarily due to the development of more modern agents that could be used in the short term immediately following transplantation (known as induction agents) while standard immunosuppression with oral medications (calcineurin inhibitor, antiproliferative and steroids) was established. These agents have a defined role for induction of immunosuppression although they are not used routinely for maintenance immunosuppression.

The 3 most commonly used induction agents are:

- Basiliximab (Simulect©), a monoclonal antibody interleukin-2 receptor antagonist (IL-2RA), which inhibits CD25;

- alemtuzumab (Campath©), another monoclonal antibody that inhibits CD52 and causes lymphocyte depletion

- rabbit anti-thymocyte globulin (Thymoglobulin©) a polyclonal anti-T cell antibody [78]

Basiliximab and alemtuzumab have been routinely used for induction immunosuppression however basiliximab is now the most widely used induction agent in solid organ transplantation. Anti-thymocyte globulin is more commonly used for the induction of 'high immunological risk' renal transplants (where patients are highly sensitized and have increased risk of acute rejection) or the treatment of steroid resistant rejection [78].

1.5.1 Basiliximab

Basiliximab is a murine-human chimeric monoclonal antibody to the interleukin-2α receptor expressed on the surface of T cells. Basiliximab has a high affinity for the IL-2α receptor and therefore competes with IL-2 and blocks the receptor from signalling. Inhibition of IL-2 signalling prevents the upregulation and proliferation of T-cells upon which IL-2 co-stimulation is dependent. Basiliximab is the most widely used antibody induction agent in renal and simultaneous pancreas-kidney (SPK) transplantation, and is used routinely in this unit as induction therapy in

combination with tacrolimus, MMF and prednisolone as maintenance immunosuppression.

1.5.2 Alemtuzumab

Alemtuzumab is a monoclonal antibody directed at the surface glycoprotein CD52. Its exact mechanism remains unclear however its effect is to cause significant leukopenia by means of antibody-derived cell lysis of lymphocytes which leads to the depletion of T cells and B cells. It has never been licensed for organ transplantation and there have been no phase II/III trials performed in solid organ transplantation [79]. Nevertheless its use as induction therapy in transplantation has been increasing world-wide (off-label) and there have therefore been clinical trials conducted to examine its efficacy, although the results have been conflicting [80, 81]. A recent multi-centred randomised trial in the UK, the 3C study, compared alemtuzumab-based induction therapy followed by low dose tacrolimus, mycophenolate and no steroids with standard basiliximab-based therapy (basiliximab induction with standard dose tacrolimus, mycophenolate and prednisolone) in kidney transplant patients. This large multicentre study reported a significant reduction in biopsy proven acute rejection at 6 months (7% alemtuzumab vs 16% basiliximab group, hazard ratio 0.42, log-rank $p<0.0001$) [82]. The long term follow up on graft and patient outcomes is still awaited but this could potentially reduce the burden of tacrolimus on

transplant patients, reducing nephrotoxicity and other side effects such as new onset diabetes after transplantation. A smaller study by *Hanaway et al* in the New England Journal of Medicine in 2011 examined induction with alemtuzumab compared with conventional induction therapy (basiliximab for low immunological risk transplants or rabbit antithymocyte globulin [rATG] for high immunological risk kidney transplant recipients). Similar to the 3C study, there was a significantly lower rates of acute rejection in the alemtuzumab group compared with the basiliximab group at 6 months (3% vs 15%, $p<0.001$) and at 12 months (5% vs 17%, $p<0.001$). At 3 years the rate of biopsy proven acute rejection in the low-risk group remained significantly lower in the alemtuzumab group compared with the basiliximab group (10% vs 22%, $p=0.003$). There was no significant difference between the alemtuzumab group and the rATG group in the high-risk patients (18% vs 15%, $p=0.63$) suggesting that the superiority of alemtuzumab was restricted to low immunological risk transplants only [83].

1.5.3 Rabbit anti-thymocyte globulin (rATG)

Anti-thymocyte globulin (ATG) is a polyclonal antibody derived from the sera of rabbits (but also horses and on rare occasions goats) with human thymocytes or T cells. The precise mechanism of action of ATG is not fully understood, however its administration brings about T cell depletion by

complement-dependent lysis in the blood compartment but it is also thought to bring about apoptosis and phagocytosis by macrophages in the lymphoid tissue [84]. The effects of ATG are more wide-reaching however and B lymphocytes are also targeted by ATG, as it contains a number of antibodies against B cell antigens. There have been both *in vitro* and *in vivo* studies that have shown how ATG can modulate various other parts of the immune system such as intracellular adhesion molecules (ICAM), plasma cells, monocytes and dendritic cells, all of which might play a role in the human response to ATG administration [85, 86].

1.6 Immunosuppression in the Future

There have been remarkable developments in immunosuppressive drugs since the early days of transplantation such that today organ transplantation is highly successful with very significant improvements in short and long-term outcomes. Nevertheless, the immunosuppressive agents carry significant side effects, require close therapeutic monitoring and are expensive for the NHS. The continued drive to develop new immunosuppressive drugs has been more lacklustre in recent years with very few new drugs coming on to the market for some time now. There is undoubtedly scope for further improvements of the current immunosuppressant regimens, perhaps drugs that do not require to be

monitored, a single monotherapy rather than a combination of drugs, fewer side effects and so on but it is less certain if, and when, these advances are likely to happen or whether an entirely new approach to preventing rejection, such as the induction of immune tolerance, will replace standard immunosuppression.

A summary diagram of the different targets of the most common modern immunosuppressants is shown below.

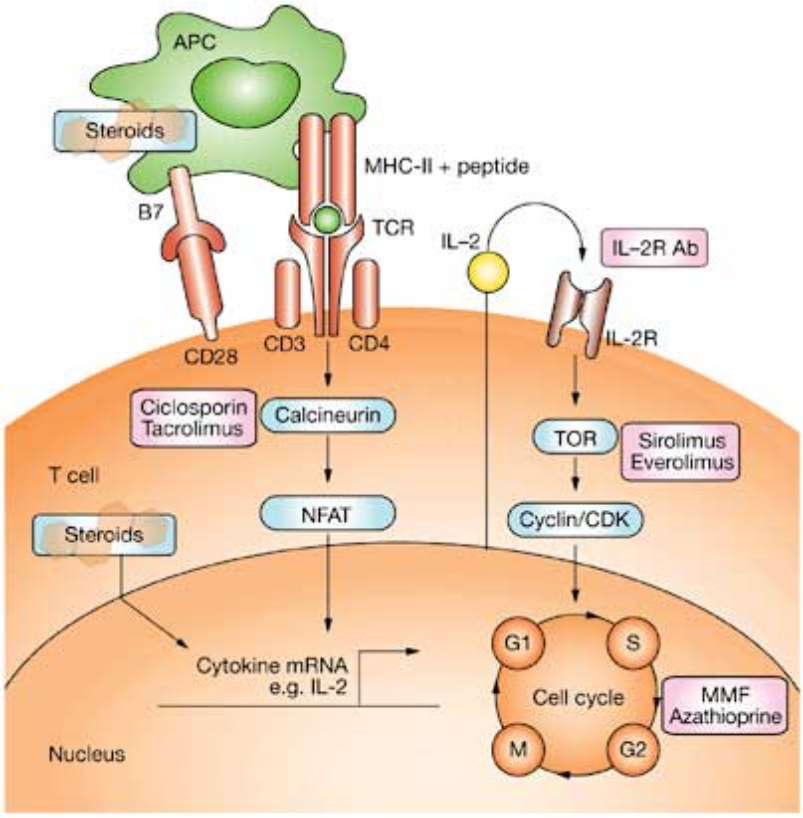

Figure 1.4 Cellular targets of modern immunosuppressant drugs demonstrating interaction between the T cell receptor and the intracellular cascade that follows for steroids, calcineurin inhibitors, mTOR inhibitors and anti-prolific agents.

Kobashigawa et al, *Nat Clin Pract Cardiovasc Med, 2006* [87]

1.7 Unit Protocols for Immunosuppression in Liver, Kidney and SPK Transplants

The transplant unit in Edinburgh has standard protocols for induction and immunosuppression for liver, kidney and SPK transplantation.

1.7.1 Liver Transplant Immunosuppression

Liver transplant patients do not routinely receive induction therapy with basiliximab in the unit. Standard immunosuppression is with tacrolimus in the immediate release formulation Prograf® given at a dose of 0.05 mg/kg twice daily. Azathioprine is given at a dose of 1 mg/kg daily. Hydrocortisone 100 mg twice daily is given intravenously on day 1 until the patient is able to take prednisolone orally which is then given as 20 mg daily on a reducing dose at 5 mg intervals a month at a time. Prednisolone is eventually stopped, with the exception of some conditions such as autoimmune hepatitis. Patients with renal impairment are prescribed mycophenolate mofetil 1g twice-daily instead of azathioprine.

1.7.2 Kidney Transplant Immunosuppression

The standard immunosuppression protocol consists of induction with basiliximab and then 'triple therapy' consisting of tacrolimus, mycophenolate mofetil and prednisolone for maintenance. Basiliximab is given intravenously in theatre (20 mg) and then again on day 4 post transplant. Tacrolimus is given at 0.05 mg/kg in the form of Prograf® twice-daily. Mycophenolate mofetil is given as 1g twice-daily if tolerated and prednisolone is given as 20 mg on a reducing dose, every 4 weeks until a maintenance dose of 5 mg once daily is achieved. Methylprednisolone 500 mg is given in theatre and also at 24 hours post transplant. The target tacrolimus trough concentration range for standard risk transplants is between 5-7 μg/L.

Some kidney transplant patients are considered intermediate risk and these include SPK recipients, previous transplants, sensitised patients (with a calculated reaction frequency of >20%, flow crossmatch positive, HLA-DR mis-match or a non-favourable match. The immunosuppression protocol is essentially the same for these patients however the trough concentration is kept at between 10-14 μg/L for the first 3 months following transplantation

(and are therefore likely to require higher doses of tacrolimus in the region of 0.1 mg/kg twice daily) and then 5 – 10 μg/L after 3 months.

Rabbit antithymocyte globulin (rATG) induction therapy is infrequently used in this unit however it may deemed appropriate in some high immunological risk patients. It is used at clinician discretion on a case by case basis alongside tacrolimus, MMF and prednisolone.

1.7.3 SPK Immunosuppression

The protocol for SPK transplant patients is the same as for intermediate risk kidney transplant patients as described above. Induction is with basiliximab 20 mg on day 0 and day 4 and the tacrolimus trough concentrations are kept higher for the first 3 months.

1.7.4 Adjusting Tacrolimus Dose in Response to Out of Range Tac Level

When a tacrolimus trough level is below the expected value then the dose of tacrolimus will be increased by the transplant physician, normally by 0.5 mg per dose. If the trough concentration is significantly lower than the

therapeutic range then the dose may be increased by 1 mg per dose but this is at the discretion of the transplant team. For tacrolimus trough concentrations that are higher than the therapeutic range a reduction in the dose of tacrolimus by 0.5 mg will usually be made.

For a trough concentration that is very high, it would be confirmed firstly that the trough concentration was a true C_0 value by checking the time that the tacrolimus was taken and the time that the blood sample was taken. If this confirmed an excessively high trough concentration then often the next dose will be omitted, followed by a reduction in the routine dose deemed appropriate by the clinical team.

1.8 Therapeutic Drug Monitoring and Tacrolimus

The narrow therapeutic range and the reported patient variability pose challenges to transplant clinicians as tacrolimus trough levels require close monitoring and adjustments to ensure effective immunosuppression whilst minimising the risk of significant systemic side effects and toxicity. These include nephrotoxicity leading to renal impairment, neurological toxicity leading to seizures, impairment of glucose tolerance resulting in new onset diabetes after transplantation (NODAT), myositis, gastrointestinal disturbances such as diarrhoea, hypertension, increased risk of malignancy

such as skin cancers as well as increased risk of cardiovascular disease [88, 89]. The requirement to keep tacrolimus within a narrow therapeutic window has resulted in therapeutic drug monitoring being utilised to monitor tacrolimus levels and make dose adjustments based on tacrolimus trough (C_0) concentrations from whole blood.

A variety of methods can (and have) been used to monitor the trough levels of different immunosuppressant drugs including immunoassays and chromatography based methods [90]. While immunoassays are widely used in therapeutic drug monitoring generally, they fall short in monitoring immunosuppressant medication due to the lack of specificity for the parent drug such as tacrolimus or ciclosporin, as they tend to overestimate the drug levels [91]. The alternative to the immunoassay technique are chromatographic techniques. These are much more selective and have higher sensitivity and specificity than immunoassays. Gas chromatography has been used but it requires the drug to be volatile in order to be measured requiring additional steps in the process making it less efficient and potentially more costly. Liquid chromatography with ultraviolet detection is another method which has been used for therapeutic drug monitoring (TDM), but there are limitations of specificity with certain compounds requiring extensive sample preparation. A newer technique is now available which couples liquid chromatography to a mass spectrometer, so called

liquid chromatography tandem mass spectrometry (LC-MS/MS) [91]. The LC-MS/MS method is favoured due to its high sensitivity, specificity, short analysis time and therefore increased throughput and lower cost. Additionally, it can be used to monitor levels of tacrolimus, ciclosporin, sirolimus and everolimus simultaneously making it efficient and cost effective. The LC-MS/MS method is used in the hospital laboratory at this centre for measuring the tacrolimus trough levels in transplant patients.

1.9 Inter patient variability in tacrolimus trough levels

Therapeutic drug monitoring is required for patients taking tacrolimus not only because it is effective within a narrow therapeutic range, but also because the dose of the drug required in order to achieve that therapeutic level, without causing toxicity, varies considerably from person to person and can be influenced by a number of different factors which include, but are not limited to, age, gender, weight, ethnic group, post-operative day (in the immediate transplant period before steady state is reached), number of days following starting tacrolimus therapy, liver metabolic function, haematocrit, albumin, creatinine clearance, corticosteroid dose, concomitant drug therapies which are inducers of cytochrome P450, time taken in relation to food and patient adherence with medication [43, 92].

Additionally, underlying disease process requiring a transplant (e.g. diabetes with associated GI tract complications) and the type of organ transplanted (liver, kidney, pancreas, small bowel etc.) can influence the pharmacokinetics of tacrolimus [93, 94].

Most patients will, therefore, require several dose adjustments in the early post-transplant period until they reach a 'steady state' (a consistent blood concentration of the drug). This also means that the early post-transplant period is the time with the highest risk of acute rejection which can have a negative impact on long term graft survival [95].

Despite the numerous factors that influence tacrolimus trough levels, current practice is to prescribe the initial dose of tacrolimus based on the weight of the patient and the desired initial tacrolimus level for each type of transplant. While this method remains adequate for most patients, publications in the late 1990s have shown that different ethnic groups, such as black ethnic origin, required significantly higher doses of tacrolimus in order to achieve comparable therapeutic levels [96-98]. Given the presumed genetic basis for these observed differences among different ethnic groups, researchers began to consider the possible genes which could influence drug pharmacokinetics as an explanation for these variations. It was known that tacrolimus was transported out of the enterocyte by the drug transporter P-glycoprotein, thus reducing the amount available for systemic absorption,

and also that it was almost completely metabolised in the liver by cytochrome P450 [45, 99].

In 2002 *Macphee et al* investigated the expression of different single nucleotide polymorphisms (SNPs) of the multi-drug resistance gene (MDR-1) which encodes for P-glycoprotein and the CYP3AP1 pseudogene which was closely related to CYP3A5 activity in renal transplant recipients of different ethnicity [100]. The authors showed that patients who possess a G allele at the 44 locus of CYP3AP1 had a significant reduction in tacrolimus concentrations compared with those of the AA genotype. The effect was noted in all patients, although G allele expression was more common in black transplant recipients. This suggested that SNPs of genes relating to hepatic cytochrome P450 (3A5) activity rather than ethnicity itself are responsible for the differences seen between individuals in the tacrolimus exposure following standard dose administration. *Macphee et al* also examined a SNP of the MDR-1 gene, namely the C3435T transition, where expression of a T allele (much more common in Caucasians) is associated with decreased P-glycoprotein expression which should therefore increase systemic absorption from the gastrointestinal tract. A modest reduction in tacrolimus concentrations was noted in transplant recipients who expressed the CC genotype (no T allele expression and therefore no reduction in P-glycoprotein activity). This was a novel finding as the consensus at the time

was that gut absorption of tacrolimus, influenced by P-glycoprotein expression, was the major contributor of systemic bioavailability [101]. *Macphee et al* demonstrated that the influence of P-glycoprotein on tacrolimus bioavailability was lower than previously predicted and that cytochrome P450 3A5 activity had the greater influence.

1.10 Genetic Polymorphisms and Tacrolimus Pharmacokinetics

The impact of the polymorphisms of genes related to drug transport and metabolism on tacrolimus exposure in transplant patients led to further research in this field [102-104]. Two main SNPs are thought to play a major role – the CYP3A5 6986A>G transition and the ABCB1 3435 C>T transition (ATP-binding cassette sub-family B member 1) which encodes P-glycoprotein. As the evidence base for CYP3A5, in particular, started to increase, researchers started to look other potential SNPs which might influence tacrolimus pharmacokinetics. In 2011 *Elens et al* described a new functional CYP3A4 polymorphism on intron 6, named CYP3A4*22, which influenced the pharmacokinetics of tacrolimus in renal transplant

patients[105]. This is a topical subject and there is an ongoing search for further SNPs that influence tacrolimus pharmacokinetics.

1.10.1 ABCB1 3435 C>T

ABCB1 is a gene which encodes for the ATP-dependent drug efflux transporter molecule P-glycoprotein. It is located on chromosome 7q21.12 and consists of 29 exons which span a genomic region of 209.6 kilobases (kb). The messenger RNA (mRNA) consists of 4872 base pairs which, in turn, results in a protein of 1280 amino acids named P-glycoprotein [106].

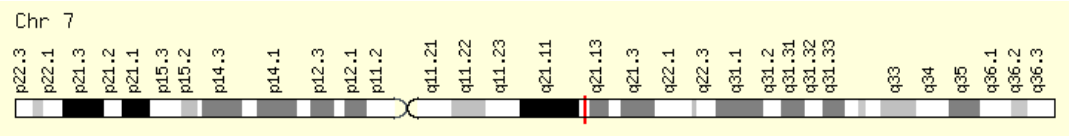

Figure 1.5 ABCB1 position on Chromosome 7
(www.genecards.org - GC07M087504)

P-glycoprotein (P-gp) is a transmembrane protein which functions as an ATP-dependent efflux pump for xenobiotic compounds with broad substrate specificity. It can be found expressed in many body tissues (adrenal gland, gut, bile ducts, lung, brain, ovaries and numerous different cells of the immune system) [102, 107]. The P-gp protein actively pumps certain molecules (of which tacrolimus is one), out of the cell and therefore normal

physiologic expression of P-gp can result in reduced drug uptake in certain key tissues where it is widely expressed. Therefore P-gp plays a vital role in the bioavailability of various drugs including chemotherapy agents, antibiotics, immunosuppressants and anti-retroviral protease inhibitors[108]. Pre-clinical studies with P-gp knockout mice have shown significantly higher concentrations of both ciclosporin and tacrolimus in the brain [109, 110].

Of the numerous SNPs of ABCB1 three have been widely studied – C1236T (rs1128503), C3435T (rs1045642) and G2677T/A (rs2032582).

The C3435T mutation is found on exon 26 at the 3435 position and results in a 'wobble' rather than an amino acid change whereby a small mutation occurs in the codon resulting in a different base pair however the amino acid produced is the same. The wild type sequence is AGAT**C**GTGA and the mutation is AGAT**T**GTGA so the 'wobble' is at the 3435 position C>T. It has been suggested that this mutation may exert an effect by linkage disequilibrium [111], reducing the stability of mRNA leading to a reduction in ABCB1 mRNA [112] or by affecting the timing of folding and insertion of P-gp into the cell membrane which, in turn, reduces its substrate specificity [113]. The C3435T mutation has been shown to decrease the amount of P-gp expressed in the duodenum of healthy volunteers with those of the CC

genotype expressing the most P-gp and those of the TT genotype expressing the least [114].

C3435T and G2677T/A SNPs have been reported to play a role in tacrolimus pharmacokinetics [100, 115, 116].

To date there have been numerous studies examining the relationship between the C3435T SNP and the pharmacokinetics of calcineurin inhibitors. Most studies have looked at adult renal transplant patients although there have been reports into liver, heart, lung and paediatric renal transplantation too. A small number of studies have shown that a TT allele expression of the C3435T SNP was associated with a higher dose corrected tacrolimus level [tacrolimus trough (C_0)/dose value], which meant that there was a lower dose requirement to reach therapeutic target levels [100, 117-120]. *Hesselink et al* reported such findings in a study of 136 renal transplant patients and found that patients with the TT genotype had a 23% higher dose corrected tacrolimus level and a 19% lower tacrolimus dose requirement when compared with the CC genotype patients [119].

There are, however, more than 20 studies which have not demonstrated an effect of the C3435T mutation of ABCB1 on tacrolimus pharmacokinetics which suggest, perhaps, that when looked at in isolation, the C3435T mutation of ABCB1 does not exhibit as significant an effect as first thought

[103, 104, 116, 121-137]. There are some studies that suggest the C3435T mutation of ABCB1 may have more of an effect when combined or linked with other SNPs rather than in isolation. In a study of 44 renal transplant patients, there was a cumulative effect of three SNPs of ABCB1 (C3435T, C1236T and T-129C) suggesting that patients who express more variant alleles of ABCB1 SNPs have reduced P-glycoprotein functionality leading to greater systemic drug absorption as less of the substrates are actively pumped out of the enterocyte by P-gp [111][125]. In this study patients who had less than 3 of the variant ABCB1 alleles (3435T, 2677T, -129C) had a significantly reduced dose corrected tacrolimus level when compared with patients who expressed the variant allele of all 3 SNPs.

1.10.2 CYP3A5 6986 A>G

The cytochromes P450 are a superfamily of enzymes which play a significant role in the metabolism of drugs, steroid synthesis and the synthesis of cholesterol and other lipids. Their name is derived from the spectral absorbance peak when bound with carbon monoxide at 450 nm. These enzymes use haem iron (hence why they are often known as haemeoxygenases) in order to oxidise compounds and make them water soluble and easier to clear.

The cytochrome P450 enzymes are monooxygenases due to the fact that they catalyse only one atom of molecular oxygen into the substrate and reduce the other one to water. The cytochromes P450 proteins form part of an electron transport system where they will accept 2 electrons from nicotinamide adenine dinucleotide phosphate (NADPH) sequentially to cytochrome P450, catalysed by cytochrome P450 reductase. This process is characterised as follows, where RH is the substrate:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+ \qquad [138]$$

The human genome project has identified 57 different genes and 58 pseudogenes which encode for the cytochrome P450 proteins. These have been assigned agreed nomenclature by the human cytochrome P450 nomenclature committee such that each enzyme name starts with CYP, followed by a numerical family number, a letter designating the sub-family and then a number designating the polypeptide if appropriate. The different CYPs are shown in the table below [139].

Table 1.1 Different classifications of the currently identified cytochrome P450 subgroups.

sterols	**xenobiotics**	**fatty acids**	**eicosanoids**	**vitamins**	**unknown**
1B1	1A1	2J2	4F2	2R1	2A7
7A1	1A2	4A11	4F3	24A1	2S1
7B1	2A6	4B1	4F8	26A1	2U1
8B1	2A13	4F12	5A1	26B1	2W1
11A1	2B6		8A1	26C1	3A43
11B1	2C8			27B1	4A22
11B2	2C9				4F11
17A1	2C18				4F22
19A1	2C19				4V2
21A2	2D6				4X1
27A1	2E1				4Z1
39A1	2F1				20A1
46A1	3A4				27C1
51A1	3A5				
	3A7				

Cytochromes P450 are the key component of phase I drug metabolism in humans, found primarily in hepatocellular microsomes, but can also be found in many other tissues such as the prostate, kidney and gut [140].

Cytochromes P450 metabolise thousands of endogenous and exogenous substances with considerable variation in the number of substrates any one cytochrome P450 protein will act upon. Overall, cytochrome P450 enzymes are involved in the metabolism of approximately 75% of the drugs commonly used today [141].

Tacrolimus undergoes extensive oxidative metabolism by liver microsomes when it is given orally or intravenously with only a small amount of drug excreted unchanged in the urine [142]. The cytochrome P450 enzymes involved in the metabolism of tacrolimus belong to the CYP3A subfamily, primarily CYP3A4 and CYP3A5.

The gene which encodes for CYP3A5 is located on chromosome 7 at position 7q22.1 and consists of 13 exons and spans 31.8 kilobases. It is closely related to a cluster of cytochrome P450 genes and pseudogenes on 7q22.1, one of which is the pseudogene CYP3A5P1 which is very similar to CYP3A5. The CYP3A5 protein consists of 502 amino acids with a molecular weight of 52.5kD.

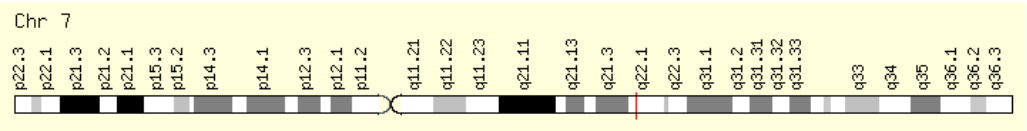

Figure 1.6 CYP3A5 position on Chromosome 7

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CYP3A4 and CYP3A5 are the two major isoforms of the CYP3A family which are expressed in humans. While CYP3A4 is found in all humans and expressed predominantly in the liver and the small intestine, CYP3A5 is only expressed in those individuals who carry at least one copy of the A allele

(which is known as CYP3A5*1). Although the CYP3A5*1 (A allele) is the wild type it is, somewhat unusually, expressed less frequently than variant allele CYP3A5*3 or the G allele. Those individuals who are homozygotes for the G allele (and are therefore designated CYP3A5*3/*3), will only make a negligible amount of CYP3A5 which will not play a role in tacrolimus metabolism, while conversely those who carry the CYP3A5*1 allele will produce functional CYP3A5 which will extensively metabolise tacrolimus in vitro [143-145].

CYP3A4 differs from CYP3A5 in that although there are numerous genetic polymorphisms of CYP3A4, there is no SNP which results in an absence of CYP3A4 functionality. In contrast, only those individuals who polymorphically express a CYP3A5*1 allele (either as a *3/*1 heterozygote or a *1/*1 homozygote) will make any functional CYP3A5. This SNP is a result of an A>G transition at the 6986 position of intron 3 of the CYP3A5 gene. Therefore, those who carry a CYP3A5*1(A) allele are termed ‘expressers’ of CYP3A5 and those who do not are termed ‘non-expressers’ [143, 146].

Tacrolimus is transformed into eight different metabolites [M-I to M-VIII] and consist of three mono-demethylated metabolites, three di-methylated metabolites, one mono-hydroxylated metabolite and a final metabolite which undergoes multiple different reactions [147, 148]. Of these 8 metabolites, there are 4 primary metabolites which clear the majority of

tacrolimus [13-O-desmethyl tacrolimus (13-DMT or M-I), 15-O-desmethyl tacrolimus (15-DMT or M-III), 31-O-desmethyl tacrolimus (31-DMT or M-II) and 12-hydroxy tacrolimus (12-HT or M-VI)] [145].

In-vitro studies of both human and animal models have shown the formation of the M-I metabolite (13-O demethylation) was the most significant metabolic pathway in the first stage of tacrolimus metabolism when cultured with liver microsomes fortified with NADPH under aerobic conditions [149]. These in vitro studies suggest that tacrolimus goes through sequential metabolism from M-I to M-VII before proceeding to more polar metabolites, catalysed by CYP3A4/5.

In vitro porcine models have shown that CYP3A5 produces both desmethyl and hydroxyl metabolites of tacrolimus whereas CYP3A4 only results in desmethyl metabolites suggesting that CYP3A5 plays the more significant role in the metabolism of tacrolimus [150].

There is considerable variation in the expression of CYP3A5 across different ethnic groups. Approximately 15-20% of Caucasians express CYP3A5 either as a *3/*1 heterozygote (13-15%) or as a *1/*1 homozygote (3-5%). Conversely approximately 85% of individuals of African (Black) ethnicity express CYP3A5 as *1/*3 (60%) or *1/*1(25%) [151]. In the South Asian ethnic group, approximately 38% of patients express the *1/*3 genotype and

13% express the *1/*1 genotype [152]. Variation has also been reported in Chinese patients, however the approximate distribution of genotypes in these patients show around 11-15% of the *1/*1 genotype, 35-40% of the *1/*3 genotype and 55-60% of the *3/*3 genotype [120, 133].

Furthermore, differences in the genotype distribution and allele frequencies of CYP3A5 were noted even across different Caucasian populations, different Asian groups and even across different regions of the one country such as China [153]. To date no study explored the allele frequencies or genotype distribution of the different SNPs in a Scottish population.

In stark contrast to the equivocal nature of the impact of ABCB1 polymorphisms on tacrolimus pharmacokinetics, the polymorphisms associated with CYP3A5, namely the A6986G transition, have been consistently shown to impact on tacrolimus metabolism and clearance across different organ transplants including kidney, liver and heart as well as in healthy volunteers [111]. In renal transplantation nearly all studies have shown an increased dose requirement and a significantly reduced dose corrected tacrolimus level (suggesting lower tacrolimus exposure) in those individuals who are carriers of at least one CYP3A5*1 allele (CYP3A5 expressers) compared with individuals who are CYP3A5*3 homozygotes (CYP3A5 non-expressers) [154]. Most studies have shown that patients who are CYP3A5 expressers require nearly twice as much tacrolimus in order to

achieve the same therapeutic trough levels (C_0) as CYP3A5 non-expressers [104, 119, 122, 124, 131, 137, 152, 155-157]. A study by *Op den Buijsh et al* examined 63 Caucasian renal transplant patients and found that the dose corrected level (C_0/D) was 2-fold lower in CYP3A5 expressers compared with non-expressers [123]. Similarly, a study by *Hesselink et al* in 136 renal transplant recipients reported that CYP3A5 expressers had a 68% higher dose requirement by day 3 after transplantation compared with non-expressers of CYP3A5 [119].

Individuals who are CYP3A5*1 allele carriers may have different pharmacokinetics of tacrolimus and given the narrow therapeutic index of the drug, this could have a significant clinical impact with an increased risk of rejection if there are delays in reaching therapeutic level post-transplant and low systemic exposure to tacrolimus [158]. There is evidence that CYP3A5 expressers take a longer time to reach therapeutic tacrolimus levels, even as part of a therapeutic drug monitoring (TDM) strategy, with some patients still not achieving therapeutic C_0 levels as far as 2 weeks post transplant [103][125]. Some studies described increased acute rejection rates within the first 3 months following transplantation in CYP3A5 expressers [159, 160] although this was not seen in other reports [119]. However, a recent meta-analysis by *Tang et al* concluded that CYP3A5 expressers run a higher risk of acute rejection within the first month post

transplantation because of the longer time to achieve therapeutic tacrolimus levels and the reduced initial tacrolimus exposure [161].

The correlation between CYP3A5 expression and other clinical outcomes, such as renal function and the nephrotoxic effects of tacrolimus have been investigated [162]. As for acute rejection there are some conflicting data. Most studies did not find any association between CYP3A5 A6986G genotype and renal function [119] [163] [164] [125] [127]) although a study by *Fukudo et al* found that recipient non-expression of CYP3A5 (*3/*3 homozygotes) had greater nephrotoxicity compared with expressers [165]. The authors suggested that CYP3A5 expression in the kidney may have had a protective effect but further work in this area is needed before any definitive conclusions can be drawn.

There appears to be an association between CYP3A4/CYP3A5 and salt dependant hypertension [166] and CYP3A5*1 allele carriers renal transplant recipients demonstrated a sustained elevation of their mean arterial blood pressure when compared with CYP3A5*3 homozygotes [163]. There have been no studies to date examining a correlation with the development of diabetes after transplantation (NODAT) whilst graft survival and patient survival appear to be unaffected by CYP3A5 expression [135, 162].

While the clinical impact of the CYP3A5 SNP is highly variable and considerably less well established, the impact on tacrolimus pharmacokinetics is well established in in all organ transplants and appears to be independent of the patient's ethnic group (although expression of CYP3A5 is very closely related to ethnicity), gender and age [154].

*1.10.3 CYP3A4*22 15389 C>T*

CYP3A4 is expressed in all individuals and is found primarily in the liver and small intestine with very little expression in the renal tissue [167]. Similar to CYP3A5, CYP3A4 is polymorphically expressed, although a polymorphism that results in total loss of CYP3A4 activity has not yet been identified. CYP3A4 metabolises tacrolimus in a NADPH dependent fashion similar to CYP3A5, although when CYP3A5 is expressed it appears to have a more dominant role than CYP3A4 does.

CYP3A4 is found in the same cluster of genes on chromosome 7 (7q22.1) as CYP3A5 and is 23.7kb in size. It encodes a protein consisting of 503 amino acids and has a molecular weight of 57.3kDa.

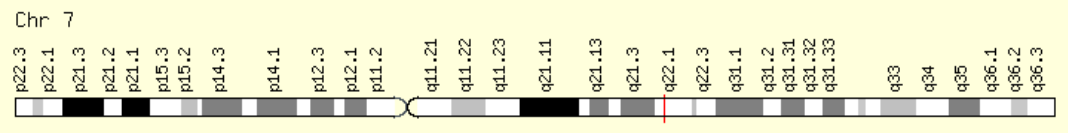

Figure 1.7 CYP3A4*22 position on Chromosome 7

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Until recently, the most studied SNP of CYP3A4 was the A>G transition at position -392 in the nifedipine specific element (NFSE) of the 5'-flanking promotor region of the CYP3A4 gene [111]. There are some *in vitro* studies which suggest that the CYP3A4 A-392G polymorphism is associated with increased CYP3A4 activity [168] although this is not widely supported [169, 170]. The evidence suggesting that CYP3A4 A-392G SNP influences tacrolimus pharmacokinetics is scarce, but the data are so limited that is difficult to determine whether this is a true effect or whether it is artefactual and is secondary to expression in different ethnicities and/or linkage disequilibrium with CYP3A5 A6986G [104, 119, 123].

Interest in CYP3A4 activity was re-ignited when *Elens et al* reported the discovery of a functional new CYP3A4 polymorphism which had been termed CYP3A4*22. This SNP was found on intron 6 of the CYP3A4 gene and consisted of a C>T transition at the 15389 position (rs35599367) and was associated with reduced CYP3A4 activity resulting in increased systemic exposure to tacrolimus in renal transplant patients [171]. This study reported that the variant (T) allele frequency was low (3.5%) but it was also

not in linkage disequilibrium with CYP3A5*3. The expression of the variant CYP3A4*22 T allele corresponds with significantly increased tacrolimus C_0 levels when corrected for dose and a reduced dose requirement to achieve therapeutic levels compared with the wild type CYP3A4*1/*1 genotype (CC) [105]. T allele carriers had a significantly higher C_0 level (20.5 µg/L) compared with the CC non expresser genotype (14.9 µg/L), (p=0.05) despite similar doses of tacrolimus (both groups in the region of 13 mg/day) [105].

In vitro studies using hepatic tissue have also demonstrated that expression of the CYP3A4*22 variant is associated with reduced CYP3A4 activity in response to statin drugs, as well as a reduction in CYP3A4 mRNA expression [172, 173].

Elens et al used the breakdown of midazolam into its hydroxylated metabolite 1-OH-MDZ as a drug probe to assess the *in vivo* activity of CYP3A4*22 and concluded that those patients who carried the variant T allele of CYP3A4*22 had 20% lower CYP3A4 activity compared with those who did not [174]. Using a similar method, *Jonge et al* suggested that the renal transplant patients who were non-expressers of CYP3A5 (*3/*3) but who expressed the CYP3A4*22 variant genotype had significantly higher exposure to tacrolimus at 12 months when steady state had been achieved. They reported that the mean total oral clearance of tacrolimus was 36.2% lower in those patients who carried the variant CYP3A4*22 (T) allele and this

translated into a 50% reduction in tacrolimus dose requirement in order to achieve a therapeutic C_0 level [175]. Furthermore, these patients had greater tacrolimus exposure, reduced dose requirements and achieved supra-therapeutic tacrolimus levels with potential adverse effects related to tacrolimus toxicity [176].

While *Elens et al* have consistently shown a significant influence of the CYP3A4*22 genotype on tacrolimus pharmacokinetics, *Moes et al* and *Santoro et al* showed only a trend towards reduced tacrolimus exposure in their respective cohorts (the latter a Brazilian population where CYP3A5 expression is more prevalent) [177, 178]. These inconsistencies may be explained by the very low allele frequency of CYP3A4*22 with only 8% of the Caucasian population expressing a T allele variant of CYP3A4*22. Similarly, the effect of CYP3A4*22 expression appears to be more pronounced in CYP3A5*3/*3 non-expressers so in populations where CYP3A5*1 expression is greater, the impact of CYP3A4*22 expression may be reduced.

The effect of CYP3A4*22 requires further investigation but there is increasing evidence suggesting that it may play a significant role in tacrolimus pharmacokinetics in the small number of individual who express the variant allele. However, the clinical impact of the expression of CYP3A4*22 is still unclear. *Elens et al* have shown that renal transplant patients who expressed the CYP3A4*22 variant allele and were taking

ciclosporin immunosuppression had significantly increased rates of delayed graft function (DGF) and a 20% lower creatinine clearance compared with the CYP3A4*1/*1 wild type [179]. Somewhat surprisingly, a recent longitudinal study suggested that there may be a protective effect of tacrolimus over-exposure in the CYP3A4*22 variant group with creatinine clearance on average 21% higher compared with the wild type CYP3A4*1/*1 genotype [180].

Given that evaluation of this SNP is still in early stages, further studies are required before the clinical impact of the CYP3A4*22 polymorphism in relation to tacrolimus exposure is fully understood.

1.11 Pharmacogenomics and organ transplantation

Transplantation poses an interesting challenge, as the pharmacokinetics of tacrolimus will be influenced by each patient's specific genotype as well as the genotype of the transplanted organ. Multiple factors can play an important role including the genotype of the recipient and the donor, the disease process requiring transplantation in the first place and the type of tacrolimus preparation.

In renal transplantation, the recipient's own genotype is likely to have the predominant influence on tacrolimus pharmacokinetics. While there is evidence of some CYP3A5 expression in the kidney, the hepatic and gut expression are far more influential. Therefore, the recipient's native cytochrome P450 SNPs of CYP3A4(*22) and CYP3A5, as well as ABCB1 polymorphisms and P-gp expression account for most differences. Studies examining the impact of the donor genotype in renal transplantation are scarce however *Glowacki et al* showed that donor genotype of both CYP3A5 and ABCB1 had no impact on tacrolimus pharmacokinetics [181].

In liver transplantation, things are somewhat different. The majority of cytochrome P450 activity takes place in liver tissue and therefore the donor genotype, particularly that of CYP3A5, could exert a much more significant role, possibly even more than the recipient's own CYP3A5 genotype. There is some evidence to support this hypothesis. *Wei-lin et al* found that the donor's CYP3A5 genotype in association with the recipient's ABCB1 genotype significantly affected tacrolimus pharmacokinetics in Chinese liver transplant patients [120]. *Yu et al* also found that donor liver expression of CYP3A5*1 allele was associated with a reduced dose corrected tacrolimus level (C_0/D) at 1 month but critically, the donor genotype had a greater influence than the recipient genotype [182]. This was also seen in a Caucasian population in an Italian study that suggested that the donated

liver genotype played a more significant compared to the recipient genotype. Recipients who received a liver graft from a CYP3A5 expresser had significantly higher dose requirements, whilst the recipients who carried at least one CYP3A5*1 allele only tended towards higher dose requirements (without reaching statistical significance) [183].

A recent meta-analysis of 8 studies which included 694 adult liver transplant recipients found that donor livers that carried a CYP3A5*1 allele had significantly reduced C_0/D ratios compared with those livers that did not express CYP3A5 (*3/*3 homozygotes) [184]. To date, there have been no studies in UK liver transplant patients examining the role of CYP3A5 or ABCB1 polymorphisms in the donor or recipient and the influence on tacrolimus pharmacokinetics. Furthermore, there have no studies examining the impact of donor or recipient CYP3A4*22.

There is a lack of data on ABCB1 polymorphisms or CYP3A4/3A5 polymorphisms in simultaneous pancreas-kidney (SPK) transplant recipients. It is conceivable that these patients have an identical pharmacokinetic profile to renal transplant patients based on the genotype expression of CYP3A4/CYP3A5/ABCB1 polymorphisms. However due to the higher incidence of gastroparesis and gut dysmotility secondary to diabetic autonomic neuropathy, the Absorption, Distribution, Metabolism and Excretion (ADME) parameters may be different. In particular the ABCB1

C3435T polymorphism which encodes P-gp may be more relevant here as the transit of tacrolimus and the rate of gut absorption may be altered in SPK patients who have gastroparesis or gut dysmotility. As the expression of P-gp differs between the proximal and distal gut [56] the expression of a T allele in of the C3435T polymorphism of ABCB1 may have a more pronounced effect.

1.12 Summary

The last 20 years of tacrolimus use in organ transplantation have clearly demonstrated multiple factors which can influence its pharmacokinetics and the narrow therapeutic window in which tacrolimus is effective means these factors require thorough investigation. By understanding the influence of genetic polymorphisms on tacrolimus pharmacokinetics across different organ transplant groups, we can begin to move towards individualised dosing plans for every patient, minimising the risk of sub-therapeutic or supra-therapeutic tacrolimus exposure and the sequelae that come with that. We hope this study will re-enforce work already done in renal transplant patients, and add to the currently small evidence base in liver transplant patients, particularly in relation to donor genotypes. There is currently no published data on the influence of genetic polymorphisms in

SPK transplantation and we hope to provide some of the earliest data in this transplant group. As use of modified release tacrolimus (Tac-QD) becomes more widespread it will be important to understand how genetic polymorphisms might affect tacrolimus exposure and we hope to add valuable data to this field.

1.13 Aims of the thesis

This study aims to describe the distribution of genetic polymorphisms of ABCB1, CYP3A5 and CYP3A4*22 in a representative sample of the Scottish population and investigate to what extent these polymorphisms affect the pharmacokinetics of tacrolimus in patients who have undergone, liver, kidney or combined pancreas-kidney transplantation. We aim to achieve this by:

1. Performing a genotype analysis of CYP3A5 A6989G, ABCB1 C3435T and CYP3A4 C15389T in a large sample of Scottish population who have donated their DNA to Generation Scotland bioresource. We will compare this genotype analysis to previously published data in different countries.

2. Performing a donor and recipient genotype analysis of these three SNPs in a cohort of liver transplant patients and investigate the impact of the expression of these polymorphisms in donor and recipient on tacrolimus dose requirements and C_0 levels and relate these to clinical outcomes.
3. Performing a genotype analysis of the SNPs in a cohort of renal transplant patients and correlate the expression of these polymorphisms with the tacrolimus dose requirements and C_0 levels and the clinical outcomes.
4. Performing a genotype analysis in a cohort of simultaneous pancreas-kidney (SPK) transplant patients and investigate the same pharmacokinetic and clinical outcomes.
5. Investigating a cohort of stable renal transplant patients who have been converted from twice-daily tacrolimus (Tac-BD) to once-daily modified release tacrolimus (Tac-QD) in relation to dose requirements and tacrolimus C_0 levels following conversion. We will investigate a smaller sub-cohort of these patients where stored DNA is available for genotype analysis and determine what impact the three SNPs of interest on Tac-QD pharmacokinetics. Finally, we will analyse the intra-patient variability and determine if there is any difference in intra-patient variability between Tac-BD and Tac-QD.

We will explore whether increased intra-patient variability has any impact on graft survival in this cohort of patients.

1.13.1 Hypotheses

1. The genotype analysis of the Scottish population in this study will reveal that the genotypes and allele frequencies of CYP3A5, ABCB1 3435 C>T and CYP3A4*22 are similar to other Caucasian populations that have previously been studied.
2. In liver transplant patients – for CYP3A5 and CYP3A4*22 which are cytochrome P450 enzymes found primarily in liver tissue – the donor genotype will have a greater influence than the recipient on tacrolimus pharmacodynamics.
3. Patients who express the CYP3A5 enzyme (genotype GA or AA) or for liver transplant patients where the donor expresses CYP3A5 will require a significantly higher dose of tacrolimus to achieve a therapeutic concentration and are likely to take longer to reach a therapeutic concentration compared with non-expressers of CYP3A5.
4. ABCB1 genotype is less likely to significantly change tacrolimus pharmacodynamics in any of the patient groups.

5. Patients who express the CYP3A4*22 T allele (genotype CT or TT) or for liver transplant patients where the donor expresses a T allele may require a reduced dose of tacrolimus in order to achieve a therapeutic trough concentration.
6. Patients who express CYP3A5 or liver transplant patients where the donor expresses CYP3A5 may have increased rejection due to a likely delay in achieving a therapeutic trough concentration.
7. Graft and patient survival is unlikely to be significantly influenced by the different genotypes of CYP3A5, ABCB1 and CYP3A4*22
8. Renal transplant patients who are converted to once-daily tacrolimus are likely to require an increased dose if they are CYP3A5 expressers and may require a reduced tacrolimus dose if they express a T allele of CYP3A4*22.
9. Conversion from twice-daily tacrolimus to once-daily tacrolimus in renal transplant patients is likely to reduce intra-patient variability. Patients with high intra-patient variability are likely to have greater graft loss compared with those of low intra-patient variability.

Chapter II

Materials and Methodology

2 MATERIALS AND METHODS

There were 3 distinct cohorts of patients that were included in this thesis. The first is a large sample of the healthy Scottish population who were genotyped for the 3 SNPs of interest in order to describe the distribution in a Scottish population. The second group were patients who had undergone liver, renal or combined kidney/pancreas transplant in the Edinburgh transplant unit and the third were a group of renal transplant patients who had been converted from the standard twice-daily Tac-BD formulation of tacrolimus to the modified release, once-daily Tac-QD. The clinical and laboratory methodology for each of these groups is described below.

2.1 Generation Scotland Methodology

DNA samples from a total of 5889 subjects were genotyped for CYP3A5 6986 A>G transition, ABCB1 3435 C>T transition and the CYP3A4*22 intron 6 C>T transition using the Taqman® drug metabolism genotyping assay and real-time PCR technique described below in the laboratory methodology section. The DNA for analysis was available from two distinct sources. The first source was stored DNA in the Histocompatibility and Immunogenetics

laboratory involved in the tissue typing of transplant patients. We analysed DNA samples from 305 kidney transplant recipients, 48 simultaneous pancreas-kidney transplant recipients, 252 liver transplant recipients and 385 deceased organ donors.

The second cohort of DNA samples for analysis was obtained from the Generation Scotland Bioresource. The cohort of samples used in this study was from the Generation Scotland 3D (GS:3D) study where healthy blood donors consented for the storage of their DNA following blood donation. The DNA from this cohort was obtained from the leukocyte filters used as part of the blood donation process [185]. A total of 4899 GS:3D samples were used in this study. In addition to the genotype of each sample, basic demographic (gender, ethnic group and age) data was collected for the entire cohort.

2.2 Patient selection process

All adult patients (> 18 years of age) who received a kidney or a simultaneous kidney-pancreas transplant between January 2008 and August 2012 and were prescribed tacrolimus in the form of Prograf® (Tac-BD) were included in the

analysis. Patients who were prescribed Advagraf® (Tac-QD) modified release or those who were not prescribed tacrolimus were excluded from the study. For the liver transplant study the same inclusion/exclusion criteria were applied however patients were transplanted between January 2007 and August 2012.

Patients without a stored DNA sample were excluded. In the liver transplant cohort, patients where no recipient and donor DNA sample was stored were excluded. Figure 2.1 (below) shows the numbers of patients included in each of the 3 organ categories relative to DNA and clinical data availability.

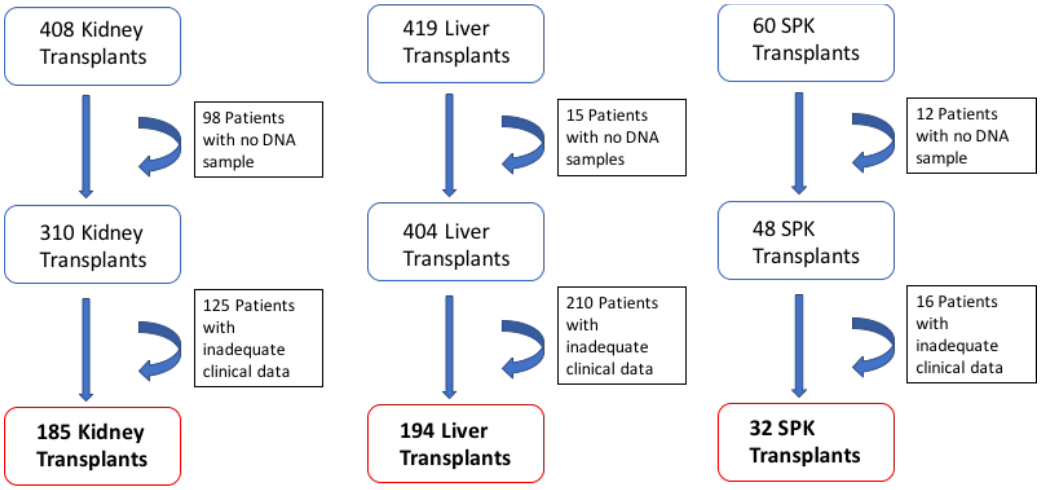


Figure 2.1 Flow diagram demonstrating the inclusion and exclusion of patients within this study. Several patients are excluded due to missing data from patient case files.

2.3 Patients converted to Tac-QD

In the final results chapter (Chapter 6) a different cohort of patients were used. 103 patients receiving a kidney transplant between April 1983 and April 2011 and converted from twice-daily Tac-BD (Prograf®) to once-daily Tac-QD (Advagraf®) were investigated. Of those 103 patients, 43 had stored DNA for genotyping and all of them had adequate clinical data. A more detailed methodology for this patient cohort is given at the beginning of chapter 6.

2.4 Laboratory Methodology

Frozen human deoxyribonucleic acid (DNA), stored at -20°C, was used for the laboratory experiments. The DNA was extracted from whole blood and prepared and stored as part of the normal clinical practice within the transplant unit. We used a technique called Magtration® described by *Obata et al* in 2001, where magnetic particles are used to selectively bind to DNA and are then extracted [186]. The device used in our laboratory is the 12GC Magtration® System (Precision System Science Company, Ltd.) All DNA extractions had taken place prior to the start of this study.

2.4.1 Materials Required for Laboratory Experiments

1. Aliquot of genomic DNA stored at -20°C
2. Aliquot of sterile water stored at -20°C
3. TaqMan® SNP Drug Metabolism Genotyping Assay from Applied Biosystems™ (Life Technologies) for each of the three different SNPs – ABCB13435 C>T(rs1045642, assay ID C_7586657_20, product code 4362691), CYP3A5 6986A>G (rs776746, assay ID C_26201809_30, product code 4362691) and CYP3A4*22 intron 6 C>T (rs35599367, assay ID C_59013445_10, product code 4351379).
4. TaqMan® genotyping mastermix (which contains reaction mix, AmpliTaq® Gold, dNTPs and ROX passive reference dye) from Applied Biosystems ™ (Life Technologies), product code 4381656.
5. MicroAmp® Optical 96-well reaction plate (skirted) from Applied Biosystems™ (Life Technologies), product code N8010560.
6. MicroAmp® Optical adhesive film cover from Applied Biosystems™ (Life Technologies), product code 4360954.
7. MicroAmp® Optical film compression pad from Applied Biosystems™ (Life Technologies), product code 4312639.
8. Pipettes, tips and eppendorfs.
9. DNA storage boxes.

2.4.2 Non-consumable equipment

1. Veriti ™ PCR 96 well Thermal Cycler (Applied Biosystems™).
2. ABI Prism® 7500 Real-Time PCR machine. (Applied Biosystems™)
3. NanoDrop™ mass spectrophotometer (ThermoScientific™)
4. Vortex mixer (Fisher Scientific™).

2.4.3 Planning and Preparation

A 96 well plate template was created in a word document and divided into 3 sections of 32 wells each. Each section of the plate would have 29 subject DNA samples, 1 sample of a known genotype (positive control) which was kindly donated by St George's Hospital in London (courtesy of Prof. McPhee), and two non-template controls (NTCs) which were DNA-free samples of sterile water (negative controls).

Each sample was genotyped 3 times on every plate each with a different assay (CYP3A5, ABCB1 and CYP3A4*22). Samples were assigned a unique identifier that had no immediately recognisable patient identifiable information.

2.4.4 Standardising the DNA concentration

Once the 29 samples for the run were identified, they were removed from the freezer and allowed to thaw. The NanoDrop™ spectrophotometer was initialised, blanked and then checked with sterile water. A 2μL drop of DNA was placed on the device and the spectrophotomer initiated on the nucleic acid setting. The concentration of the DNA was then entered into a pre-prepared excel spreadsheet which automatically calculated the required volume of sterile water to be added in order to achieve a concentration of 10ng/μL. This process was repeated for each of the samples, ensuring that the spectrophotometer was cleaned and reset between each sample to prevent cross contamination of DNA.

2.4.5 Preparing the DNA and Sterile Water

Each assay requires a total volume of 11.25 μL of DNA and sterile water. Therefore, for each assay a total of 2 μL of DNA (10ng/μL) and 9.25 μL of sterile water was used and as the genotyping assay was set up for n=4 samples (one additional sample to allow for errors) a total of 8 μL of DNA and 37 μL of sterile water was placed into a pre-labelled Eppendorf for each

DNA sample to be genotyped. The labelled DNA samples were kept refrigerated at 4°C until they were ready to be used.

2.4.6 Preparing the Genotyping Assays

Three Eppendorfs were prepared and labelled CYP3A5, ABCB1 and CYP3A4*22. Each assay was removed from the freezer and allowed to thaw, centrifuged to 1500 rpm and then swirled using the Vortex machine as per the manufactures guidelines. To each of the pre-labelled assay Eppendorfs 437.5 μL of genotyping mastermix was added followed by 43.75 μL of the corresponding drug metabolising assay to give a total volume of 481.25 μL per Eppendorf which was enough for 35 individual wells on the plate (32 + 3 extra). Once the assay was added to the genotyping mastermix, each Eppendorf was centrifuged to 1500 rpm and then swirled using the Vortex machine.

A clean unused MicroAmp® 96 well plate was placed in a cool rack and 11.25 μL of the DNA/sterile water mix is added to the corresponding well for the CYP3A5 assay based on the previously prepared plate layout. This is repeated for the ABCB1 and CYP3A4*22 section of the plate, again adding the same volume of DNA/sterile water mix. For the 2 NTCs 11.25 μL of

sterile water alone is added to the appropriate well in the corresponding genotype section.

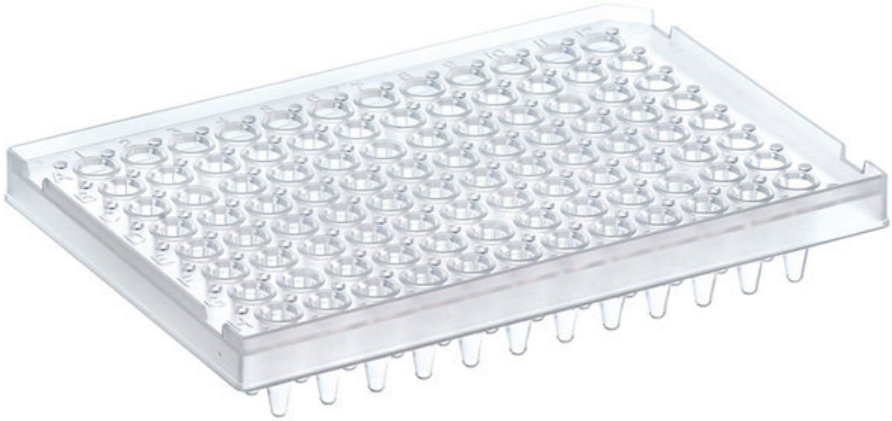
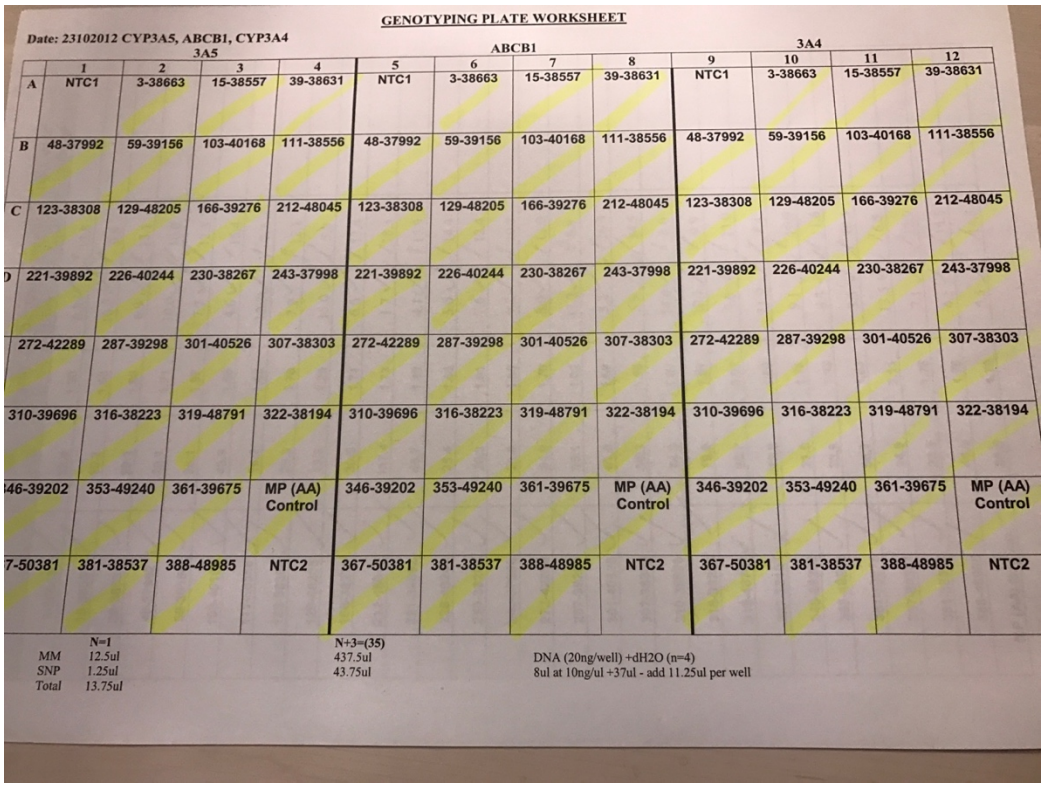


Figure 2.2 A MicroAmp® 96 well Optical Reaction Plate manufactured by Applied Biosystems™ used to carry out the PCR reaction for genotyping which can then be read on the ABI Prism® 7500 RT-PCR machine.

2.4.7 Plate layout

An example of a plate layout showing 29 subjects genotyped for each of the drug-metabolising assays. The NTCs and positive controls are also shown in this layout.



GENOTYPING PLATE WORKSHEET

Date: 23102012 CYP3A5, ABCB1, CYP3A4

	3A5				ABCB1				3A4			
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC1	3-38663	15-38557	39-38631	NTC1	3-38663	15-38557	39-38631	NTC1	3-38663	15-38557	39-38631
B	48-37992	59-39156	103-40168	111-38556	48-37992	59-39156	103-40168	111-38556	48-37992	59-39156	103-40168	111-38556
C	123-38308	129-48205	166-39276	212-48045	123-38308	129-48205	166-39276	212-48045	123-38308	129-48205	166-39276	212-48045
D	221-39892	226-40244	230-38267	243-37998	221-39892	226-40244	230-38267	243-37998	221-39892	226-40244	230-38267	243-37998
	272-42289	287-39298	301-40526	307-38303	272-42289	287-39298	301-40526	307-38303	272-42289	287-39298	301-40526	307-38303
	310-39696	316-38223	319-48791	322-38194	310-39696	316-38223	319-48791	322-38194	310-39696	316-38223	319-48791	322-38194
	346-39202	353-49240	361-39675	MP (AA) Control	346-39202	353-49240	361-39675	MP (AA) Control	346-39202	353-49240	361-39675	MP (AA) Control
	367-50381	381-38537	388-48985	NTC2	367-50381	381-38537	388-48985	NTC2	367-50381	381-38537	388-48985	NTC2

	N=1	N+3=(35)
MM	12.5ul	437.5ul
SNP	1.25ul	43.75ul
Total	13.75ul	

DNA (20ng/well) +dH2O (n=4)
8ul at 10ng/ul +37ul - add 11.25ul per well

Figure 2.3 Diagram of the plate layout used demonstrating the unique identifier for each sample, the non-template controls (NTC) and the known genotype controls (MP) as laid out in each 96 well plate. The CYP3A5, ABCB1 and CYP3A4*22 assay is run on each plate simultaneously as shown in the layout above.

The pipette tip was changed between each patient to ensure that there is no cross contamination.

Once the DNA/sterile water mix is added to the plate for each of the subjects and the NTCs and positive controls have been plated out, 13.75 μL of genotype assay is added to each well with the first 4 x 8 block of the plate being the CYP3A5 assay, the middle 4 x 8 block being ABCB1 and the final 4 x 8 block for CYP3A4*22. The total volume in each well is then 25 μL (11.25 μL of DNA/sterile water, 13.75 μL of assay/mastermix).

The plate was then sealed with a MicroAmp® optical film lid and kept refrigerated at 4°C until ready for the PCR run. The PCR was run on the Veriti® thermocycler and the pre and post plate reads were done on the ABI Prism® 7500 RT-PCR machine. The standard operating procedures for the drug metabolism assays from Applied Biosystems™ describe this method of running the experiment as an alternative to running the entire PCR on the ABI Prism® 7500 RT-PCR system.

2.4.8 Pre-Read on ABI Prism® 7500 RT-PCR

A new file was created for each run of the plate and named "pre-read [date]". The system was set for 'alleic discrimination'. The volume in each well was set to 25 μL. On the very first run a template for each of the genotyping assays was created which could then easily be used for all subsequent runs on the RT-PCR machine. The creation of this template involved assigning

the reporter dyes, named VIC and FAM to an allele for each of the drug metabolism assays. For CYP3A5, the 6986G allele was assigned to the FAM reporter dye and the 6986A allele was assigned to the VIC reporter dye. For ABCB1 the 3435C allele was assigned to the FAM reporter dye and the 3435T allele was assigned to the VIC reporter dye and for CYP3A4*22 the C allele was assigned to the VIC reporter dye and the T allele assigned to the FAM reporter dye.

Once the template was loaded and the pre-read file created with all of the subject numbers entered and the NTCs specified, the pre-read run was initiated. This essentially scans the pre-PCR plate and ensures that no reporter dyes are detected at this stage. Once the pre-read was complete it was saved and this file was later used for the post-read run.

2.4.9 Running the PCR

The Veriti® thermocycler was programmed to run the cycle as described in the manufacturers standard operating procedure document for the 3 assays. All 3 assays were run on precisely the same thermocycler program. The plate was placed into the thermocycler and the MicroAmp® compression pad placed on top, shiny side down. The programme was then initiated as shown in the table below.

Table 2.1 PCR Settings used for the Veriti® Thermocycler

AmpliTaq Gold Enzyme Activation	PCR (50 cycles)		Hold
HOLD	Denature	Anneal/Extend	Until ready for post-read
10 mins at 95°C	15 secs at 92°C	90 secs at 60°C	4°C

Once the thermocycler program was complete the plate was removed from the thermocycler, ready to be read on the ABI Prism® 7500 RT-PCR machine.

2.4.10 Post-Read on ABI Prism® 7500 RT-PCR

The plate was placed in the RT-PCR machine and the previously created pre-read file opened. The machine was set for 'automatic-calling' and the quality set to 90%. This means that any wells that do not have a quality result of at least 90% (which is deemed by the manufacturer as the appropriate cut-off) will not have a result called and ensures a high quality of results. Two-cluster calling was also selected. Once these settings are entered and checked, the plate is scanned for each assay in turn, selecting only the wells that are relevant to that particular assay and the results are generated.

Once all 3 assays have been scanned the results are displayed both graphically and as a list which are then recorded next to the patients anonymised dataset number in a spreadsheet that was kept separate to the clinical data set.

The graphical output of the RT-PCR machine is shown below in Figures 2.4, 2.5 and 2.6 and demonstrates the distribution of the alleles for each of the drug metabolising genes in this study.

2.4.11 CYP3A5 Output

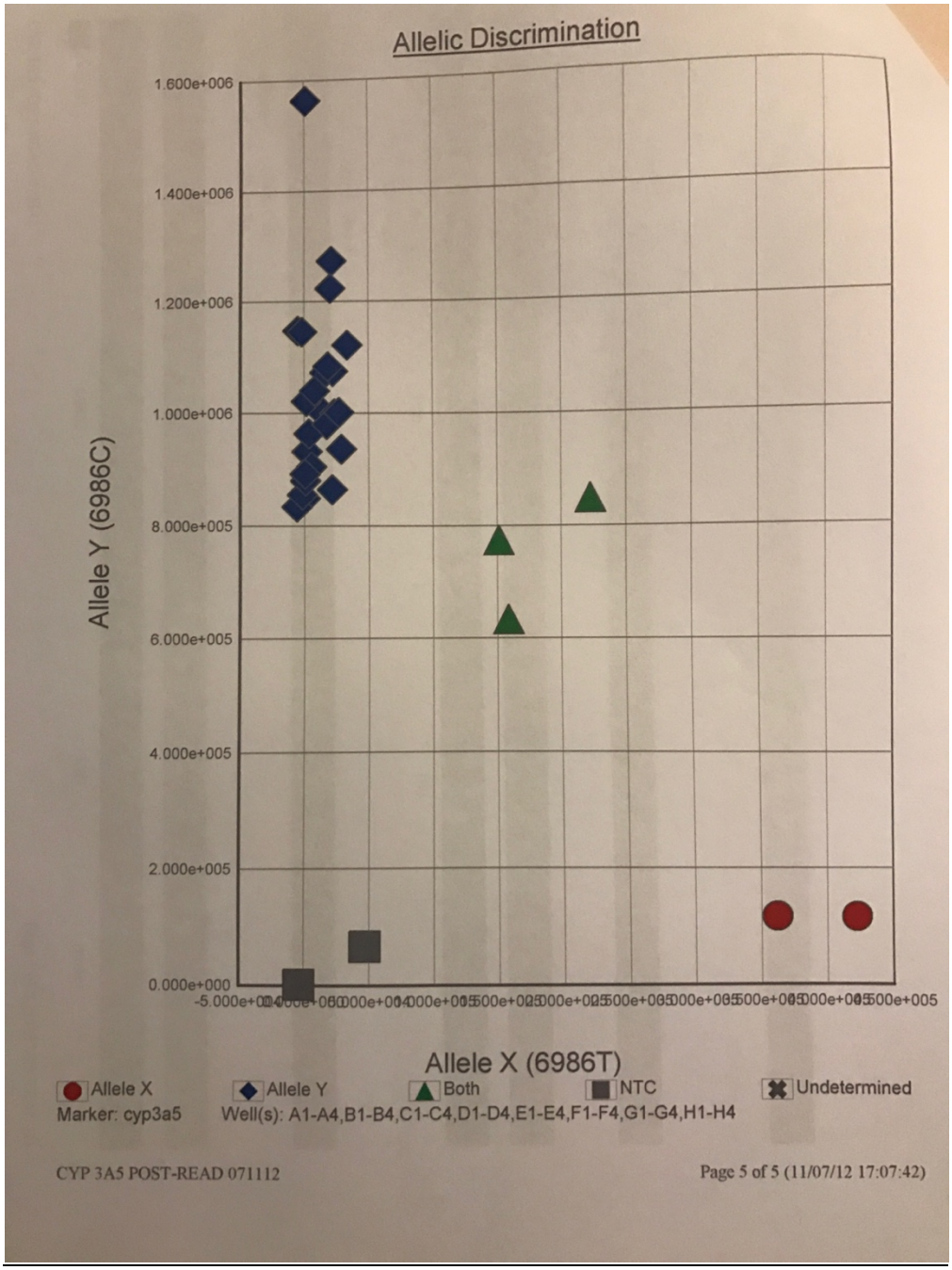


Figure 2.4 RT - PCR output for CYP3A5 Alleles. Allele X is the wild type A allele (*1/*1) represented by red dots and allele Y is the mutant G allele (*3/*3) and both alleles present GA (*3/*1) are represented by the green triangle. The NTCs are grey squares.

2.4.12 ABCB1 Output

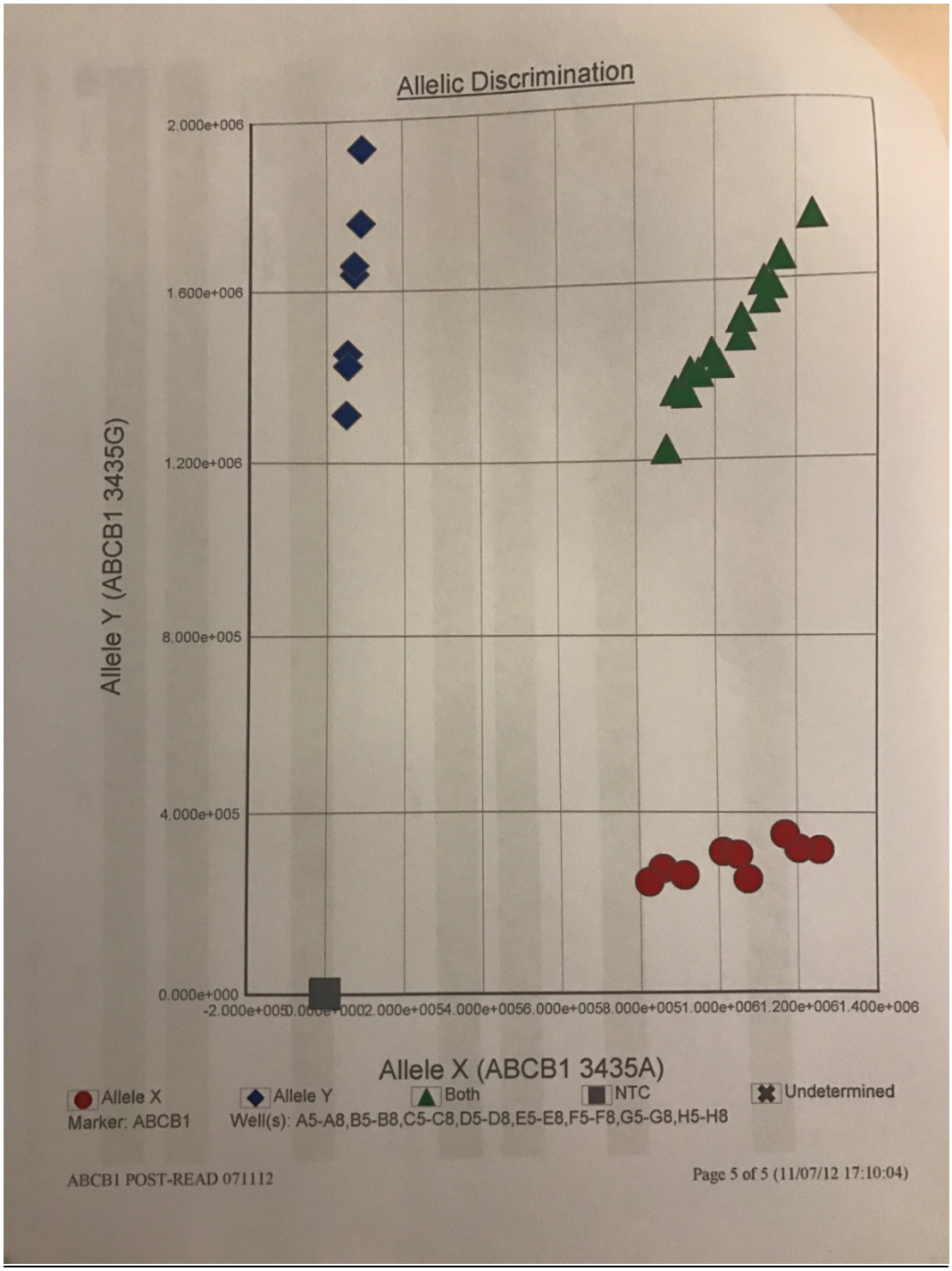


Figure 2.5 RT-PCR output for ABCB1 Alleles. CC represented by the blue squares, CT by the green triangle and TT by the red dots, again the grey squares are the NTCs.

*2.4.13 CYP3A4*22 Output*

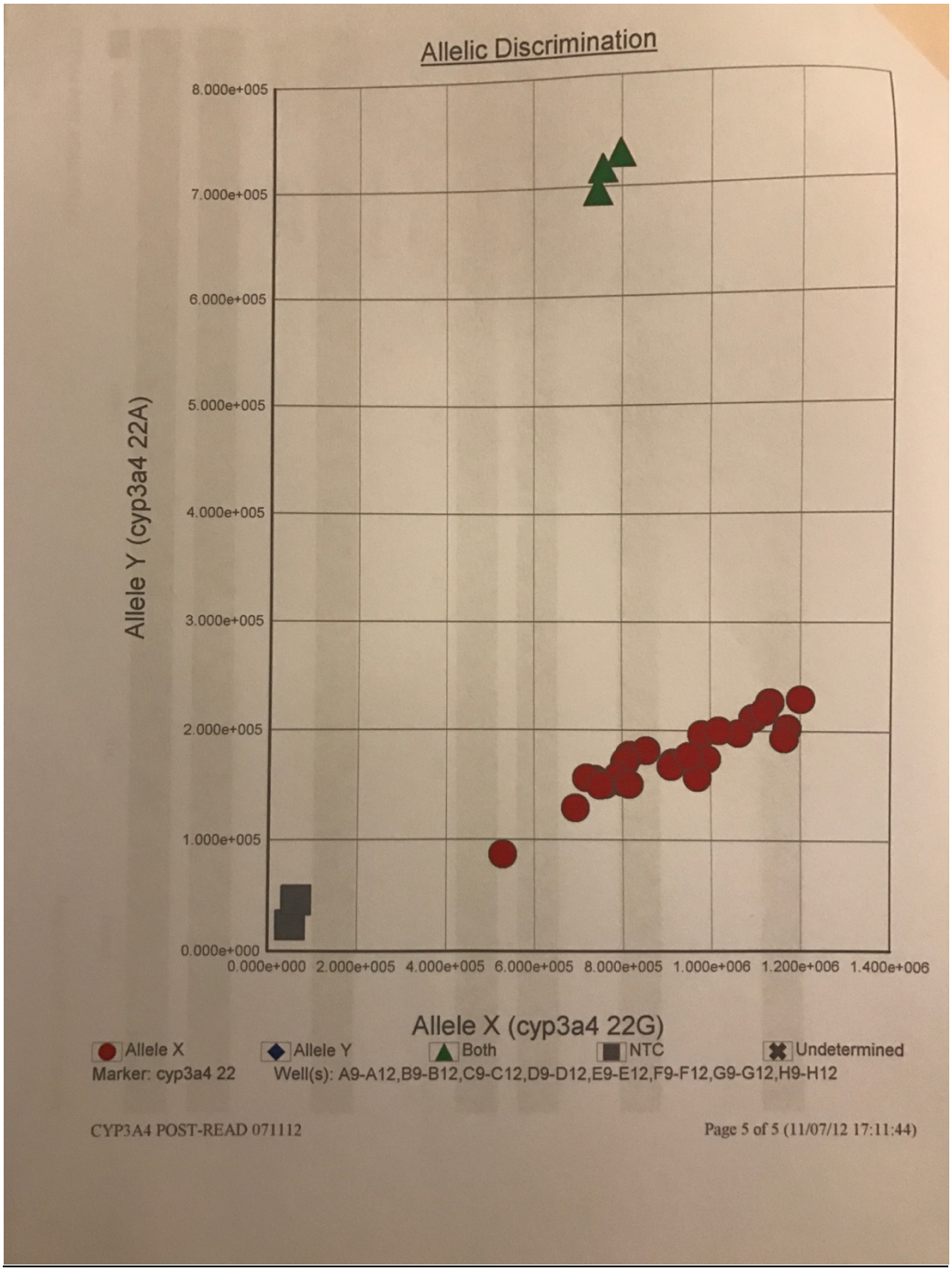


Figure 2.6 RT - PCR output for CYP3A4*22 Alleles. The CC genotype represented by the red dots and the CT allele represented by the green triangles, note there are no TT genotypes in this run, a reflection of the rarity of this genotype. Grey squares are NTCs.

2.5 Large Volume Genotyping

A total of 203 patients had their genotype for CYP3A5, ABCB1 and CYP3A4*22 determined by the method described above and genotyping of the remaining 787 samples was out-sourced to the Clinical Research Facility at the Western General Hospital, the Wellcome Trust Clinical Research Facility (WTCRF) due to the clinical demands for the PCR machine. The same preparation process was followed. However, the subsequent process was slightly different as WTCRF used a PicoGreen® assay on the NanoDrop® fluorescent spectrophotometer. This assay binds selectively to only double stranded DNA (dsDNA) and gives a more accurate concentration by eliminating single strand DNA (ssDNA) and other contaminants such as proteins and extraction buffers.

The WTCRF standardised the concentration of the DNA for the assay at 50 ng/μL as compared to the 10 ng/μL used in the Royal Infirmary of Edinburgh.

The WTCRF used a 7900HT RT-PCR machine from Applied Biosystems™ which is a high-throughput system that is fully automated. The samples were run on 384 well plates rather than 96 well plates for maximum throughput. The thermocycler settings were the same as used on the Veriti® machine.

The same research facility was used to determine the genotypes of the Generation Scotland cohort and the process was exactly the same as used for the transplant patients.

2.5.1 Hardy-Weinberg Equilibrium

Hardy-Weinberg equations are used to describe and predict the genotype and allele frequencies of a non-evolving population from one generation to the next. Given a set of 5 assumptions – a large sample is used and there is no genetic drift, there is no gene flow between populations (from migration), mutations are negligible, individuals are mating randomly and natural selection is not operating on that population – then a population's genotype and allele frequencies will remain unchanged from one generation to the next. A significant deviation from Hardy-Weinberg equilibrium would suggest systematic genotyping errors or other biases that could invalidate the results and therefore it is recommended to perform a Hardy-Weinberg analysis for any large population based genetic study. The most commonly used statistical model used to detect a departure from Hardy-Weinberg equilibrium is the goodness-of-fit χ^2 test and this is the model used in this study. This is the standard model used in large unrelated populations as

analysed in our data. All of the assays that were run for CYP3A5, ABCB1 and CYP3A4*22 were found to be in Hardy-Weinberg equilibrium.

2.6 Clinical Data Collection

The clinical data for the studies were acquired from a combination of clinical case notes and electronic patient records and laboratory systems. A proforma was generated to collect patient data from the case notes and the electronic patient records. Once the proforma was complete the data was then entered into an anonymised excel spreadsheet which did not contain the patient genotypes but did have the matching patient identifier for the genotypes held on a separate secure drive in the tissue typing laboratory.

The clinical data consisted of tacrolimus dose and trough level at 14 different time points up to and including 12 months, 3 time points in the first, second and third week, 1 month, 2 months, 3 months, 6 months and 12 months. Patient demographic data included gender, age, ethnicity, primary disease, transplant graft number, renal replacement therapy, HLA mis-match, virology status for cytomegalovirus (CMV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and the donor virology for the same viruses. Donor and recipient blood group and

relevant recipient pre-transplant co-morbidity (hypertension, diabetes and established cardiovascular disease) data were collected.

Clinical outcome data included duration of cold ischemic time, renal function, biopsy proven acute rejection (BPAR), post-transplant infections, new onset diabetes after transplant, significant cardiovascular events, development of post-transplant malignancies, graft and patient survival. The full list of collected clinical data items is included in the appendix.

Once the clinical data collection was completed the 2 excel spreadsheets (one containing only the genotype of CYP3A5, ABCB1 and CYP3A4*22 and the patient identifier and the other containing only clinical data and the identifier) were merged to form a single database. All patient specific identifiers were removed and only the study identifier remained, allowing for a completely anonymised dataset containing both clinical and genotype data.

2.6.1 Laboratory Tacrolimus Assay

The clinical laboratory in this unit measure the trough concentration of tacrolimus in whole blood samples using a liquid chromatography tandem

mass spectrometry technique, which is used widely in transplant units. In this centre the laboratory has both a low and a high quality-control (QC) concentration and results should be within 2 standard deviations of this value. The low QC mean is 2.86 ± 0.76 μg/L and the high QC mean is 16.6 ± 2.2 μg/L for the assay in our clinical laboratory and the quality control checks are performed in line with the standard operating procedures for the laboratory.

2.7 Data Analysis

All data was analysed on IBM® SPSS® version 21 statistical analysis software. All graphs were drawn on either GraphPad Prism® version 7.0a or Microsoft® Excel version 15.30. Continuous variables were analysed using one-way analysis of variance (ANOVA) test and categorical variables analysed by Chi-square test unless otherwise stated. Graft and patient survival was determined by Kaplan-Meier curves and log-rank test in univariate analyses and by Cox Regression in multivariate analyses. Continuous variables are expressed as the mean±standard deviation unless otherwise stated. Error bars on graphs represent the standard error of the mean unless otherwise stated.

Chapter III

Distribution of Polymorphisms in Scotland

3 DISTRIBUTION OF POLYMORPHISMS IN SCOTLAND

3.1 Background

It is widely recognised that there are considerable differences in the pharmacokinetics and pharmacodynamics of certain drugs between different ethnic groups and wider populations. Variations in polymorphisms of genes involved with drug metabolism, such as cytochrome P450 or drug transporters such as MDR-1 (ABCB1), can play a pivotal role in these observed differences [153]. This is particularly relevant in transplantation as expression of certain genetic polymorphisms can influence the pharmacokinetics of immunosuppressant drugs and therefore have a significant clinical impact.

There is much variation of the expression of these genetic polymorphisms between different ethnic groups and geographical populations and these variations go some way in explaining the observed differences in the pharmacokinetics of certain drugs between ethnic groups.

Within the United Kingdom the study of these polymorphisms has been undertaken predominantly in renal transplant patients in the London area. London has an ethnically more diverse population than Scotland, with some 40% of people living there being born out with the UK. It cannot therefore be assumed that the distribution of the genotypes of interest for this study will be the same in Scotland, nor that the distribution of these polymorphisms within a renal transplant population is representative of the population as a whole. To date there have been no studies examining the distribution of single nucleotide polymorphisms (SNPs) of CYP3A5, CYP3A4*22 or ABCB1 in a Scottish population.

Scotland has primarily a Caucasian population although smaller numbers of people from many different ethnic groups live here. Previously published data has shown that expression of CYP3A5 (AG/AA or *1*3/*1*1) occurs in 15-20% of Caucasians with only minor variations in allele frequency across Europe. Conversely around 85% of individuals of sub-Saharan African ethnicity (Black), will express functional CYP3A5 while those individuals from an Asian ethnic group fall somewhere in the middle, with nearly 50% of them expressing a copy of the A (*1) allele of CYP3A5 [143, 187]. Within the Asian population itself there are significant differences between Eastern Asian countries (Japan, Korea, China); South East Asian countries (Thailand, Vietnam, Singapore) and South Asian countries such as India [153].

CYP3A4*22 involvement with tacrolimus pharmacokinetics has only recently been investigated and the published literature is considerably less than that for CYP3A5. Studies to date have shown that individuals who express a T allele and therefore have the CT or the TT genotype require lower doses of tacrolimus in order to achieve therapeutic levels. The T allele frequency in Caucasians is around 4- 5% and represents a C>T transition in intron 6 resulting in reduced mRNA expression of CYP3A4 and therefore lower enzyme activity [105, 172]. The T allele frequency of CYP3A4*22 has been reported as 1% in individuals of African ethnic origin (Black) [188]. There is currently very little data on the allele frequency of CYP3A4*22 T allele in any Asian ethnic groups however a paper by *Okubo et al* examining a Japanese cohort of 53 patients did not find any expression of a CYP3A4*22 T allele however in the same study a cohort of 41 Caucasian patients found T allele expression was 6.1%. They estimated the expression of a CYP3A4*22 allele in a Japanese population at less than 1% but further studies of this polymorphism in Asian ethnic groups is required to fully evaluate its expression this population [173].

There are several polymorphisms of ABCB1 which can alter the expression of P-gp in the enterocyte, however the most commonly studied SNP in terms of tacrolimus pharmacokinetics is the 3435 C>T transition. Expression of a T allele occurs commonly in Caucasians with approximately 28% expressing

the CC genotype, 48% expressing the CT genotype and 24% expressing the TT genotype [189]. Subjects of African ethnic origin (Black) have a different distribution of these genotypes with 68% expressing the CC genotype, 31% expressing the CT genotype and only 1% expressing the TT genotype [190]. The reported distribution in an Asian (Chinese) population shows CC expression at 32%, CT expression at 48% and TT expression at 20% [191]. In an Indian population, however, the distribution is yet again different with CC genotype reported at 19.7%, the CT genotype reported at 51.4% and the TT genotype reported at 28.9% [192].

The current published literature demonstrates wide variation in the expression of SNPs of the three genes of interest in this study between different ethnic and geographical groups. The aim of this study was to establish the expression of the described polymorphisms of CYP3A5, CYP3A4*22 and ABCB1 in a representative sample of the healthy Scottish population and compare it with expression of the polymorphisms of the same genes in kidney transplant recipients, liver transplant recipients, combined kidney-pancreas transplant recipients and deceased organ donors.

3.2 Study Methods

DNA samples from 5889 subjects were genotyped for CYP3A5 6986 A>G transition, ABCB1 3545 C>T transition and the CYP3A4*22 intron 6 C>T transition using the Taqman® drug metabolism genotyping assay and real-time PCR technique. The DNA for analysis was available from 2 distinct sources. The first source was frozen, stored DNA in the Histocompatibility and Immunogenetics (H&I) laboratory involved in the tissue typing of transplant patients. There were a total of 990 DNA samples available for genotyping from the H&I laboratory: 305 kidney transplant recipient samples, 48 simultaneous pancreas-kidney transplant recipient samples, 252 liver transplant recipient samples and 385 deceased organ donor samples.

The second source of DNA samples for analysis was a bio-resource facility called Generation Scotland. Generation Scotland is a bio repository that collects basic demographic data (age, gender, ethnicity) and stores DNA samples from volunteers. The cohort of samples used in this study was from the Generation Scotland 3D (GS:3D) study, where healthy blood donors were asked to allow the storage of their DNA following blood donation for use in the bio-repository. The DNA from this cohort was obtained from the leukocyte filters used as part of the blood donation process and all participants had given written consent [185]. A total of 4899 GS:3D samples

were used in our analysis. In addition to the genotype, basic demographic data was collected for the entire cohort (gender, ethnic group and age).

3.2.1 Genotyping

All genomic DNA samples were standardised to a 10ng/μl concentration and genotyped for each of the 3 genetic polymorphisms using the TaqMan® drug metabolism genotyping assay as described in detail in Chapter 2.

3.2.2 Missing Data

The majority of subjects have a complete dataset in this study however there are small numbers where data is missing both in terms of demographic and genotyping data. There are 104 (1.8%) subjects where the age was not determined and 107 (1.8%) where the gender was not recorded. The majority of missing data of these variables occurred in the organ donor group where historically recording of these data occurred on paper forms could result in erroneous omissions. There were 10 patients (0.2%) where the ethnic group was not recorded.

Failure to identify the genotype of a subject can occur when the quality of the genomic DNA is poor, is contaminated or by chance. In order for a genotyping run to be considered successful 95% of the subjects must have their genotype identified. Of the 5889 samples tested there were 122 (2.1%) of the subjects where the CYP3A5 genotype was not determined, 136 (2.3%) where the CYP3A4*22 genotype was not determined and 210 (3.6%) where the ABCB1 genotype was not determined.

3.2.3 National Census Data

Comparisons of demographic data was made with the 2011 national census data for Scotland and the rest of the United Kingdom using data from the Office for National Statistics which is freely available.

3.3 Chapter Results

3.3.1 Gender

There were a total of 3214 (55.6%) male subjects and 2568 (44.4%) female subjects overall in this study, excluding the 107 subjects (1.8%) where the gender information was unavailable. This differs significantly from the 2011 census data for Scotland where the population had 2567444 males (48.5%) and 2727959 females (51.5%), $p<0.0001$, Chi-square test. The England and Wales census data and the UK as a whole are very similar to the Scottish census data and thus also differ, in terms of gender distribution, to this study cohort (Table 3.2).

The kidney transplant cohort of patients has significantly more men (208, 68.2%) compared to women (97, 31.8%), $p<0.0001$ Chi-Squared test, but this in itself would be unlikely to change the overall gender distribution significantly given the overall size of the cohort. There were also more men in the Generation Scotland cohort (2696, 55.2%) compared with women (2189, 44.8%), which is why this overall number is higher in the cohort. Within the transplant cohort as a whole, there were 518 males (57.7%) compared with 379 (42.3%) females. These data suggest that males are over-

represented in our cohort of subjects compared with the census data of both Scotland the rest of the United Kingdom.

3.3.2 Age

The subjects in this study were categorised into 11 different age brackets starting at <17 and then 17 – 25 years and then increasing in 4 year age brackets up to and including >66 years of age, shown in Table 3.1 below. The majority of the subjects in the Generation Scotland cohort were in the 41 – 45 years age bracket (788, 16.1%). There was variation in age across the different groups within the transplant cohort of patients also shown in Table 3.1. The highest number of kidney transplant patients were in the 41 – 45 years age bracket (45, 14.8%), as were the SPK patients (13, 27.1%) similar to the Generation Scotland cohort, however in the liver transplant cohort most patients were in the 56 – 60 years age bracket (53, 21.0%). Most of the organ donors were in the >66 year age group (43, 11.2%) but this may not be representative of the true age spread in the organ donor group as there are 94 donors (24.4%) where the age was unavailable or not recorded.

The age distribution differs between the different groups in the transplant cohort and reflects the disease burden of each group (SPK patients tend to be younger, liver disease patients tend to be older) and this therefore also

differs from the Generation Scotland group who are a sample of the healthy population. The age distribution of the Generation Scotland group is also different from the Census data for both Scotland and England and Wales, primarily due to the extremes of age that are obviously captured in census data but will not be blood donors. Age clearly has no influence on genotype expression however the census data is also recorded in Table 3.1 below to give a complete detail of demographics in our patient cohort compared with the Scottish population and the rest of the United Kingdom.

Table 3.1 Age distribution of the subjects in this study grouped by different cohorts. Comparison is shown with the census data from 2011 for Scotland, England and Wales and the UK as a whole to compare and highlight any differences

Age	Generation Scotland	Kidney Transplant	Liver Transplant	SPK Transplant	Organ Donor	Entire Cohort	Scottish Census	England and Wales Census	UK Census
<17	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	6 (1.2%)	6 (0.1%)	978476 (18.5%)	11265853 (20.1%)	12244329 (20.0%)
17 - 25	543 (11.1%)	21 (6.9%)	10 (4.0%)	1 (2.1%)	40 (10.4%)	615 (10.6%)	640631 (12.1%)	6741757 (12.0%)	7382388 (12.0%)
26 - 30	315 (6.4%)	16 (5.2%)	8 (3.2%)	6 (12.5%)	9 (2.3%)	354 (6.1%)	344546 (6.5%)	3851963 (6.9%)	4196509 (6.8%)
31 - 35	384 (7.8%)	20 (6.6%)	3 (1.2%)	7 (14.6%)	22 (7.6%)	436 (7.5%)	316102 (6.0%)	3600075 (6.4%)	3916177 (6.4%)
36 - 40	626 (12.8%)	29 (9.5%)	8 (3.2%)	10 (20.8%)	36 (12.4%)	709 (12.3%)	353009 (6.7%)	3848216 (6.9%)	4201225 (6.8%)
41 - 45	788 (16.1%)	45 (14.8%)	17 (6.7%)	13 (27.1%)	31 (10.7%)	894 (15.5%)	399450 (7.5%)	4116330 (7.3%)	4515780 (7.4%)
46 - 50	729 (14.9%)	40 (13.1%)	23 (9.1%)	5 (10.4%)	30 (10.3%)	827 (14.3%)	408462 (7.7%)	4032081 (7.2%)	4440543 (7.2%)
51 - 55	633 (12.9%)	30 (9.8%)	45 (17.9%)	5 (10.4%)	23 (7.9%)	736 (12.7%)	366170 (6.9%)	3486811 (6.2%)	3852981 (6.3%)
56 - 60	517 (10.6%)	41 (13.4%)	53 (21.0%)	1 (2.1%)	31 (10.7%)	643 (11.1%)	325697 (6.2%)	3157221 (5.6%)	3482918 (5.7%)
61 - 65	300 (6.1%)	26 (8.5%)	51 (20.2%)	0 (0.0%)	20 (6.9%)	397 (6.9%)	325887 (6.2%)	3310236 (5.9%)	3636123 (5.9%)
>66	61 (1.2%)	30 (9.8%)	34 (13.5%)	0 (0.0%)	43 (14.8%)	168 (2.9%)	836973 (15.8%)	8665369 (15.5%)	9502342 (15.5%)

3.3.3 Ethnic Groups

The participants in this study were mostly Caucasian accounting for 99.3% of the Generation Scotland group and 98.1% of the Transplant cohort. While the Scottish population is primarily Caucasian this is a significantly higher percentage of Caucasians than the general population of Scotland in the 2011 Census (96.0%) and even more so than in England and Wales where the Caucasian population is 85.9%. $p<0.0001$, Chi-Square test. This data suggests that Caucasians may be over-represented in this study compared with the general population of Scotland but also shows that England and Wales is a more diverse population in terms of ethnicity than Scotland.

There was a single black subject in this study who was in the liver transplant cohort however even in the largest cohort in this study, the Generation Scotland Group, there were no other black subjects. The percentage of black people resident in Scotland compared with the rest of the population in the 2011 census was low at 36178 (0.7%) however this is still considerably higher than the number in this study (0.02%) and therefore it is reasonable to conclude that black subjects are under-represented in this study when compared with the 2011 National Census data for Scotland. This difference

is even more marked when compared with the census data for England and Wales where 3.3% of the population are of the black ethnic group and the UK overall where the proportion of the population that is black sits at 3.0%.

Asian subjects are also under-represented in this study when compared with the census data for Scotland, England and Wales and the UK as a whole. There were a total of 25 Asian subjects overall in this study, 7 from the Generation Scotland cohort (0.1%) and 18 as part of the transplant patient cohort (1.8%). The 2011 Scottish census data shows that 2.7% of the Scottish population were of the Asian ethnic group and in England and Wales this figure rises to 7.5%, giving the overall UK census figure as 6.9% of the population described as Asian in the 2011 census.

These findings are summarised in the table below comparing the gender and ethnic groups of the transplant cohort, the Generation Scotland data and the 2011 census data for Scotland, England and Wales and the UK as a whole.

Table 3.2 Gender and ethnic groups of the Generation Scotland cohort and the transplant cohort and compared with the census data for 2011 in Scotland, England and Wales and the UK as a whole.

		Generation Scotland	Transplant Cohort	Census Data Scotland	Census Data Eng/Wales	Census Data UK
Gender						
	Male	2696 (55.6%)	518 (52.3%)	2567444 (48.5%)	27573376 (49.2%)	31028143 (49.1%)
	Female	2189 (44.4%)	379 (38.3%)	2727959 (51.5%)	28502536 (50.8%)	32154035 (50.9%)
	Unknown	14 (0.3%)	93 (9.4%)			
Ethnic Group						
	Caucasian (White)	4864 (99.3%)	971 (98.1%)	5084407 (96.0%)	48151715 (85.9%)	55010359 (87.1%)
	White: Scottish	4317 (88.1%)	691 (69.8%)	4445678 (83.9%)	no separate data	no separate data
	White: Other British	463 (9.5%)	127 (12.8%)	417109 (7.9%)	no separate data	no separate data
	White: Irish	47 (1.0%)	1 (0.1%)	54090 (1.02%)	no separate data	no separate data
	White: Other	37 (0.8%)	152 (15.4%)	167530 (3.2%)	no separate data	no separate data
	Black	0 (0.0%)	1 (0.1%)	36178 (0.7%)	1864890 (3.3%)	1904684 (3.0%)
	Asian	7 (0.1%)	18 (1.8%)	140678 (2.7%)	4213531 (7.5%)	4373339 (6.9%)
	Asian: Chinese	3 (0.1%)	2 (0.2%)	33076 (0.64%)	393141 (0.7%)	433150 (0.7%)
	Asian: Indian	3 (0.1%)	16 (1.6%)	32706 (0.62%)	1412958 (2.5%)	1451862 (2.3%)
	Asian: Other	1 (0.02%)	0 (0.0%)	74266 (1.4%)	2407432 (4.3%)	2488327 (3.9%)

3.4 Genotype Distribution

3.4.1 CYP3A5

The distribution of polymorphisms of the CYP3A5 genotype across the entire cohort which included the Generation Scotland subjects as well as all of the transplant patients revealed 5038 (87.4%) were CYP3A5 non-expressers of the GG (*3/*3) genotype and therefore made no functional CYP3A5. There were a total of 701 subjects (12.2%) who were CYP3A5 heterozygotes of the GA (*3/*1) genotype, expressing a single copy of the wild type allele and therefore making functional CYP3A5. A considerably smaller number of subjects, 28 in total (0.5%), were homozygotes for the wild type A allele (*1/*1).

A similar genotype distribution is seen in the cohort of White – Scottish subjects with 4306 (87.7%) of the GG genotype, 582 (11.9%) of the GA genotype and 22 (0.4%) were of the AA genotype, which is to be expected given the majority of the subjects in this study are of the White – Scottish ethnic group. The other Caucasian ethnic groups have similar proportions although there is some slight variation between the different Caucasian groups as shown in Table 3.3.

The near exclusively Caucasian cohort makes interpretation of the genotype in other ethnic groups difficult due to their very small numbers however in the largest non-Caucasian group (Asian-Indian), n=19, the genotype distribution of CYP3A5 was different from the Caucasian group. In this group the GG (*3/*3) genotype was less common with only 10 subjects (52.6%) who were CYP3A5 non-expressers, however there were 6 (31.6%) subjects who expressed the GA genotype and 3 (15.8%) who were AA homozygotes. The single Black subject in this study was of the AA (*1/*1) genotype.

3.4.2 ABCB1

The distribution of the 3435 C>T transition of ABCB1 differs somewhat from the CYP3A5 polymorphism. In the overall cohort there were 1182 (20.8%) subjects who expressed the CC genotype, 2796 (49.2%) who expressed the CT genotype and was therefore the most common genotype expressed and 1701 (30.0%) subjects of the TT genotype. As with the distribution of the CYP3A5 genotype the distribution in the White – Scottish ethnic group very closely resembled that of the overall cohort with 1021 (21.1%) being of the CC genotype, 2372 (49.0%) of the CT genotype and 1443 (29.8%) subjects who expressed the TT genotype.

While most of the other Caucasian ethnic subgroups in this study followed a similar pattern, those patients that identified as White – Irish had a different distribution with 4 subjects (8.3%) were of the CC genotype, 20 (41.7%) were of the CT genotype and 24 (50.0%) were of the TT genotype. There is no published literature specifically examining the distribution of this particular SNP of ABCB1 at this time and therefore it is difficult to know if this difference is truly reflective of the Irish population or whether it is anomaly due to small numbers.

As before there are very small numbers of non-Caucasian subjects and only the Asian – Indian group has sufficient numbers to give any meaningful description with 4 subjects (22.2%) expressing the CC genotype, 8 (44.4%) were of the CT genotype and 6 (33.3%) were of the TT genotype. The single black subject in this study expressed the CC genotype of ABCB1.

*3.4.3 CYP3A4*22*

The CYP3A4*22 SNP has been described much more recently than either CYP3A5 or ABCB1 and, as such, the literature regarding the genotype distribution in different populations and ethnic groups is more limited. In this study the overall cohort there were 5185 (90.1%) subjects who expressed

the CC genotype (wild-type), 554 (9.6%) who expressed the CT genotype and only 14 (0.2%) subjects expressed the TT genotype of CYP3A4*22.

As with the other polymorphisms the White – Scottish subjects almost exactly mirrors the overall genotype distribution [4410 (90.2%) CC, 472 (9.7%) CT, 9 (0.2%) TT]. Other Caucasian sub-groups are very similar. Interestingly, with the exception of a single subject in the group 'other' who expresses the CT genotype, there is no expression of the T allele in any of the non-Caucasian ethnic sub-groups. Published data on this polymorphism is relatively scarce however it does suggest that T allele expression is found almost exclusively in Caucasians [193].

Table 3.3 Distribution of CYP3A5, ABCB1 and CYP3A4*22 genotypes among Caucasians in the study cohort as a whole.

	CYP3A5			ABCB1			CYP3A4*22		
	GG (*/3*3)	**GA (*3/*1)**	**AA (*1/*1)**	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**
White - Scottish	4306 (86.0%)	582 (11.6%)	22 (0.4%)	1021 (20.4%)	2372 (47.4%)	1443 (28.8%)	4410 (88.1%)	472 (9.4%)	9 (0.2%)
White - Other British	500 (84.7%)	74 (12.5%)	1 (0.2%)	107 (18.1%)	292 (49.5%)	168 (28.5%)	514 (87.1%)	57 (9.7%)	5 (0.8%)
White - Irish	41 (85.4%)	4 (8.3%)	1 (2.1%)	4 (8.3%)	20 (41.7%)	24 (50.0%)	44 (91.7%)	4 (8.3%)	0 (0.0%)
White - Other	153 (81.0%)	29 (15.3%)	0 (0.0%)	40 (21.2%)	85 (45.0%)	50 (26.5%)	166 (87.8%)	18 (9.5%)	0 (0.0%)
Caucasian Total	5000 (85.7%)	689 (11.8%)	24 (0.4%)	1172 (20.1%)	2769 (47.5%)	1685 (28.9%)	5134 (88.0%)	551 (9.4%)	14 (0.2%)

3.5 Genetic Distributions in the Transplant Cohort

Given that the incidence of diabetes, renal failure and liver disease can vary between different ethnic groups, we analysed the transplant cohort separately so they could be compared with the healthy cohort of volunteers from the Generation Scotland project.

3.5.1 CYP3A5

There was some minor variation between the different transplant groups of the CYP3A5 genotypes however none of these were significant (Chi-squared test). The kidney transplant group had 241 (79.0%) subjects with the GG (*3/*3) non-expresser genotype while in the SPK cohort this was 36 (75.0%), 218 (86.5%) in the liver transplant cohort and 325 (84.4%) of the organ donor group. There were 4218 (86.1%) of the Generation Scotland cohort who had the GG (*3/*3) genotype of CYP3A5.

The GA (*3/*1) genotype of CYP3A5, which does result in functional CYP3A5 activity, was seen in 48 (15.7%) of the kidney transplant cohort, 9 (18.8%) of the SPK cohort, 27 (10.7%) of the liver transplant patients and 47 (12.2%) of

the organ donors. There was no significant difference among any of the transplant groups in the expression of this genotype or the Generation Scotland cohort where 570 (11.6%) expressed the GA (*3/*1) genotype (Chi-squared test).

The AA (*1/*1) genotype of CYP3A5 was expressed in 9 (3.0%) of the kidney transplant patients, 1 (2.1%) of the SPK cohort, 2 (0.8%) of the liver transplant patients and was not found in any of the liver donors. The Generation Scotland cohort had only 16 (0.3%) subjects with the AA genotype which was significantly lower than that of the transplant cohort ($p>0.001$, Chi-Squared test).

3.5.2 ABCB1

The distribution of the ABCB1 genotype was broadly similar in the transplant cohort with 63 (20.7%) of the kidney transplant patients, 9 (18.8%) of the SPK cohort, 59 (23.4%) of the liver transplant group and 77 (20.0%) of the organ donors all expressing the CC genotype. This compared with 974 (19.9%) of the Generation Scotland group who had the CC genotype and was not found to be significantly different on Chi-Squared test.

The distribution of the CT genotype of ABCB1 was also found to be similar between the transplant groups - kidney 143 (46.9%), SPK 24 (50.0%), liver 108 (42.9%) and organ donor 187 (48.6%). This was again found to be comparable to the Generation Scotland cohort of 2334 (47.6%) who expressed the CT genotype.

The distribution of the TT genotype of ABCB1 was unsurprisingly similar between the transplant groups – kidney 97 (31.8%), SPK 15 (31.3%), liver 76 (30.2%) and organ donor 96 (24.9%). There was no significant difference between the distribution of the TT genotype among the transplant groups or compared with the Generation Scotland cohort where there were 1417 (28.9%) subjects of the TT genotype.

*3.5.3 CYP3A4*22*

The T allele is so infrequently expressed that demonstrating a statistical difference between the transplant groups would be difficult and, indeed, there is no significant difference in the percentage of individuals with any given CYP3A4*22 genotype across the different transplant groups. The most common CC genotype is found in 276 (90.5%) of the kidney transplant patients, 44 (91.7%) of the SPK patients, 225 (89.3%) of the liver transplant

cohort and 327 (84.9%) of the organ donor cohort. This is comparable with the Generation Scotland cohort where 4313 (88.0%) express the CC genotype of CYP3A4*22.

The CT genotype is seen in 25 (8.2%) of the kidney transplant patients, 3 (6.3%) of the SPK patients, 16 (6.3%) of the liver transplant patients and 38 (9.9%) of the organ donors which was again comparable with the Generation Scotland cohort expression of the CT genotype, 472 (9.9%) subjects.

The TT genotype of CYP3A4*22 is very rarely expressed and was found only in 2 (0.7%) of the kidney transplant patients and only 1 (0.3%) of the organ donors. None of the SPK patients or the liver transplant patients expressed the TT genotype of CYP3A4*22. The Generation Scotland cohort also had a very low incidence of this genotype with only 11 (0.2%) subjects expressing it.

Table 3.4 below summarises the genotype distribution of the different transplant groups as well as the Generation Scotland cohort for the 3 different SNPs.

Table 3.4 Genotype distribution of CYP3A5, ABCB1 and CYP3A4*22 between the different cohorts of Generation Scotland subjects and the sub-groups of the transplant subjects.

	CYP3A5			ABCB1			CYP3A4*22		
	GG (*/3*3)	**GA (*3/*1)**	**AA (*1/*1)**	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**
GENERATION SCOTLAND	4218 (86.1%)	570 (11.6%)	16 (0.3%)	974 (19.9%)	2334 (47.6%)	1417 (28.9%)	4313 (88.0%)	472 (9.6%)	11 (0.2%)
KIDNEY TRANSPLANT	241 (79.0%)	48 (15.7%)	9 (3.0%)	63 (20.7%)	143 (46.9%)	97 (31.8%)	276 (90.5%)	25 (8.2%)	2 (0.7%)
SPK TRANSPLANT	36 (75.0%)	9 (18.8%)	1 (2.1%)	9 (18.8%)	24 (50.0%)	15 (31.3%)	44 (91.7%)	3 (6.3%)	0 (0.0%)
LIVER TRANSPLANT	218 (86.5%)	27 (10.7%)	2 (0.8%)	59 (23.4%)	108 (42.9%)	76 (30.2%)	225 (89.3%)	16 (6.3%)	0 (0.0%)
ORGAN DONOR	325 (84.4%)	47 (12.2%)	0 (0.0%)	77 (20.0%)	187 (48.6%)	96 (24.9%)	327 (84.9%)	38 (9.9%)	1 (0.3%)
TOTAL	5038 (85.5%)	701 (11.9%)	28 (0.5%)	1182 (20.1%)	2796 (47.5%)	1701 (28.9%)	5185 (88.0%)	554 (9.4%)	14 (0.2%)

3.6 Allele Frequencies

3.6.1 CYP3A5 allele frequencies

It is also possible to describe the distribution of alleles of a particular gene in a population by the allele frequency. The genotype could not be determined for 122 subjects (2.1%) and in order to express the allele frequency accurately these patients were removed from the analysis leaving 5767 individuals for whom the frequency can be calculated. Taking the single nucleotide polymorphism of CYP3A5 as an example, there are 2 alleles (G or A) but a total of 3 possible genotypes (GG, GA or AA). Looking at the overall cohort we can see that 5038 individuals (85.5%) express the GG (*3/*3) genotype, 701 (11.9%) express the GA (*3/*1) genotype and 28 (0.5%) express the AA (*1/*1) genotype. The total number of alleles in this cohort is (5767 x 2) = 11534. Of these there are 10777 G alleles and 757 A alleles, giving a G allele frequency of 0.934 and an A allele frequency of 0.066.
The allele frequency for the 4910 White – Scottish ethnic group individuals with a determined genotype is 0.936 for the G allele of CYP3A5 and 0.064 for the A allele frequency. The allele frequency for the 575 White – other British ethnic group individuals was comparable with a G allele frequency of 0.934 and an A allele frequency of 0.066, suggesting that the White –

Scottish population is similar to the rest of the Caucasian population of the UK. Although the other ethnic groups are less well represented, the largest non-Caucasian group [Asian – Indian (n=19)] had a significantly different allele frequency. In this group the G allele frequency was 0.684 and the A allele frequency was 0.316. Despite the small numbers in this study, this frequency is very similar to previously published work in Indian populations where the G allele frequency has been shown to be between 0.635 and 0.683 [194, 195].

3.6.2 ABCB1 allele frequencies

The allele frequencies of ABCB1 are quite different from the cytochrome p450 3A5 and 3A4*22 SNPs, as reflected in the distribution of the different genotypes already discussed in this chapter. The ABCB1 genotype could not be determined for 210 individuals who were excluded from the allele frequency calculations. This gives a total of 5679 specimens for analysis and a total of 11358 alleles (either C or T). There were 5160 C alleles giving a C allele frequency of 0.454 and 6198 T alleles giving a T allele frequency of 0.546 for the entire cohort.

The C allele frequency of the 4836 White – Scottish ethnic group was 0.456 and the T allele frequency was 0.544. The 567 White – other British

individuals had a C allele frequency of 0.446 and a T allele frequency of 0.554, comparable to that of the White – Scottish population. Interestingly the allele frequency seen in the White – Irish ethnic group is different with a C allele frequency of 0.292 and a T allele frequency of 0.708. As there are only 48 individuals in this group, this may not be a true representation of the Irish population, but nevertheless it is an interesting observation worthy of further evaluation in a larger sample. Again, the other ethnic groups are very small in number but the distribution in the largest of the non-Caucasian ethnic shows that the C allele frequency of the Asian – Indian ethnic group was 0.444 whilst the T allele frequency was 0.556, similar to the White – Scottish and the White – other British ethnic groups.

*3.6.3 CYP3A4*22 allele frequencies*

CYP3A4*22 is a more recently discovered polymorphism and as such there are few studies investigating population distribution of the CYP3A4*22 genotype. The allele frequency of the T allele appears to be very low in Caucasian populations that have been studied so far [175, 179, 180, 193]. In the overall cohort there were 5753 subjects with determined genotypes suitable for analysis. The C allele frequency of the cohort as a whole was 0.949 and the T allele frequency 0.051.

In the White - Scottish ethnic group of the cohort, again the values are comparable with a C allele frequency of 0.950 and a T allele frequency of 0.050. Similarly in the White – other British ethnic group the C allele frequency is 0.942 and the T allele frequency is 0.058. The White – Irish ethnic group is very similar for the other Caucasian groups in terms of the allele frequency and has a C allele frequency of 0.958 and a T allele frequency of 0.042.

The Asian – Indian ethnic group had no individuals who expressed a T allele in this study giving it a C allele frequency of 1.000. It is unknown if this is a true representation of this ethnic group, but does suggest that the T allele frequency is either very low or possibly absent in this ethnic group. Further evaluation of this is required in order to draw reasonable conclusions.

The allele frequencies of the different Caucasian groups in this study are summarised in Table 3.5 below and also include the allele frequencies for the small number of Asian – Indian subjects in this study.

Table 3.5 Allele frequencies of CYP3A5, ABCB1 and CYP3A4*22 in different ethnic groups across all subjects within this study.

	CYP3A5		**ABCB1**		**CYP3A4*22**	
	G (*3)	A (*1)	C	T	C	T
Overall	0.934	0.066	0.454	0.546	0.949	0.051
White - Scottish	0.936	0.064	0.456	0.544	0.950	0.050
White - Other British	0.934	0.066	0.446	0.554	0.942	0.058
White - Irish	0.935	0.065	0.292	0.708	0.958	0.042
White - Other	0.920	0.080	0.471	0.529	0.951	0.049
Asian - Indian	0.684	0.316	0.444	0.556	1.000	0.000

3.7 Allele Frequencies of other Populations

This study has revealed the allele frequencies of a large sample of almost exclusively Caucasian Scottish individuals however it is worth exploring the allele frequencies of other populations as a comparison. *Kurose et al* published a comprehensive review in 2012 of the allele frequencies of numerous different genes involved in drug metabolism across several different countries [153]. Table 3.6 below summarises those allele frequencies.

The table is grouped by continent and demonstrates the similarities between neighbouring regions where the ethnic groups will be similar such as Japan, Korea and China. Among these 3 countries the CYP3A5 G allele frequencies are similar, 0.762, 0.759, 0.737 respectively but quite different from the majority of the European countries where the G allele frequencies are in the region of 0.9. The Singaporean distribution is interesting in that the Malay, Chinese and Indian Singaporean ethnic sub-groups have different G allele frequencies of CYP3A5 at 0.61, 0.76 and 0.59 respectively.

The African countries report much lower G allele frequencies of CYP3A5 as might be expected with South-Africa and Ethiopia having very G allele frequencies of 0.145 and 0.205 respectively. Interestingly Zimbabwe and Tanzania have a higher G allele frequency reported than might be expected at 0.776 and 0.640 respectively. African-Americans are reported as having an G allele frequency of 0.357.

Across European countries which all have predominantly Caucasian populations the allele frequency of CYP3A5 still tends to be in the region of 0.9 with some minor variation among different countries. Interestingly, there are only minor differences between Scandinavian, central European, Eastern European or Mediterranean countries. Portugal is the only

European country where the G allele frequency of CYP3A5 drops below 0.9, at 0.875.

The allele frequencies of ABCB1 shown in table 3.6 below also show some variation among the different countries with African countries tending to have a lower T allele frequency in the region of 0.1 – 0.2 compared to both Caucasian populations and indeed Asian populations where the T allele frequency of ABCB1 is between 0.4 and 0.6.

The minor allele frequency (T allele) of CYP3A4*22 is only shown for a few countries in table 3.6 below as it has only been discovered more recently and there are few publications where it has been studied. Scotland has an almost identical allele frequency to France for CYP3A4*22, at 0.051 and 0.050 respectively. Data from the Netherlands reports an allele frequency slightly higher at 0.08 and Greece higher still at 0.1. Studies from America however reveal a lower allele frequency at 0.04 and lower still for African Americans at 0.01. There are 2 papers where Chinese subjects have been genotyped for CYP3A4*22 and both found no expression of the T allele [193, 196].

Table 3.6 Variations in allele frequencies of CYP3A5, ABCB1, CYP3A4*22 reported in different countries.

			CYP3A5 (G/*3 allele)	ABCB1 (T allele)	CYP3A4*22 (T allele)
Country	*Asia*	Japan	0.762	0.43	no data
		Korea	0.759	0.431	no data
		China	0.737	0.44	0
		Thailand	0.669	no data	no data
		Singapore (Malay)	0.61	0.52	no data
		Singapore (Chinese)	0.76	0.54	no data
		Singapore (Indian)	0.59	0.62	no data
		India	0.648	0.6	no data
	Europe	**Scotland, UK**	**0.934**	**0.546**	**0.051**
		Denmark	0.948	no data	no data
		Finland	0.91	0.37	no data
		Sweden	0.919	0.53	no data
		UK	0.926	0.42	no data
		Germany	0.942	0.48	no data
		Netherlands	0.917	no data	0.08
		France	0.948	0.43	0.05
		Greece	0.944	0.435	0.1
		Italy	0.933	0.4	no data
		Portugal	0.875	0.57	no data
		Spain	0.921	0.48	no data
		Bosnia-Herzegovina	0.932	no data	no data
		Russia	0.916	0.54	no data
		Poland	0.94	0.38	no data
	Africans	South Africa	0.145	0.11	no data
		African-Americans	0.357	0.16	0.01
		Zimbabwe	0.776	no data	no data
		Ghana	no data	0.17	no data
		Tanzania	0.64	0.22	no data
		Ethiopia	0.205	0.16	no data
	America	USA	no data	no data	0.04

3.8 Chapter Summary

The single nucleotide polymorphisms evaluated in this thesis are involved in drug metabolism and can influence the pharmacokinetics of tacrolimus, the most common calcineurin inhibitor used to prevent allograft rejection in solid organ transplantation. It is known that expression of different SNPs of a particular gene will vary depending on the population studied and the greatest difference in genotype expression and/or allele frequency is seen between different ethnic groups. To date there has been no study examining the genotype distribution of the three SNPs in a Scottish population.

The Generation Scotland cohort is a large cohort but is, interestingly, somewhat different in its ethnic make-up compared with both Scottish and UK census data from 2011. Ethnic minorities in the Generation Scotland cohort are significantly under represented when compared with this census data. The reason for this almost certainly lies with the source of DNA used for analysis. The Generation Scotland 3D study acquired its DNA from the white cell filters used during the blood donation process and it is well known that there are difficulties in recruiting ethnic minorities to donate blood, which may reflect differences in cultural practices, and as such are likely to lead to under-representation in this cohort. As one might anticipate, there are a greater number of Asian subjects in the renal transplant group

compared with the other cohorts, as a reflection of a higher incidence of renal failure in this ethnic group.

Scotland has overall a fairly homogenous Caucasian population with the vast majority described as White – Scottish. Therefore, despite the under-representation of the small ethnic minority population, Generation Scotland cohort gives us a large sample of people to evaluate. The White – Scottish population has a very similar genotype expression for the SNPs of CYP3A5, ABCB1 and CYP3A4*22 to the White – other British ethnic group. This is in keeping with other previously studied Caucasian populations for these 3 SNPs (allowing for the fact that CYP3A4*22 has only recently been discovered and published data on the expression of this SNP in different ethnic groups is very limited). The White – Irish ethnic group in this study have similar distributions of the CYP3A5 and CYP3A4*22 SNPs to other Caucasian groups however, interestingly, ABCB1 shows a slightly different genotype expression with greater numbers of TT genotype expression and fewer CC genotype expression compared to other Caucasian groups. There is no obvious explanation for this phenomenon, particularly when the cytochrome P450 SNPs are consistent with the other Caucasian groups reported elsewhere.

The low numbers of ethnic minority groups throughout this study make any meaningful evaluation of the genotype expression of CYP3A5, ABCB1 and CYP3A4*22 challenging and one can, at best, note that in the Asian ethnic groups there is greater expression of the A (*1) allele in CYP3A5, in keeping with other studies. ABCB1 expression is similar to Caucasian groups and for CYP3A4*22 all non-Caucasian subjects are of the CC genotype, the significance of which is unknown.

In conclusion this study shows that the White – Scottish population has a similar distribution of genotypes for CYP3A5, ABCB1 and CYP3A4*22 SNPs to the White – other British (representing the rest of the UK) and this is in keeping with previously studied Caucasian populations.

Chapter IV

Pharmacogenomics and Liver Transplantation

4 PHARMACOGENOMICS AND LIVER TRANSPLANTION

4.1 Background

Tacrolimus is the most commonly used immunosuppressant to prevent allograft rejection in patients who undergo liver transplantation. As with kidney transplantation, the narrow therapeutic window of tacrolimus requires therapeutic drug monitoring to ensure whole blood tacrolimus trough levels (C_0) remain within the target range in order to prevent allograft rejection but also to ensure the concentrations do not become supra-therapeutic, increasing the risk of toxicity and significant adverse events. As with renal transplant recipients, there is significant variation in dose requirements between individuals and this is, to some extent, attributable to the polymorphisms that encode key genes involved in drug metabolism [197]. Genetic polymorphisms which have been most reliably shown to influence the absorption and metabolism of tacrolimus include ABCB1 and CYP3A5 with more recently CYP3A4*22 being described. The SNP which has been shown to have the most significant impact on tacrolimus pharmacokinetics is CYP3A5 A6986G and individuals who carry the CYP3A5*1 allele and therefore make the functional protein require, on

average, double the dose of tacrolimus in order to achieve therapeutic levels [111].

Most studies to date exploring the relationship between these genetic polymorphisms and tacrolimus pharmacokinetics have been in renal transplant recipients where the impact of CYP3A5 expression, in particular, is now well established. However, liver transplant patients are especially interesting because CYP3A5 is predominantly expressed in the liver tissue. As we hypothesise that CYP3A5 is the dominant factor in tacrolimus metabolism (as it is the case for renal transplantation), and given that patients receive a new liver, this raises an obvious question: Is it the recipient or the donor genotype of CYP3A5 which has the most significant impact on tacrolimus pharmacokinetics in liver transplant patients?

4.1.1 Current literature

A paper by Shi et al in 2013 performed a genotype analysis of the 3 SNPs of interest in this study, CYP3A5 A6986G, CYP3A4*22 and ABCB1 C3435T in a cohort of 216 liver transplant recipients in Asia. The liver donors were not genotyped. They did not find a single recipient that expressed the CYP3A4*22 polymorphism and to date there are no studies that have shown

expression of the CYP3A4*22 polymorphism in Asian populations. This study did find that individuals who expressed the CYP3A5*1 allele had higher dose requirements than those of the *3/*3 genotype (non-expressers) similar to what has been previously observed in renal transplant patients [193]. An earlier paper by *Yu et al* in 2006 had found that while both liver donors and recipients who expressed CYP3A5*1 had higher dose requirements and lower tacrolimus trough concentration divided by dose (C_0/D) levels, the donor genotype appeared to have a greater influence [182]. A Korean study in 2012 found that the recipient expression (gut expression) of CYP3A5 was the most significant factor in tacrolimus C_0/D requirements in the first month. However after the first month, the donor genotype took over as the most influential factor in tacrolimus metabolism, a donor expression of CYP3A5 resulting in persistently lower C_0/D levels up to 48 months after transplant [198]. In 2011, *Muraki et al* found very similar results, the recipient CYP3A5 expression having a more significant role in tacrolimus pharmacokinetics in the early post transplant period, whilst the donor genotype played the most important role in the later post-transplant period [199]. This study also found an increase in post transplant infectious complications when both recipient and donor were CYP3A5 expressers. There have been further studies, primarily in Asian subjects, which have supported the view that both recipient and donor genotype influence the pharmacokinetics of tacrolimus in liver transplant patients [200]. While

CYP3A5 expression in both liver recipients and donors may have an influential role in tacrolimus pharmacokinetics there is no convincing evidence that the ABCB1 C3435T polymorphism has any significant impact. Furthermore, there are no published studies to date suggesting a role for the CYP3A4*22 SNP. While evidence regarding the role of recipient and donor genotype influence of CYP3A5 on tacrolimus pharmacokinetics is becoming more consistent, the clinical impact is far from certain. Some studies report no impact on rejection or renal function, whilst others suggest that CYP3A5 expression increases the risk of acute rejection, renal injury or sepsis.

4.1.2 Study aims

The aim of this chapter was to examine the impact of donor and recipient SNPs of CYP3A5, CYP3A4*22 and ABCB1 in a cohort of 194 liver transplant patients and assess their correlation with tacrolimus pharmacokinetics and clinical outcomes. Tacrolimus dose requirements, C_0 levels, C_0/D ratios and time until therapeutic C_0 level reached were evaluated for both donor and recipients. Additionally, clinical outcomes of biopsy proven acute rejection, renal function, graft survival, patient survival and biochemical liver function were investigated.

4.2 Chapter Methodology

194 liver transplants were included in this study. Stored frozen DNA samples were available for 137 liver recipients and 191 donors. DNA samples were genotyped for the CYP3A5 A6986G transition (rs776746), the ABCB1 C3435T transition (rs1045642) and the CYP3A4*22 C15389T transition (rs35599367) using the Taqman® genotyping assay and real-time polymerase chain reaction (RT-PCR) technique as described in the materials and laboratory methodology chapter. Clinical data was collected from a variety of sources including medical case notes, electronic medical records, donor information records and the unit's prospectively collected database. Genotype data was assigned study numbers with no patient identifiable information and recorded and stored separately from the clinical data. Clinical data was collected using patient identifiable information and once the dataset was complete, it was anonymised and linked with the genotype data using the unique study number. The resulting dataset had no patient identifiers and included all of the clinical data as well as the recipient and donor genotypes.

Clinical data collected were recipient demographics such as gender, age, ethnic group, height, weight, BMI, liver disease diagnosis, medical comorbidities, blood group, MELD score at listing, type of liver disease (cirrhosis/malignancy/fulminant), number of transplant, and virology status

(HCV/HBV/HIV/CMV). Donor demographics included gender, age, ethnic group, height, weight, BMI, blood group, donor type (DBD/DCD/LIVE), cause of death, region of donation, comorbidities, liver function tests and virology status (HCV/HBV/HIV/CMV). Additional information included the cold ischaemic time (CIT), graft steatosis and whether it was a whole or a split graft.

Tacrolimus dose and trough levels (C_0) were collected at a total of 14 time points: 3 time points in the first week, 3 time points in the second week, 3 time points in the third week, 2 time points in the 4^{th} week, 3 month time point, 6 month time point and 12 month time point. The time to reach the therapeutic C_0 tacrolimus level was recorded. Biopsy proven acute rejection episodes, the development of significant post operative complications and graft survival and patient survival were collated.

4.3 Chapter Results

4.3.1 Recipient Demographics

194 patients transplanted between 17-Jan-2007 and 23-Aug-2012 in the Scottish Liver Transplant Unit at the Royal Infirmary of Edinburgh were included in this study [89 female recipients (45.9%) and 105 male recipients (54.1%)]. The majority of the recipients were Caucasian (186, 95.9%) but there were 7 Asian recipients (3.6%) and 1 black recipient (0.5%). The mean age of the liver recipients was 53.39±11.82 years (range 18 – 72 years). There were 175 (90.2%) recipients who received their first liver transplant, 16 (8.2%) patients had their 2nd liver transplant whilst 3 (1.5%) patients received their 3rd liver transplant. The mean recipient weight was 77.7±20.29 kg (39.0 – 177.0 kg) with a mean recipient body mass index of 26.83±5.04 (16.38 – 41.18).

4.3.2 Indication for transplant

Cirrhosis secondary to alcoholic liver disease was the most common pathology requiring liver transplantation [51 patients (26.3%)] followed by cirrhosis secondary to hepatitis C (HCV) infection in 33 (17.0%) patients and

primary biliary cirrhosis (PBC) in 26 patients (13.4%). 15 patients (7.7%) required liver transplantation for cirrhosis secondary to primary sclerosing cholangitis (PSC). The most common cause of fulminant liver failure was paracetamol overdose which accounted for 9 patients (4.6%) requiring liver transplantation.

While the majority of the patients had a single pathological diagnosis resulting in their liver failure, there were 83 (42.8%) patients with a secondary pathology, the most common being hepatocellular carcinoma [47 patients (24.2%)]. Other secondary diagnoses include alcoholic liver disease (7 patients, 3.6%), autoimmune hepatitis (5 patients, 2.6%), primary sclerosing cholangitis (8 patients, 4.1%), hepatopulmonary syndrome (4 patients, 2.1%) and non-alcoholic fatty liver disease (4 patients, 2.1%). The full list of primary and secondary diagnoses are listed in Table 4.1 below.

Table 4.1 Primary and secondary diagnoses of patients undergoing liver transplantation in this study group.

		Number	Percentage
PRIMARY DIAGNOSIS			
	Alcoholic Liver Disease	51	26.30%
	Hepatitis C	33	17.00%
	Primary Biliary Cirrhosis	26	13.40%
	Primary Sclerosing Cholangitis	15	7.70%
	Non-Alcoholic Fatty Liver Disease	14	7.20%
	Cryptogenic	11	5.70%
	Paracetamol Overdose	9	4.60%
	Hepatic Artery Thrombosis	7	3.60%
	Autoimmune Hepatitis	6	3.10%
	Primary Non Function	5	2.60%
	Chronic Rejection	5	2.60%
	Haemachromatosis	3	1.50%
	Polycystic Liver Disease	2	1.00%
	Adult Idiopathic Ductopenia	1	0.50%
	Cholangiopathy	1	0.50%
	Giant Cell Hepatitis	1	0.50%
	Granulomatous Hepatitis	1	0.50%
	Hepatitis B	1	0.50%
	Liver Failure Post Resection	1	0.50%
	Non A-E Hepatitis	1	0.50%
	TOTAL	194	100%
SECONDARY DIAGNOSIS			
	None	111	57.20%
	Hepatocellular Carcinoma	47	24.20%
	Primary Sclerosing Cholangitis	8	4.10%
	Alcoholic Liver Disease	7	3.60%
	Autoimmune Hepatitis	5	2.60%
	Hepatopulmonary Syndrome	4	2.10%
	Non Alcoholic Fatty Liver Disease	4	2.10%
	Haemachromatosis	2	1.00%
	Agille's Syndrome	1	0.50%
	Biliary Atresia	1	0.50%
	Cryptogenic	1	0.50%
	Drug Induced Liver Disease	1	0.50%
	Primary Biliary Cirrhosis	1	0.50%
	Budd Chiari Syndrome	1	0.50%
	TOTAL	194	100%

4.3.3 Transplant Categories

Patients who undergo a liver transplant fall into two categories: fulminant hepatic failure and chronic liver failure. In this study, 14 patients (7.2%) were transplanted for acute fulminant liver failure, 9 patients (4.6%) as urgent re-transplants whilst 155 patients (79.9%) were transplanted from the chronic liver failure waiting list in accordance with nationally agreed guidelines. The mean MELD score for the liver recipients at the time of being placed on the waiting list was 19.39±7.68.

4.3.4 Donor Demographics

194 liver donors were included in this study of which 176 (90.7%) had donated following brain stem death (DBD), 16 (8.2%) who donated after circulatory death (DCD) and 2 live donor transplants (1.0%). 99 donors were male (51.0%) and 95 female (49.0%). Most were of Caucasian ethnicity [175 (90.2%)] whilst 2 (1.0%) were Asian and 17 (8.8%) did not have the ethnic group recorded. The mean age of the liver donors was 46.87±16.86 years with a mean height of 169.93±12.71 cm (71.00 – 193.00cm) and a mean weight of 75.33±16.16 kg (39.00 – 177.00 kg). The calculated mean donor body mass index (BMI) was 25.70±4.41 (16.38 – 41.18).

4.3.5 Type of Liver Allograft

173 whole liver grafts (89.2%) and 21 split liver grafts (10.8%) were transplanted. The livers are assessed visually for steatosis at the time of retrieval and 128 liver allografts had no features of steatosis (66.0%), whilst all the other had varying degrees of steatosis [24 livers (12.4%) - mild steatosis; 36 livers (18.6%) - moderate steatosis and 6 livers (3.1%) - severe steatosis].

The mean cold ischaemic time was 09:07±02:10 (hh:mm) with a range of 02:54 – 15:51. The mean second warm ischaemic time (liver out of ice until reperfusion) was 36±7 minutes with a range of 13 – 62 minutes.

The full set of donor demographics is shown in Table 4.2 below.

Table 4.2 Donor demographics of the liver transplant cohort

Donor Characteristics		Value	
Donor Type	DBD	176	90.70%
	DCD	16	8.20%
	LDLT	2	1.00%
Gender	Male	99	51.00%
	Female	95	49.00%
Ethnicity	Caucasian	175	90.20%
	Asian	2	1.00%
	Unknown	17	8.80%
Cause of Death	CVA	101	52.10%
	Anoxia	13	6.70%
	Trauma	27	13.90%
	Other	8	4.10%
	Live Donor	2	1.00%
	Unknown	43	22.20%
Virology	CMV +	85	43.80%
	CMV -	101	52.10%
	Unknown	8	4.10%
	HCV +	1	0.50%
	HCV -	191	98.50%
	Unknown	2	1.00%
	HBV +	0	0.00%
	HBV -	192	99.00%
	Unknown	2	1.00%
	HIV +	0	0.00%
	HIV -	192	99.00%
	Unknown	2	1.00%
Comorbidities	Hypertension	30	15.50%
	Diabetes	7	3.60%
	Cardiovascular Disease	10	5.20%
	Obesity	21	10.80%
Region	Local	28	14.40%
	Regional	57	29.40%
	National	98	50.50%
	Unknown	11	5.70%
Blood Group	O	107	55.20%
	A	64	33.00%
	B	16	8.20%
	AB	7	3.60%
Demographics	Age (Years)	46.87±16.86	11 - 81
	Weight (kg)	75.33±16.16	47 - 180
	BMI	25.70±4.41	18.30 - 49.12

4.3.6 Cytochrome P450 3A5 (CYP3A5) Expression

Of the 194 recipients in this study there were 136 valid samples available for analysis. 58 samples had no genotype data available because there was no stored DNA available or, occasionally the assay failed to determine the genotype.

119 (87.5%) liver transplant recipients were GG homozygotes (*3/*3), 16 (11.8%) were GA heterozygotes (*3/*1) and 1(0.7%) patient was an AA homozygote (*1/*1). Individuals with either the *3/*1 or the *1/*1 genotype make functional CYP3A5 and were therefore termed CYP3A5 expressers (n=17). *3/*3 genotype does not make any functional CYP3A5 and therefore these recipients are termed CYP3A5 non-expressers.

The liver donors were also genotyped and there were 181 valid samples for analysis. 161 donors (89.0%) were GG homozygotes (*3/*3, CYP3A5 non-expressers), 19 (10.5%) were GA heterozygotes (*3/*1, CYP3A5 expressers) and 1 (0.5%) who was AA homozygote (*1/*1, CYP3A5 expresser).

It was possible to obtain a full set of genotype data (recipient and donor) for 124 patients (63.9%) and then group them according to both recipient and donor expression of CYP3A5. Recipients were labelled expressers or non-

expressers (RE or RnE) and similarly donors were labelled as expressers or non-expressers (DE or DnE). In 96 cases (49.5%) neither the donor nor the recipient (RnE/DnE) expressed CYP3A5 (both had CYP3A5 *3/*3 genotype). In 13 (6.7%) cases the recipient expressed CYP3A5 whilst the donor did not (RE/DnE). Similarly, there were 13 (6.7%) combinations where the recipient did not express CYP3A5 but the donor did (RnE/DE). Finally, there were 2 patients (1.0%) where both the donor and the recipient were expressers of CYP3A5 (RE/DE).

4.3.7 Recipient CYP3A5 and tacrolimus dose requirements

By the end of the first week following transplantation (WK1-3 time point) liver transplant recipients who are CYP3A5 expressers required a significantly higher dose of tacrolimus (5.82±2.63 mg) compared with non-expressers of CYP3A5 (4.50±1.90 mg), p=0.015, one-way ANOVA. The dose remained significantly higher up to and including the 3 month time point with the exception of the third time point in the second week (WK 2-3) and the third time point in the third week (WK 3-3) where the difference in dose did not reach statistical significance. This is likely, in part, to be due to the smaller numbers of available data at these 2 time points compared with the rest, as only some liver transplant patients attended clinic appointments at

these time points. At 6 months and 12 months there was no significant difference in the prescribed doses of tacrolimus for the expressers and non-expressers of CYP3A5. This suggests, perhaps, that the impact of recipient CYP3A5 expression (which accounts primarily for gut expression) is less important after the early transplant period.

The tacrolimus dose requirements relative to recipient genotype are shown in Figure 4.1 below.

4.3.8 Donor CYP3A5 and tacrolimus dose requirements

Patients who receive a liver transplant from a donor who expresses CYP3A5 [*3/*1 or *1/*1 genotype] require significantly higher doses of tacrolimus in order to achieve therapeutic trough levels. As early as the second time point within the first week (WK1-2), patients transplanted with CYP3A5 expressers donor livers were prescribed significantly higher doses of tacrolimus (4.77±1.52 mg) compared with those patients with a non-expresser donor liver (3.94±1.52 mg), p=0.045, one-way ANOVA. The magnitude of the effect increased during the follow-up (Figure 4.1).

Patients who received a liver from a CYP3A5 expresser donor had significantly higher tacrolimus dose requirements at every time point from WK1-2 up to and including 12 months post-transplant, by which time they required approximately double the dose of tacrolimus (6.29±3.60 mg) compared to the liver transplant recipients of a CYP3A5 non-expresser donor (3.99±1.84 mg), $p<0.0001$, one-way ANOVA.

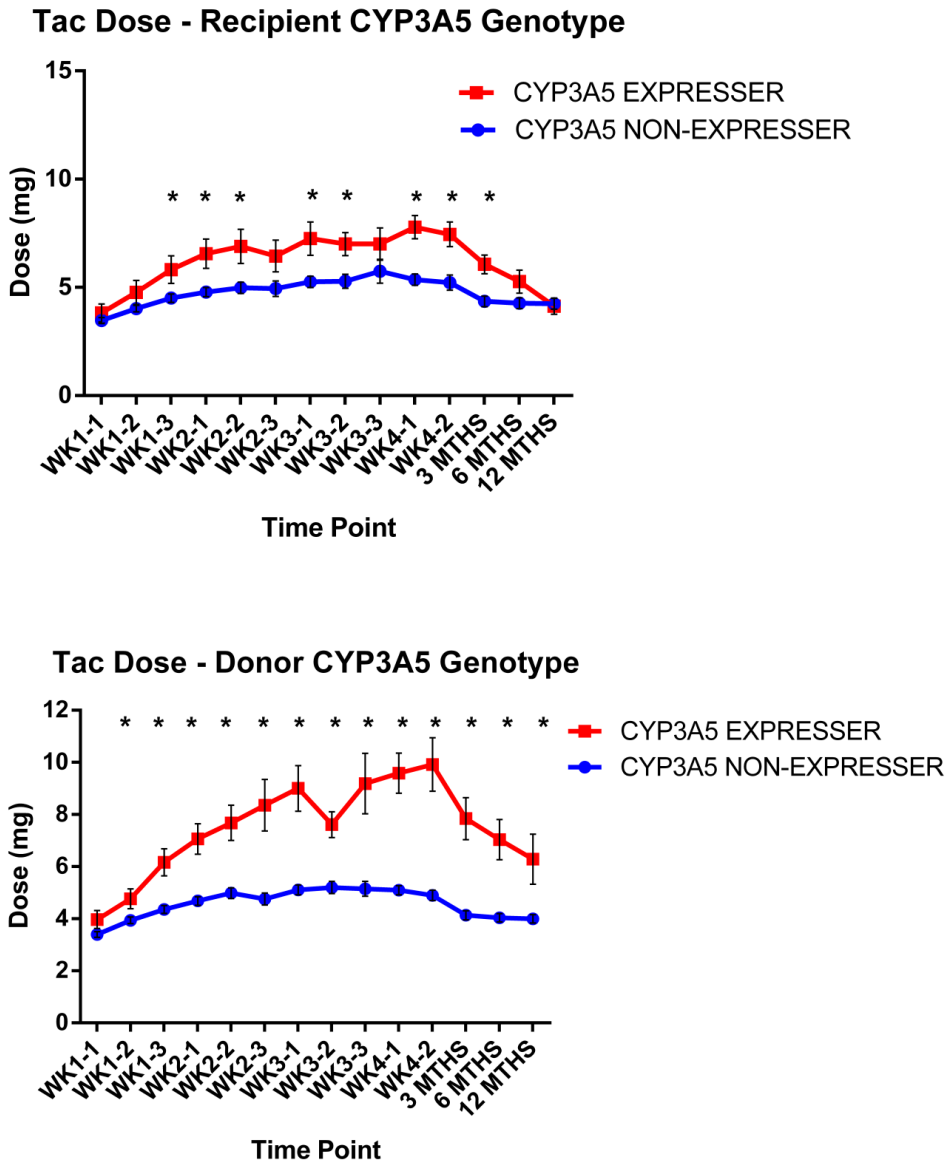


Figure 4.1 Tacrolimus dose requirements relative to CYP3A5 expression for both the donor genotype and recipient genotype at each time point. * signifies significant result $p<0.05$

4.3.9 Recipient CYP3A5 and Tacrolimus Trough Levels

The tacrolimus C_0 levels are lower in the recipient expressers of CYP3A5 up to the start of the second week (WK2-1), however these differences do not reach statistical significance. There is no significant difference between the C_0 levels at any of the subsequent time points between recipient CYP3A5 expressers and non-expressers.

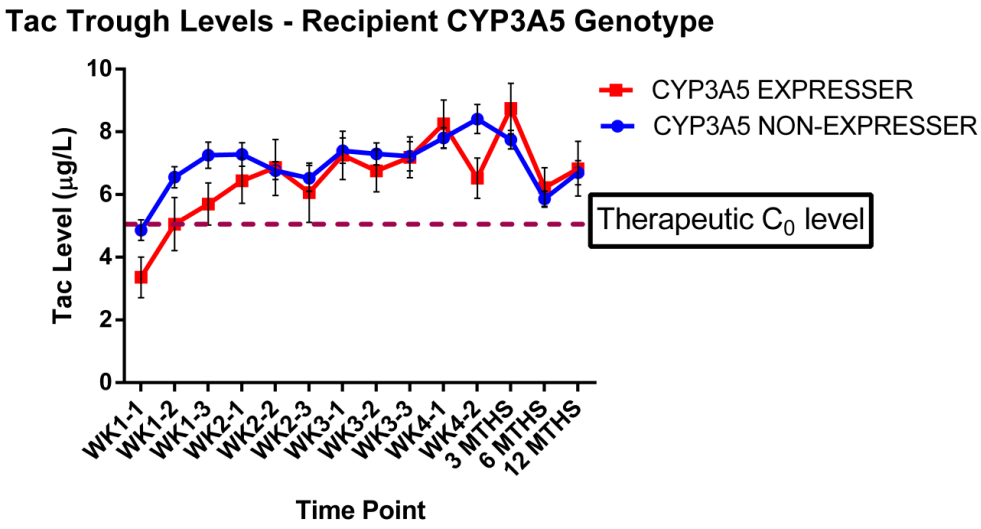
While the difference in the achieved C_0 levels does not differ significantly, it does appear that recipient CYP3A5 expressers do not achieve a therapeutic C_0 level at the first time point as shown in Figure 4.2 below. It is only at the 2nd time point (WK1-2), usually around day 5, that recipient CYP3A5 expressers breach the 5 µg/L therapeutic level.

4.3.10 Donor CYP3A5 and Tacrolimus Trough (C_0) Levels

Patients who received a liver transplant from a CYP3A5 expresser donor had a significantly lower tacrolimus trough (C_0) level (3.15±1.88 µg/L) immediately post transplantation (WK1-1) compared with those transplanted with a liver from a CYP3A5 non-expresser donor (4.96±3.66 µg/L), p=0.036, one-way ANOVA. Patients who received a liver from a

CYP3A5 expresser donor had persistently lower tacrolimus C_0 levels throughout the first week and at the beginning of the second week post transplantation (up to and including time point WK2-1). Furthermore, at time point WK2-3 patients transplanted with a CYP3A5 expresser liver had a significantly lower tacrolimus C_0 level (4.48±1.63 µg/L) compared with those transplanted with a non-expresser liver (6.69±3.18 µg/L), p=0.007, one-way ANOVA, and this was also seen at time point WK3-2 (5.18±1.43 vs 7.43±2.66 µg/L), p=0.003, one-way ANOVA.

This shows that individuals transplanted with a CYP3A5 expresser liver have significantly lower tacrolimus trough (C_0) levels and only after the dose is sequentially increased they achieve a therapeutic tacrolimus level. Figure 4.2 below demonstrates that the mean tacrolimus trough (C_0) level was below the 5 µg/L therapeutic threshold right up until the beginning of the second week (WK2-1) and remained lower than 6 µg/L up to three weeks, potentially exposing the patient to inadequate immunosuppression and an increased risk of acute rejection.



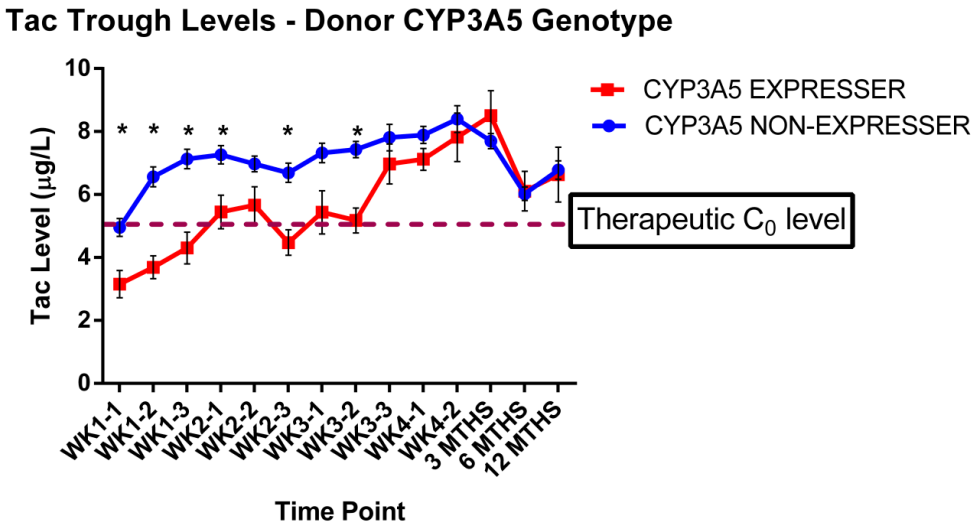


Figure 4.2 Tacrolimus trough concentration (C_0) relative to CYP3A5 expression for both donor and recipient genotype at each time point. * signifies significant result $p<0.05$

4.3.11 Dose Corrected Tac Levels relative to Recipient and Donor CYP3A5

Given that the tacrolimus trough (C_0) level is related to the dose administered then one could argue that these differences are a reflection of the different doses prescribed rather than anything else. As a means of trying to address this, the dose corrected tacrolimus level has been proposed as a measure of tacrolimus exposure. This is quite simply the tacrolimus level (C_0) divided by the dose (D) [C_0/D] and is also referred to as the 'dose-adjusted tacrolimus level', the concentration/dose ratio.

4.3.12 Recipient CYP3A5 and Dose Corrected Tacrolimus Level

Recipients who were CYP3A5 expressers had slightly lower dose corrected tacrolimus levels at the first time point (1.00±0.69 µg/L per mg) compared with CYP3A5 non-expressers (1.55±1.24 µg/L per mg) however this did not reach statistical significance, p=0.097, one-way ANOVA. Overall, the dose corrected tacrolimus level was lower at each time point for those recipients who were CYP3A5 expressers, but this only reached statistical significance at 2 time points [WK3-1 (0.98±0.35 vs 1.74±1.25µg/L per mg), p=0.027, one-way ANOVA and WK4-2 (0.92±0.34 vs 2.10±1.74 µg/L per mg), p=0.036, one-way

ANOVA]. The dose-corrected tacrolimus levels relative to recipient CYP3A5 expression are shown in Figure 4.3 below.

4.3.13 Donor CYP3A5 and Dose Corrected Tacrolimus Level

The initial dose corrected tacrolimus level at time point WK1-1 was significantly lower in patients with a CYP3A5 expresser liver (0.90±0.48 µg/L per mg) compared with patients transplanted with a non-expresser liver (1.55±1.17 µg/L per mg), p=0.031, one-way ANOVA. At every time point up to and including the 12 months time point, the dose corrected tacrolimus level (C_0/D) was significantly lower in patients with a donor CYP3A5 expresser liver when compared with patients who received a liver transplant from a CYP3A5 non-expresser liver. Figure 4.3 below shows the dose corrected tacrolimus level (C_0/D) at each time point relative to the CYP3A5 donor genotype.

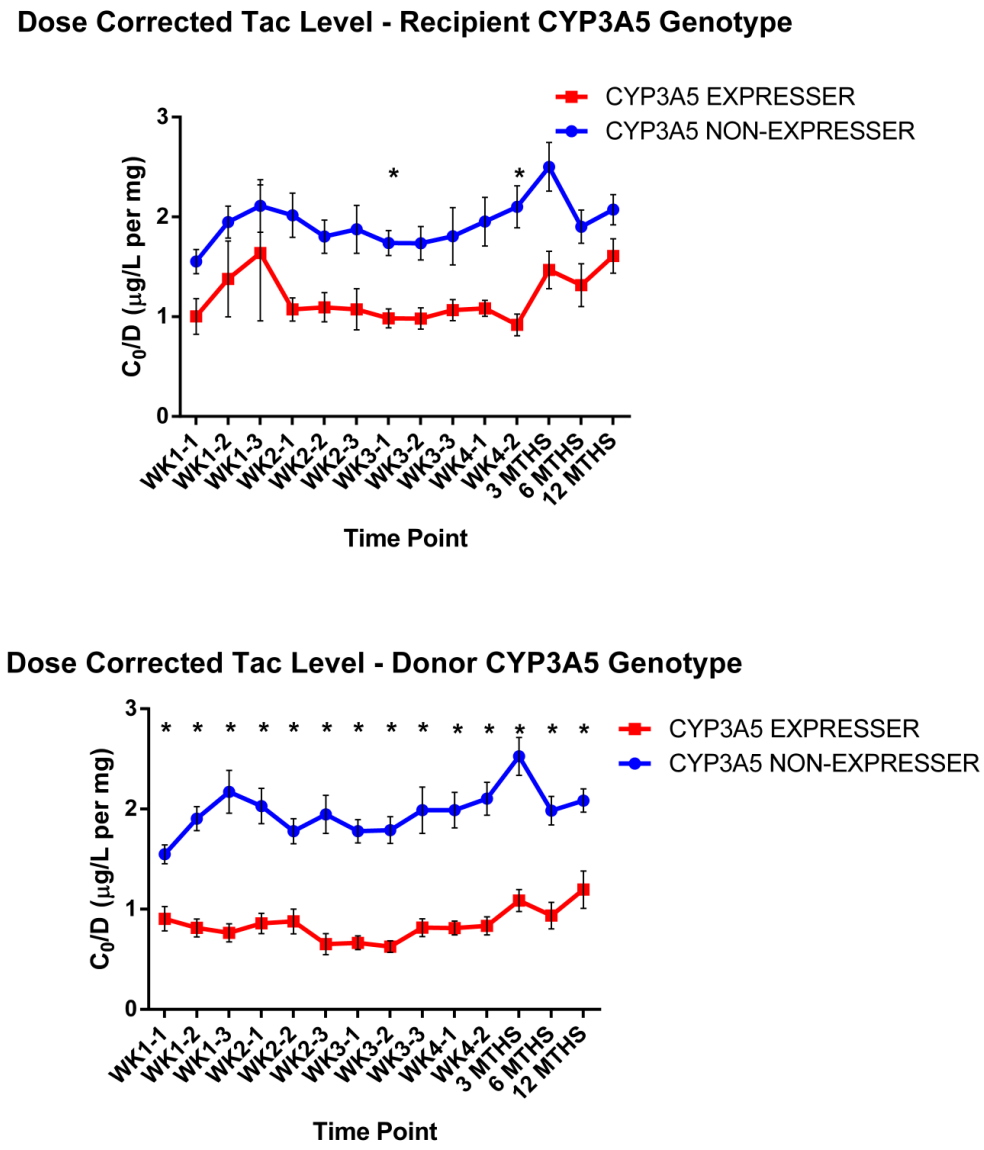


Figure 4.3 Dose corrected tacrolimus trough concentration (C_0/D) relative to CYP3A5 expression for both donor and recipient genotype at each time point. * signifies significant result $p<0.05$

4.3.14 Grouped Genotype Analysis

In order to evaluate whether the recipient or donor CYP3A5 genotype has the greater influence on tacrolimus pharmacokinetics and exposure, patients were grouped based on recipient and donor expression of CYP3A5. Four groups were defined:

- recipient and donor both non-expressers of CYP3A5 (RnE/DnE)
- recipient expresser and donor non-expresser (RE/DnE)
- recipient non-expresser and donor expresser (RnE/DE)
- both recipient and donor expressers of CYP3A5 (RE/DE).

4.3.15 Grouped CYP3A5 Genotype Analysis of Tacrolimus Dose

There were significant differences in the tacrolimus dose requirements between these 4 groups by the end of the first week (time point WK1-3) with the mean tacrolimus dose for the RnE/DnE group of 4.31±1.75 mg, 5.88±2.95 mg for the RE/DnE group, 6.30±2.25 mg for the RnE/DE group and 5.75±2.47 mg for the DE/RE group, p=0.004, one-way ANOVA.

This suggests that the DnE/RnE group require the lowest doses of tacrolimus, whilst the RnE/DE and the RE/DE groups require the highest

doses of tacrolimus in order to achieve therapeutic levels. Given that there are only 2 patients in the RE/DE group, the results from this group must be interpreted cautiously. From time point WK1-3 onwards, the doses of prescribed tacrolimus were significantly different between the 4 groups at each subsequent time point and up to and including 12 months.

Figure 4.4 below plots the mean dose at each time point for each of the genotype combinations of CYP3A5. All lines start at exactly the same place as all patients are given approximately the same starting dose of tacrolimus based on their weight but separate out and appear to support the previous findings that the donor liver genotype has a greater impact than the recipient genotype (both DE curves require the highest doses).

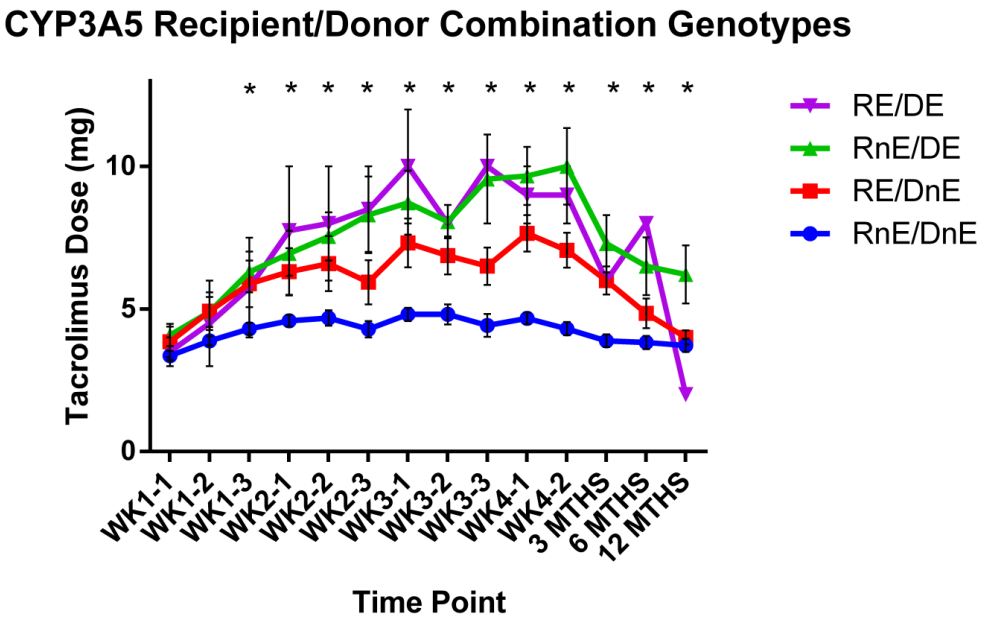


Figure 4.4 Tacrolimus dose requirements relative to CYP3A5 combination genotype of both the liver donor and recipient at each time point. * signifies significant result p<0.05 (RE = recipient expresser, RnE = recipient non-expresser, DE = donor expresser, DnE = donor non-expresser)

4.3.16 Grouped CYP3A5 Genotype of Tacrolimus Trough Levels

There was no significant difference between the tacrolimus trough levels of the 4 groups at any of the time points with the exception of WK1-2 where the mean tacrolimus trough level for the RnE/DnE group was 6.73±3.68 µg/L, for the RE/DnE group was 5.68±3.71 µg/L, for the RnE/DE group was 3.52±1.50 µg/L and for the RE/DE group was 3.35±0.78 µg/L, p=0.017, one-way ANOVA. While the statistical significance of between the groups in the early post transplant period is lacking, the graphical representation of the data in Figure 4.5 below suggests similar trends to previously described in

this chapter, where CYP3A5 expressers appear to have lower trough levels of tacrolimus. Donor expressers of CYP3A5, either in isolation or in combination with recipient expression, appear to have lower tacrolimus trough levels initially, albeit not statistically significant except at WK1-2.

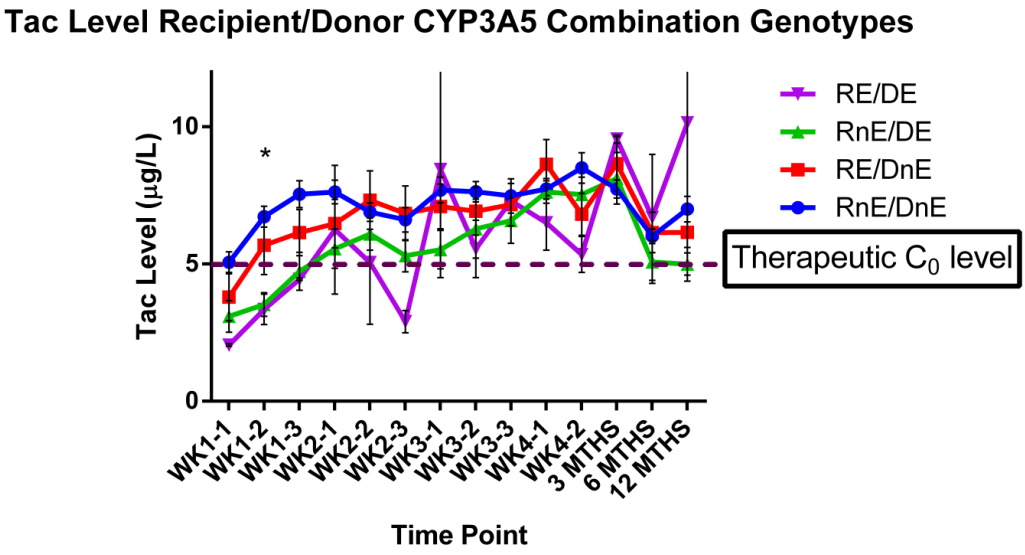


Figure 4.5 Tacrolimus trough concentration (C_0) relative to CYP3A5 combination genotype of both the liver donor and recipient at each time point. * signifies significant result $p<0.05$ (RE = recipient expresser, RnE = recipient non-expresser, DE = donor expresser, DnE = donor non-expresser)

4.3.17 Grouped CYP3A5 Genotype of Dose-Corrected Tac Trough Levels

There were significant differences in the dose corrected tacrolimus levels (C_0/D) between the four donor/recipient genotype combinations at a few time points (WK-3-1, WK3-2, WK4-2 and 12 months). Figure 4.6 below shows that the DnE/RnE group (where neither patient or recipient express CYP3A5) appears to have the greatest exposure to tacrolimus (the highest C_0/D levels).

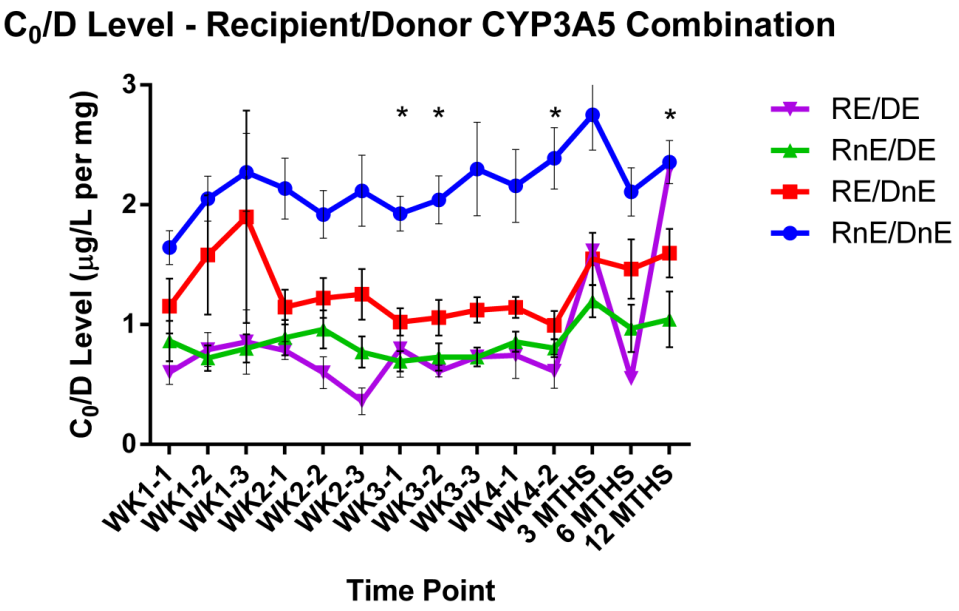


Figure 4.6 Dose corrected tacrolimus trough concentration (C_0/D) relative to CYP3A5 combination genotype of both the liver donor and recipient at each time point. * signifies significant result $p<0.05$ (RE = recipient expresser, RnE = recipient non-expresser, DE = donor expresser, DnE = donor non-expresser)

4.3.18 ABCB1 Recipient Genotype in Relation to Tacrolimus Dose

The recipient genotype of ABCB1 does not appear to influence the required dose of tacrolimus in this study. Individuals with the CC, CT or TT genotype have similar doses of tacrolimus prescribed at all time points up to and including 12 months as shown in Figure 4.7 below.

4.3.19 Donor ABCB1 Genotype in Relation to Tacrolimus Dose

The donor genotype of ABCB1 C3435T also appears to have very little effect on the tacrolimus dose requirements of liver transplant patients. There were two time points where the donor genotype of ABCB1 resulted in a statistically significant difference in the dose requirements between the groups (WK4-1 and WK4-2). At WK4-1 the mean tacrolimus dose for the donor CC genotype was 6.66±2.34 mg compared with 5.16±2.81 mg for the CT genotype and 5.90±2.83 mg for the TT genotype, p=0.028, one-way ANOVA. A post-hoc Bonferroni analysis reveals that the significant difference lies between the CC and the CT values, (p=0.029), but not between the CC and the TT values (p=0.784), nor between the CT and the TT values (p=0.558).

Similar results were seen at time point WK4-2 and a post-hoc Bonferroni analysis showed that the significant difference was between the CC and the CT values. The reason for the significant difference at these 2 time points is unclear but given it is seen in the CT genotype but not the TT genotype then it is less likely to be truly significant and may represent an outlier at these 2 time points.

Figure 4.7 below shows the 3 different genotypes of ABCB1 C3435T in relation to tacrolimus dose at all time points for both the recipient and the donors. Both recipient and donor graphs show very little difference in tacrolimus dose requirements between the 3 genotypes at any of the time points.

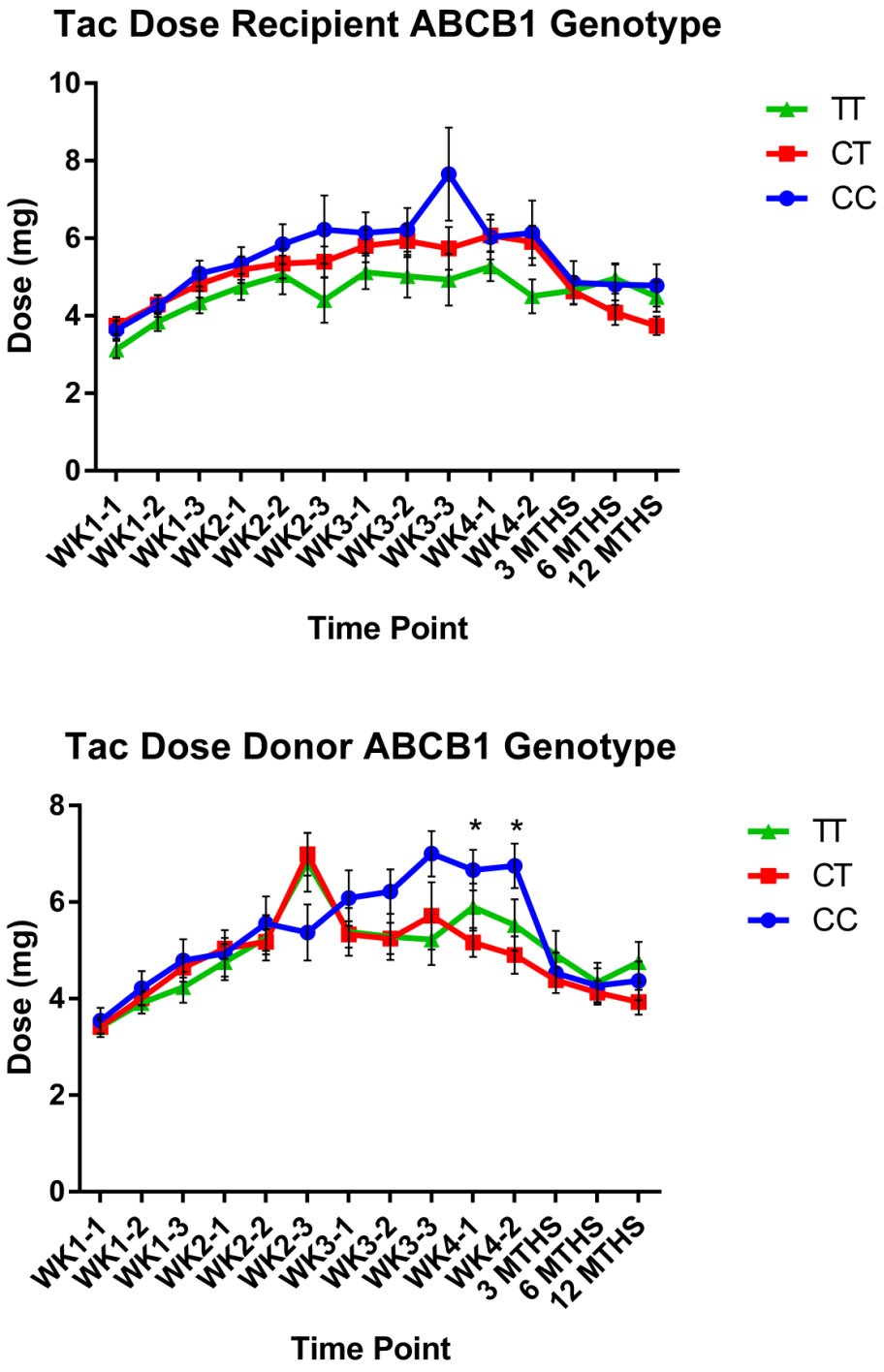


Figure 4.7 Tacrolimus dose requirements relative to ABCB1 genotype for both the donor and recipient genotype at each time point. * signifies significant result $p<0.05$

4.3.20 ABCB1 Recipient Genotype in Relation to Tac Trough Levels

Similar to the findings of the tacrolimus dose requirements in relation to recipient ABCB1 C3435T genotype, there was no significant differences in the tacrolimus trough levels at any of the time points between the CC, CT or TT recipient genotypes of ABCB1 in liver transplant patients as shown in Figure 4.8 below.

4.3.21 ABCB1 Donor Genotype in Relation to Tac Trough Levels

Similar to the recipient ABCB1 genotype, the donor genotype of ABCB1 C3435T does not have any influence on the tacrolimus trough levels at any of the time points as shown in Figure 4.8 below.

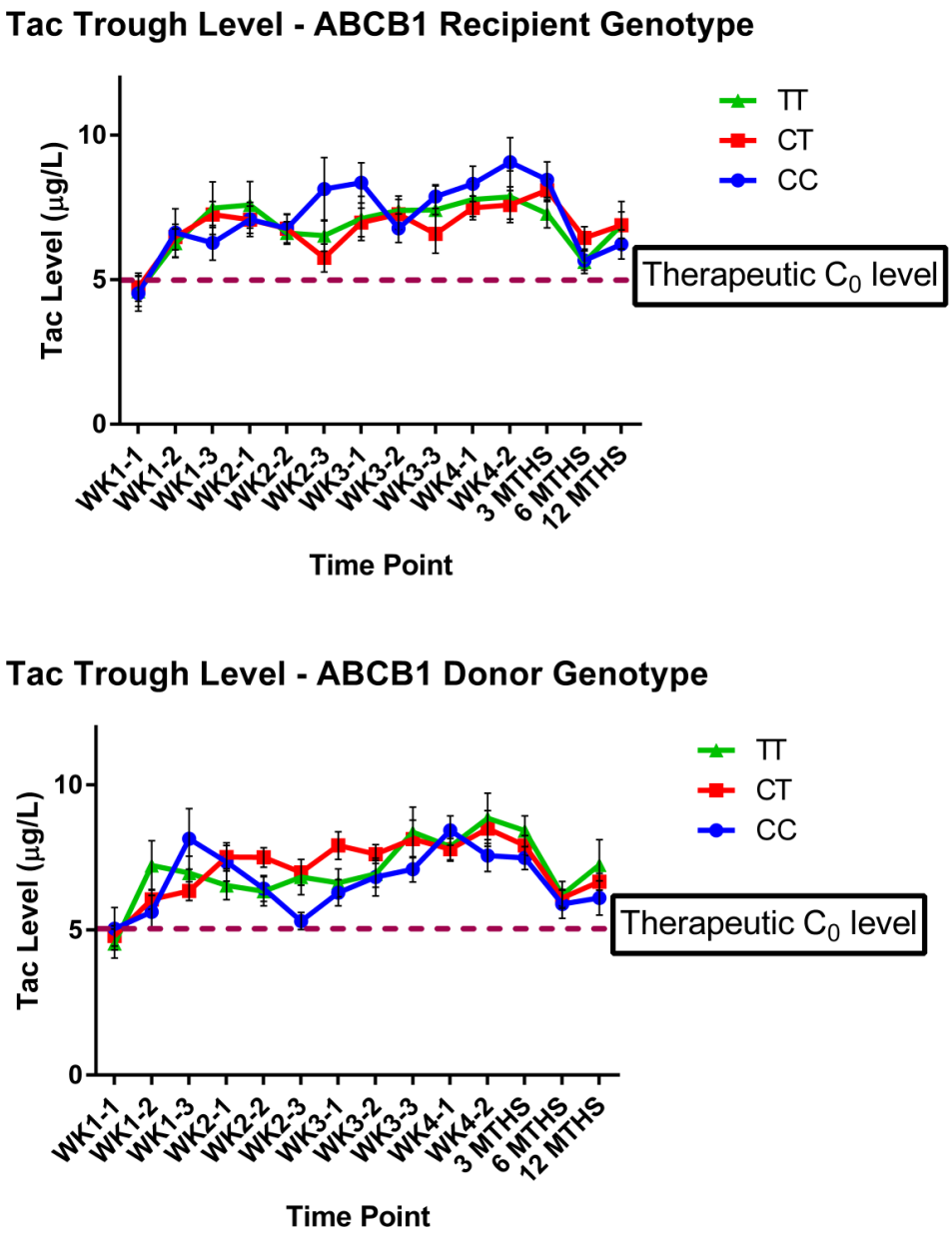


Figure 4.8 Tacrolimus trough concentration relative to ABCB1 genotype for both the donor and recipient genotype at each time point. * signifies significant result $p<0.05$

4.3.22 ABCB1 Recipient Genotype and Dose Corrected Tac Levels

Given that both the tacrolimus dose and the tacrolimus trough levels were not affected by the ABCB1 C3435T single nucleotide polymorphism, it is not surprising that the dose corrected tacrolimus levels are not influenced by the recipient ABCB1 genotype. None of the time points show any significant difference in the dose corrected levels between the CC, CT or TT genotypes of ABCB1 as shown in Figure 4.9 below.

4.3.23 ABCB1 Donor Genotype and Dose Corrected Tac Levels

There are 3 time points where the dose corrected tacrolimus levels (C_0/D) were significantly different (WK1-3, WK4-2 and 12 months) and were due to differences between CT and TT genotypes.

The differences in the C_0/D levels at these time points in isolation are unlikely to represent a true significant difference and may be due to values which are outliers. Figure 4.9 displays the dose corrected tacrolimus level (C_0/D) for both recipients and donors at each of the time points and shows that ABCB1 C3435T polymorphisms do not significantly affect the dose corrected tacrolimus level.

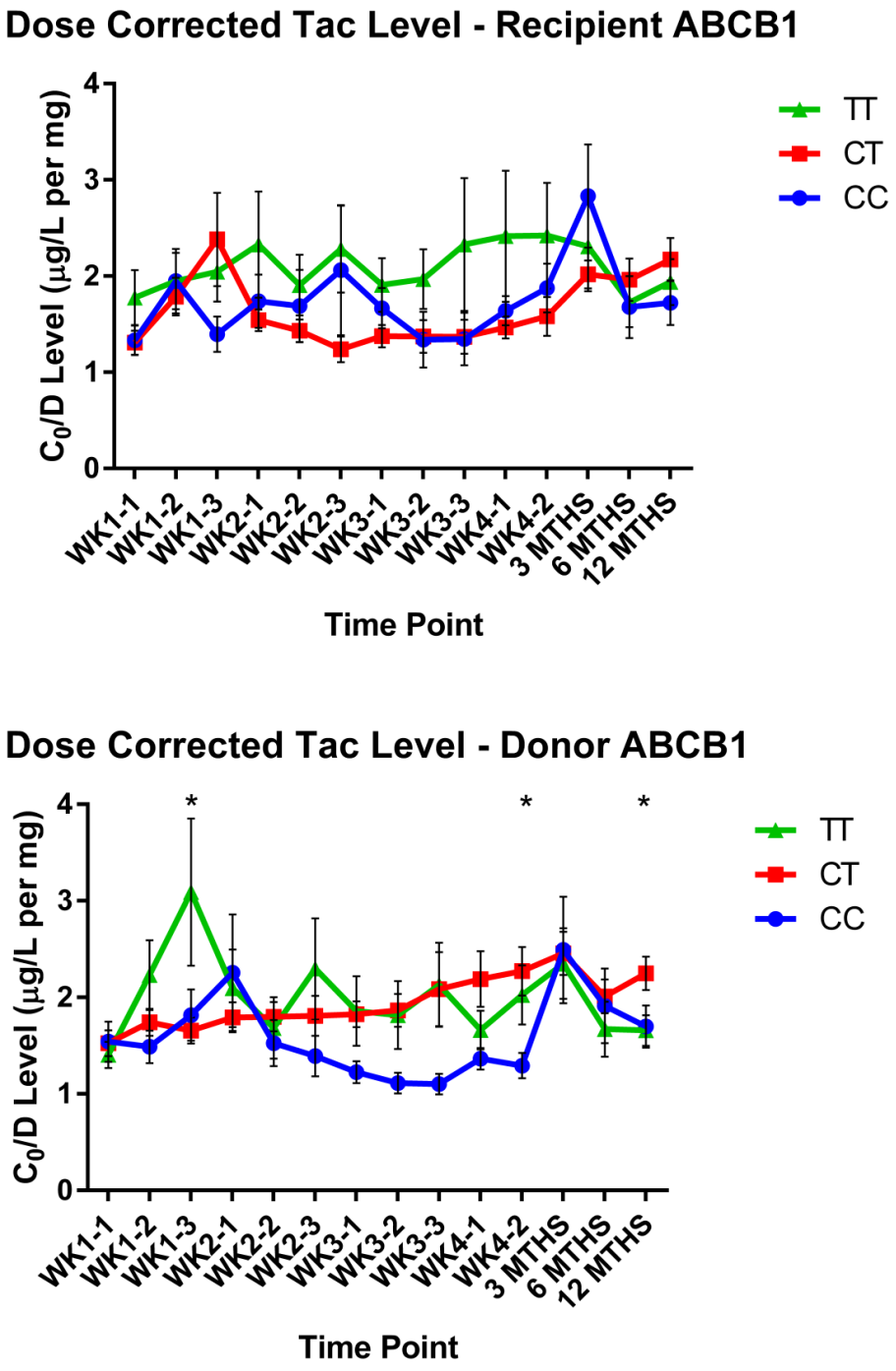


Figure 4.9 Dose corrected tacrolimus trough concentration (C_0/D) relative to ABCB1 genotype for both the donor and recipient genotype at each time point. * signifies significant result $p<0.05$

*4.3.24 CYP3A4*22 Recipient Genotype in Relation to Tac Dose*

Due to the low allele frequency of the CYP3A4*22 T allele, the genotype for CYP3A4*22 is analysed as 2 groups, those who do not express the T allele (wild type genotype CC) and those who do express the T allele (CT or TT genotype).

There was no significant difference in the tacrolimus dose requirements of liver transplant patients based on the recipient CYP3A4*22 genotype at any of the time points. (Figure 4.10)

*4.3.25 CYP3A4*22 Donor Genotype and Relation to Tacrolimus Dose*

The dose of tacrolimus was significantly lower in the CT/TT donor genotype group by WK2-3 time point with a mean dose of 5.47±2.91mg for the CC genotype and 3.44±1.42 mg for the CT/TT genotype, p=0.042, one-way ANOVA.

The dose of tacrolimus was significantly lower at the WK3-1, WK4-1, WK4-2, 3M, 6M and 12M time points too.

Figure 4.10 below demonstrates the tacrolimus dose requirements in relation to both the recipient and the donor CYP3A4*22 genotype. The donor genotype plays a much more significant role and expression of a T allele seems to result in a significant reduction in tacrolimus dose over time.

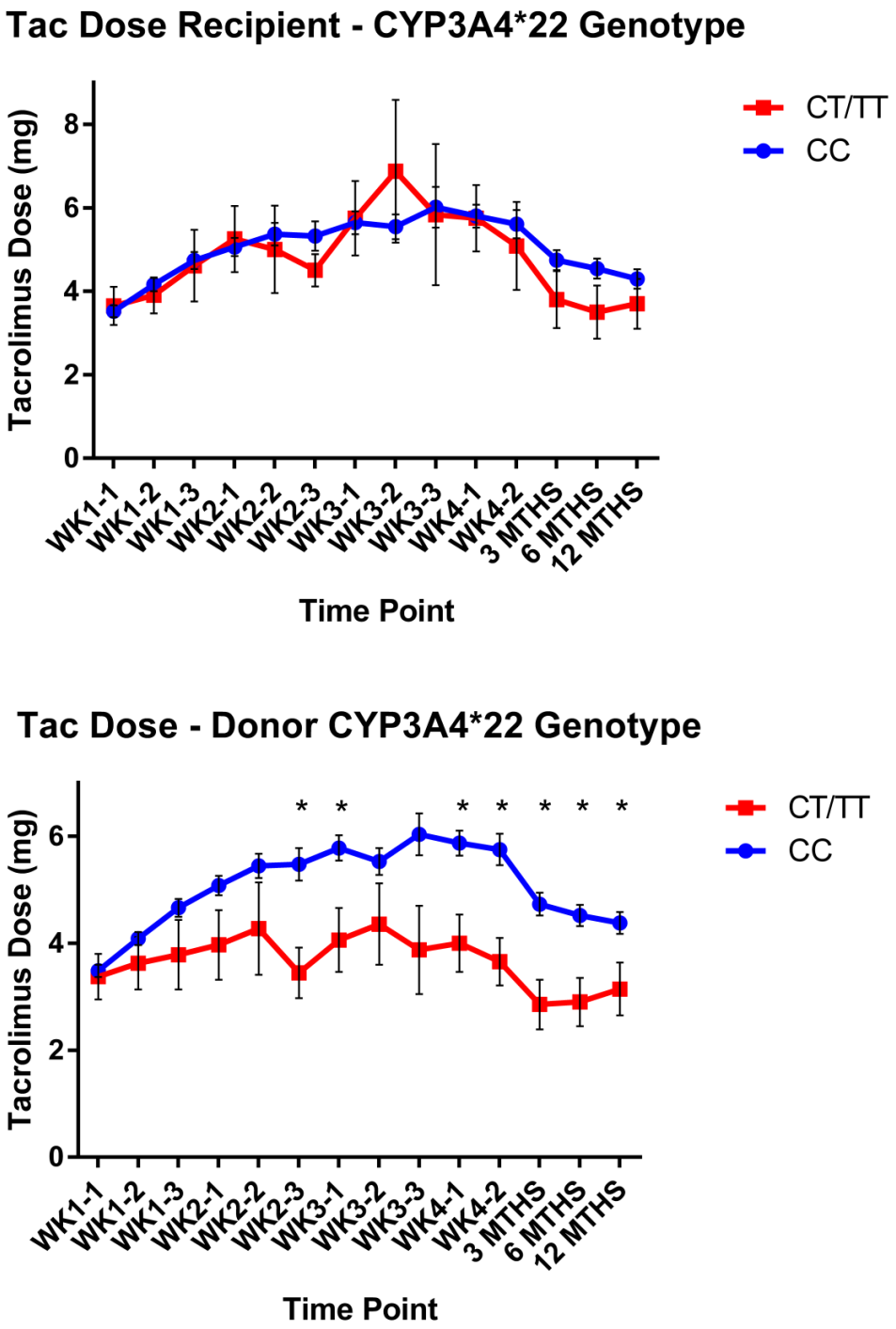


Figure 4.10 Tacrolimus dose requirements relative to CYP3A4*22 T allele expression for both the donor and recipient genotype at each time point. * signifies significant result $p<0.05$

*4.3.26 CYP3A4*22 Recipient Genotype Relative to Tac Trough Levels*

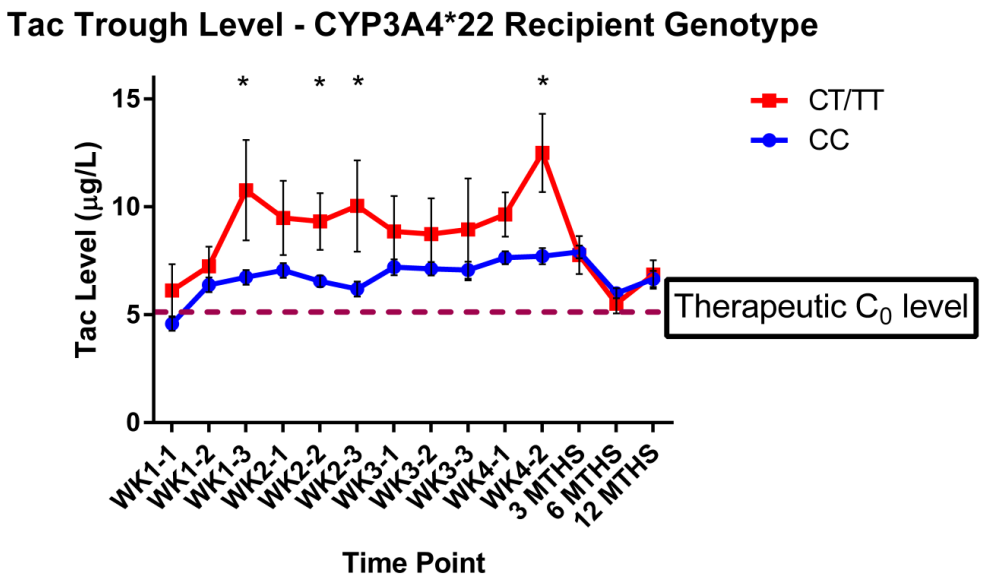
The recipient CYP3A4*22 genotype has a mixed effect on tacrolimus trough levels, with the C_0 levels significantly different between the CC and the CT/TT groups at some of the time points (WK1-3, WK2-2, WK2-3 and WK4-2) where the T allele expressers have higher tacrolimus trough levels.

Displaying graphically the mean tacrolimus trough levels of the CYP3A4*22 CC and CT/TT genotypes (Figure 4.11) does suggest that those individuals who carry the CYP3A4*22 T allele have higher tacrolimus levels than the CC genotype, but this only reaches statistical significance at certain time points.

*4.3.27 Donor CYP3A4*22 Genotype Relative to Tac Trough Levels*

There appears to be only minimal impact on tacrolimus trough levels in relation to donor CYP3A4*22 genotype with a significant difference seen at time points WK3-1 and WK3-2 however this is likely to be an anomaly rather than any significant difference.

Figure 4.11 below shows graphically the differences in tacrolimus trough levels for both donor and recipient CYP3A4*22 genotypes.



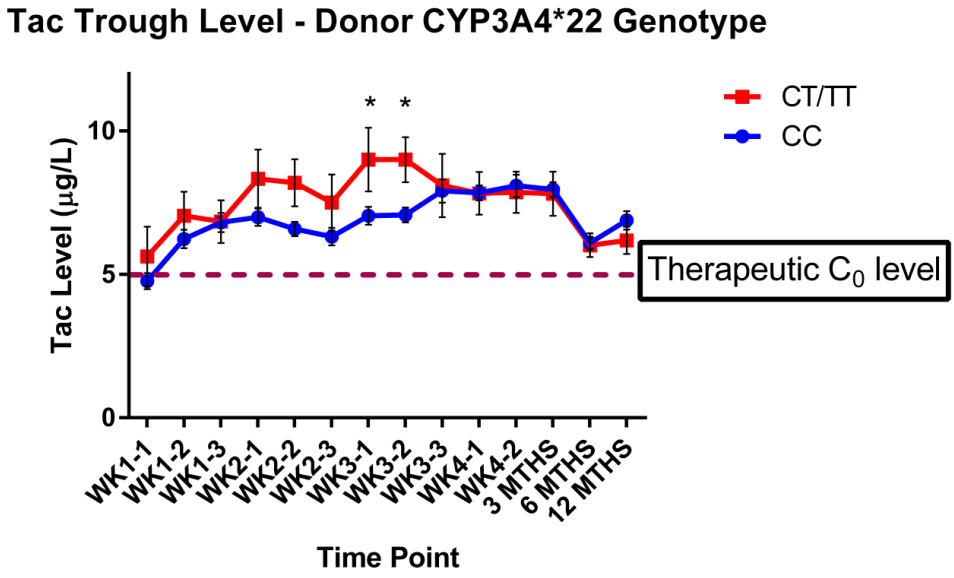


Figure 4.11 Tacrolimus trough concentration (C_0) relative to CYP3A4*22 T allele expression for both the donor and recipient genotype at each time point. * signifies significant result $p<0.05$

*4.3.28 Dose-corrected Tac Levels Relative to Recipient CYP3A4*22*

Recipients who express a T allele do not have any difference in the dose corrected tacrolimus levels compared with those of the CC genotype. The only exception noted was at the WK4-2 time point where the dose corrected tacrolimus level for the CC genotype was 1.76±1.41 µg/L per mg compared with 3.51±2.94 µg/L per mg for the CT/TT genotype, p=0.010, one-way ANOVA.

*4.3.29 Dose-Corrected Tac Levels Relative to Donor CYP3A4*22*

There was a significant difference in the dose corrected tacrolimus levels between the donor CC genotype and the CT/TT genotype at multiple time points (WK3-1, 3M, 6M and 12M). Patients transplanted with a donor liver expressing a T allele have higher dose corrected tacrolimus levels (greater tacrolimus exposure) at these time points.

Figure 4.12 below displays the dose corrected tacrolimus levels at each of the time points in relation to the donor and recipient CYP3A4*22 genotype. The donor genotype appears to have a greater influence leading to greater tacrolimus exposure in patients transplanted with donor livers expressing

the T allele compared with the CC homozygotes. While these differences only reach statistical significance at a few of the time points, the allele frequency of the T allele in this SNP is very low and therefore much larger numbers of patients may be required in order to evaluate if there is a truly sustained significant difference.

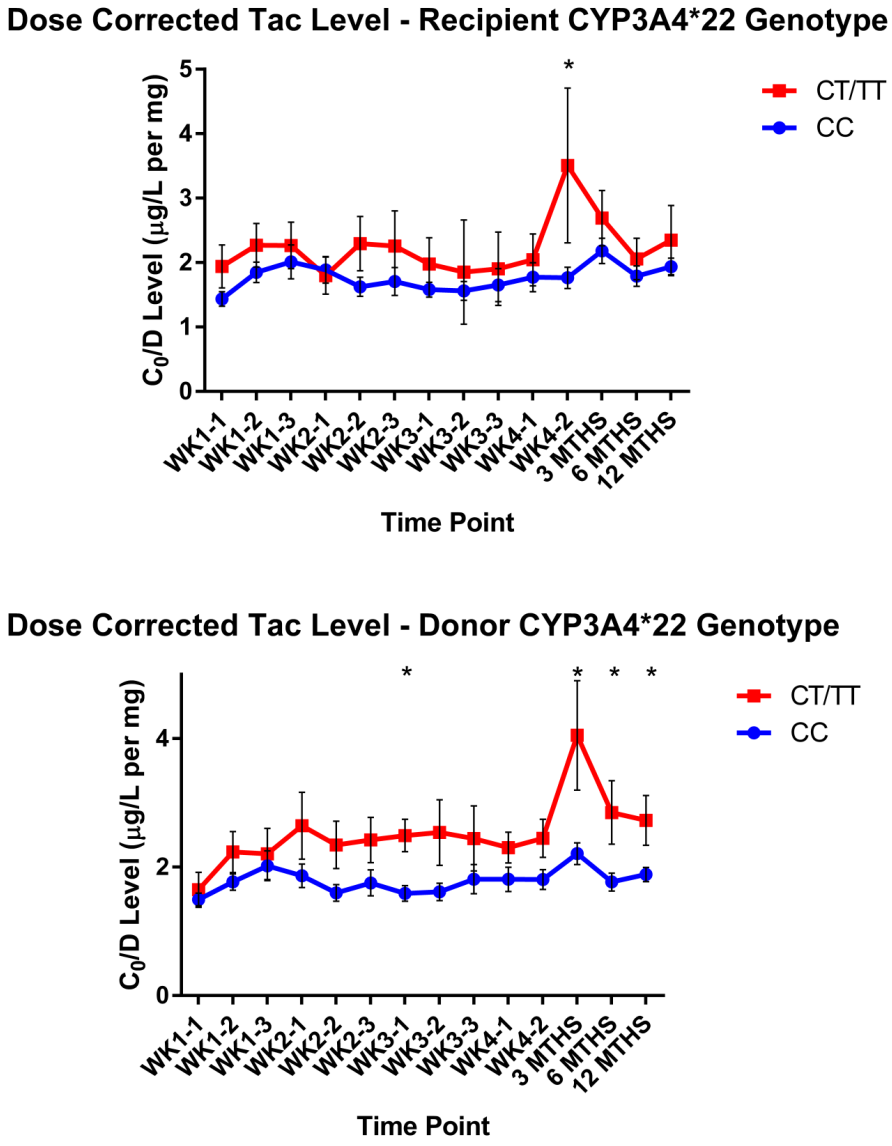


Figure 4.12 Tacrolimus dose corrected trough concentration (C_0/D) relative to CYP3A4*22 T allele expression for both the donor and recipient genotype at each time point. * signifies significant result $p<0.05$

4.3.30 Repeated Measures ANOVA

A potential pitfall of repeatedly measuring the same variables in the same subjects over a time period is that patient baseline characteristics may be misinterpreted as a true difference and also a regression towards the mean effect, whereby a result that may seem significant has, in fact, just followed on from an unusually high or unusually low result and the actual mean is not significantly different to other subjects.

A repeated measures ANOVA is a multivariate analysis that corrects for this and is considered a better statistical test than carrying out an ANOVA analysis at each time point. The repeated measures ANOVA will give an overall p value (called Wilks' lambda) and will also give a significance of the within-subject effects and the between-subject effects.

Table 4.3 below outlines the significance overall (Wilks' lambda) of the mean tacrolimus dose, the mean tacrolimus trough concentration and the mean dose-corrected trough concentration for both recipient and donor CYP3A5, ABCB1 and CYP3A4*22 genotypes. It also details any significant difference of the within-subject effects or between-subject effects for these parameters.

In general the results reflect similar findings of the ANOVA univariate analyses already presented in this chapter with the donor CYP3A5 genotype having the dominant effect on tacrolimus pharmacodynamics.

Table 4.3 Repeated measures ANOVA for donor and recipient CYP3A5, ABCB1 and CYP3A4*22 genotype for tacrolimus dose, tacrolimus trough concentration and dose corrected tacrolimus trough concentration. Overall multivariate comparison (Wilks' lambda), within-subject effects and between-subject effects are shown.

	Wilks' Lambda p value (Multivariate)			**Within-Subject Effects (p value)**			**Between-Subject Effects (p value)**		
	Tac Dose	Tac Level	Dose Corr	Tac Dose	Tac Level	Dose Corr	Tac Dose	Tac Level	Dose Corr
DONOR CYP3A5	**0.048**	0.322	0.933	**0.007**	**0.04**	0.991	**0.014**	**0.002**	**0.012**
DONOR ABCB1	0.205	0.337	0.597	0.309	0.273	0.256	0.676	0.068	0.619
DONOR CYP3A4	0.986	0.095	**<0.0001**	0.606	**0.042**	0.259	0.053	0.373	0.275
RECIPIENT CYP3A5	0.151	**0.045**	0.655	0.714	0.268	0.993	0.453	0.637	0.375
RECIPIENT ABCB1	0.359	0.496	0.485	0.111	0.332	0.536	**0.026**	0.434	0.142
RECIPIENT CYP3A4	0.195	0.119	0.753	0.72	**<0.0001**	0.932	0.776	0.647	0.74

There are some drawbacks of this type of analysis, however, which make its application to this retrospective analysis somewhat difficult. This analysis requires each field between subjects and within a subject to be filled, in short it only analyses results where every value is recorded between the subjects and cannot analyse missing data. The result is far fewer subjects where an entirely full dataset is available. A retrospective study of this kind will

almost always have some missing data therefore this analysis is likely to be underpowered as the dataset is much smaller (only 32 subjects analysed for donor CYP3A5 genotype relative to tacrolimus dose). The majority of the currently published literature performs a standard univariate method of analysing the difference of the means (ANOVA or t-test) although more complex multivariate models are now appearing in literature. Given the limitations of this repeated measures analysis with our dataset the remainder of analysis in this thesis are performed with univariate ANOVA, accepting the limitations of that statistical test.

4.3.31 Time to Reach Therapeutic Tacrolimus Levels

Following liver transplantation it is important that patients achieve a therapeutic tacrolimus trough level as soon as possible to ensure adequate immunosuppression to prevent allograft rejection. In our centre the minimum level at which tacrolimus whole blood concentration trough level is considered therapeutic is 5µg/L. We investigated the time taken for each patient to reach a therapeutic level and correlated this with the recipient and donor genotype of CYP3A5, ABCB1 and CYP3A4*22.

4.3.32 CYP3A5 Recipient and Donor Genotypes

Recipients who were non-expressers of CYP3A5 (GG or *3/*3 genotype) had a significantly shorter mean time before reaching a therapeutic tacrolimus level compared to recipients who were expressers of CYP3A5 (GA *3/*1 or AA *1/*1 genotype) [5.188±1.2491 days (0 – 21 days) vs 8.088±6.0679 days (0 – 23 days), p=0.014, one-way ANOVA]. The trend was similar for the donor genotype however it did not reach statistical significance, [5.671±5.4260 days (0 – 34 days) compared with 7.474±5.2400 days (0 – 21 days), p=0.170, one-way ANOVA]. Figure 4.13 below displays the time taken to reach the therapeutic tacrolimus trough level relative to recipient and donor expression of CYP3A5.

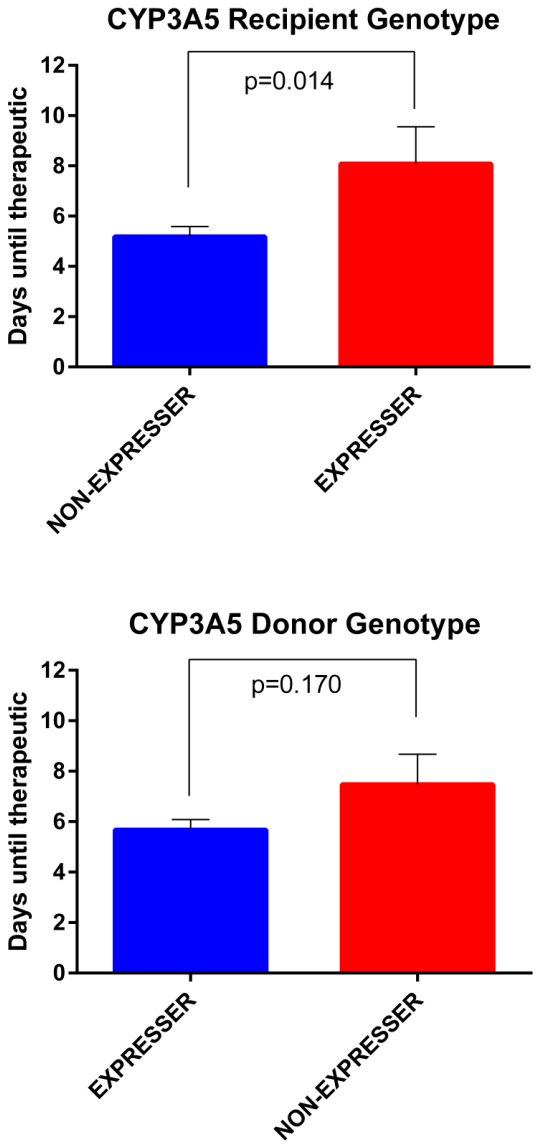


Figure 4.13 Time taken to reach a therapeutic tacrolimus trough concentration (C_0) relative to CYP3A5 expression in both the liver donor and recipient

4.3.33 CYP3A5 Donor and Recipient Grouped Genotypes

The time to reach therapeutic tacrolimus level for (RnE/DnE) patients was 5.03±4.23 days (0 – 21 days). Where the recipient was an expresser of CYP3A5 but the donor liver was not (RE/DnE), a therapeutic level was reached in 7.034±5.83 (0 – 23 days). When the recipient was a non-expresser of CYP3A5 but was transplanted with a CYP3A5 expresser liver (RnE/DE), the mean time to reaching a therapeutic tacrolimus level was 7.39±4.78 days (0 – 14 days). There were only two patients in the RE/DE group (both recipient and donor expressers of CYP3A5) and the mean time to therapeutic level was 8.50±0.71 days (8 – 9 days). None of these times were statistically different, p=0.128, one-way ANOVA.

Figure 4.14 below displays the time taken to reach a therapeutic tacrolimus level for each of the CYP3A5 donor/recipient genotype combinations.

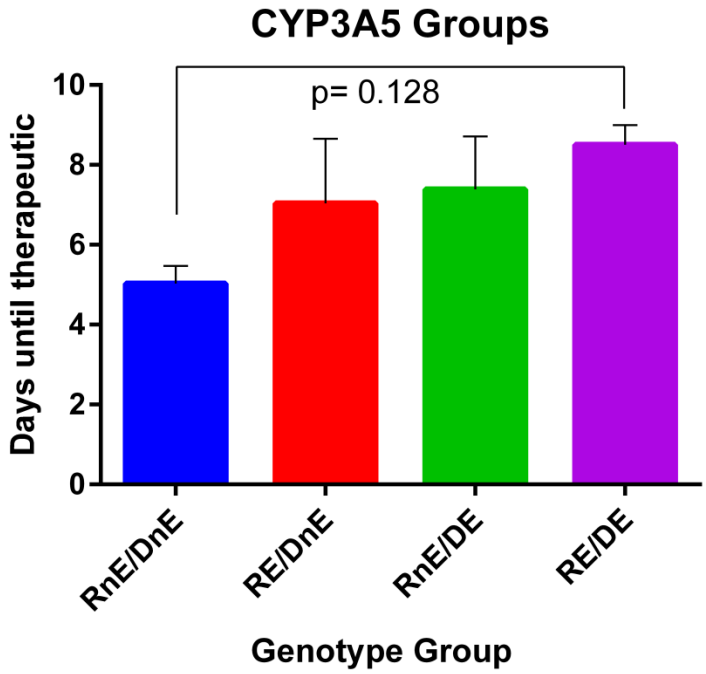


Figure 4.14 Time taken to reach a therapeutic tacrolimus trough concentration (C_0) relative to CYP3A5 grouped genotype in both the liver donor and recipient. (RE = recipient expresser, RnE = recipient non-expresser, DE = donor expresser, DnE = donor non-expresser)

4.3.34 ABCB1 Recipient and Donor Genotypes

There was no difference in the time to reaching therapeutic tacrolimus levels between any of the recipient genotypes of ABCB1 [5.17±4.27 days for CC genotype, 5.48±3.90 days for CT genotype and 6.18±5.67 days for the TT genotype, (p=0.604, one-way ANOVA).

Similar results were seen for the liver donor ABCB1 genotype [5.68±5.05 days for the CC genotype, 5.59±5.18 days for the CT donor genotype and 7.00±6.66 days for the TT donor genotype, (p=0.370, one-way ANOVA). Figure 4.15 below shows the mean time taken until a therapeutic level of tacrolimus is reached for both the recipient and donor genotypes of ABCB1.

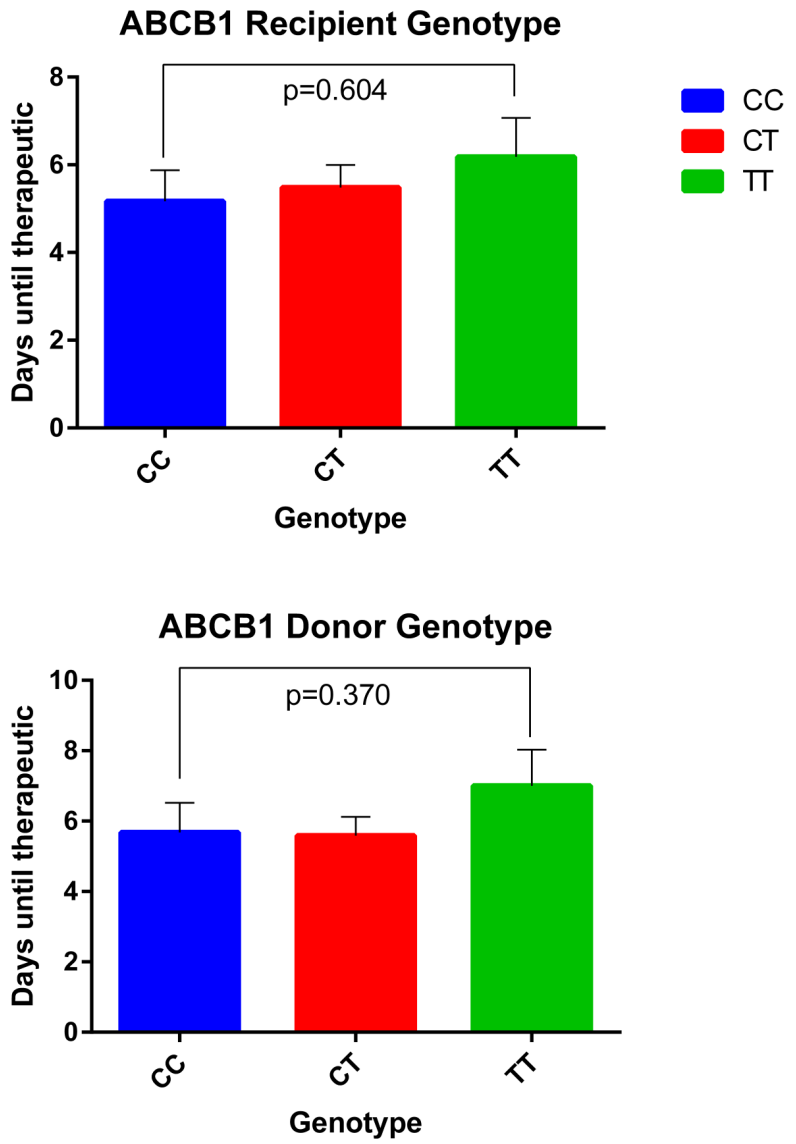


Figure 4.15 Time taken to reach a therapeutic tacrolimus trough concentration (C_0) relative to ABCB1 genotype in both the liver donor and recipient

*4.3.35 CYP3A4*22 Recipient and Donor Genotypes*

Liver transplant recipients who expressed a T allele of CYP3A4*22 appeared to reach a therapeutic tacrolimus level slightly earlier than those who did not express a T allele although this did not reach statistical significance [3.818±2.8220 days (1 – 11days) for the CT/TT genotype compared to 5.712±4.6768 days (0 – 23 days) for the CC genotype, (p=0.189 one-way ANOVA)].

In contrast, there were no differences according to the expression of a donor T allele of CYP3A4*22 [5.914±5.7060 days (0 – 34 days) for the CC genotype compared with 5.947±4.5151 days (1 – 19 days) for the CT/TT genotype, (p=0.980, one-way ANOVA)]. Figure 4.16 below shows the time to reach therapeutic tacrolimus level for both donor and recipient genotypes of CYP3A4*22.

Only recipient expressers of CYP3A5 take a significantly longer time to therapeutic tacrolimus levels compared with recipient non-expressers of CYP3A5. While there are some trends towards a longer time, the donor CYP3A5 genotype and the recipient genotype of CYP3A4*22 have no statistically significant impact.

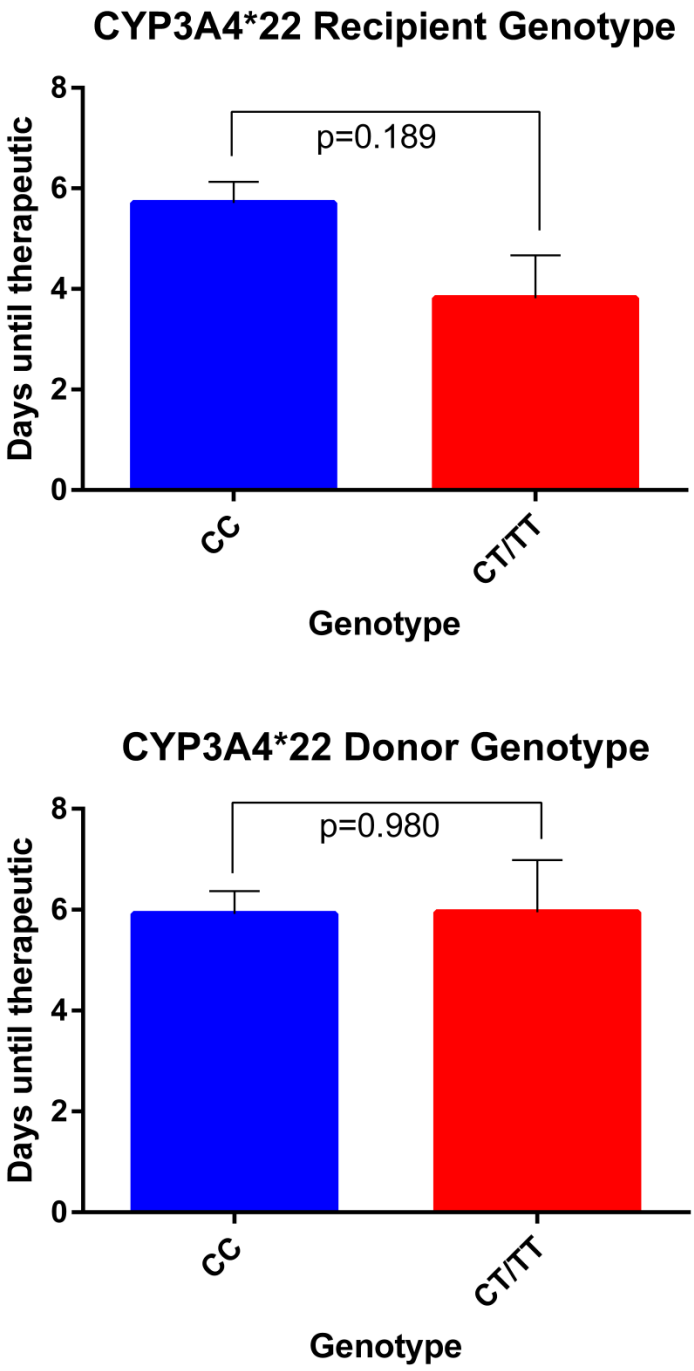


Figure 4.16 Time taken to reach a therapeutic tacrolimus trough concentration (C_0) relative to CYP3A4*22 T allele expression in both the liver donor and recipient

4.3.36 Biopsy Proven Acute Rejection in Relation to Genotype

While different SNPs can affect the pharmacokinetics of tacrolimus, the clinical implications of this are less clearly established.

The recipient CYP3A5 genotype analysis revealed that 30 out of 119 liver transplant patients (25.2%) who were GG (*3/*3) genotype [non-expressers of CYP3A5] had at least one episode of acute rejection compared with four out of 17 patients (23.5%) who were CYP3A5 expressers (GA/AA or *3/*1,*1/*1) (p=0.574, Chi-square test). We also examined the temporal relationship of BPAR with the transplant looking at three time points: 30 days, 90 days and 365 days post transplantation.

At 30 days, there were no differences between CYP3A5 expressers [2 patients (11.8%)] and non-expressers [13 patients (10.9%)]. (p=0.918, Chi-square test).

Similar findings were noted at 90 days [19 (16.1%) vs 3 (17.6%), p=0.872, Chi-square test) and 365 days respectively [29 (24.6%) compared with 3 (17.6%), p=0.530, Chi-square test]. (Figure 4.17)

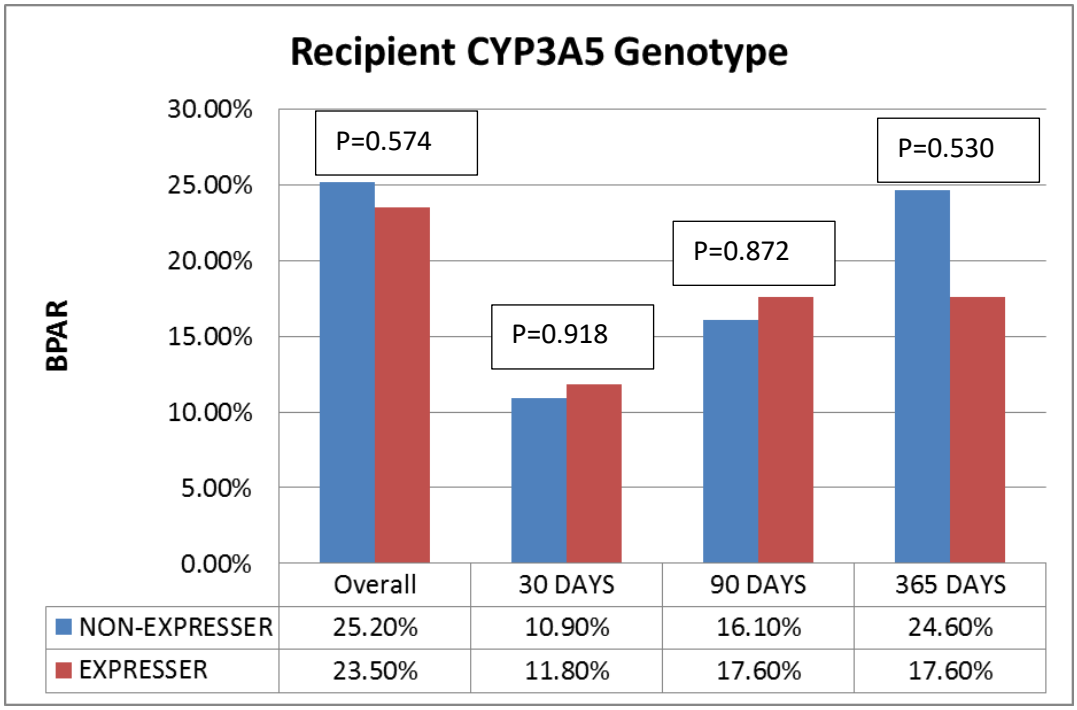


Figure 4.17 Biopsy proven acute rejection rate relative to recipient CYP3A5 expression over the entire study period and at 30, 90 and 365 days.

4.3.37 Donor CYP3A5 Expression and Acute Rejection

In contrast to the recipient CYP3A5 genotype, CYP3A5 expression in donor livers does appear to have a significant impact on acute rejection in liver transplant patients. Overall 8 (42.1%) out of 19 patients transplanted with a CYP3A5 expresser donor liver had at least one episode of biopsy proven acute rejection compared with 36 (20.9%) out of 172 patients transplanted with a CYP3A5 non-expresser donor liver, (p=0.038, Chi-square test).

This difference was not significant at 30 days [2 (10.5%) CYP3A5 expressers vs 16 (9.3%) non-expressers, (p=0.862, Chi-square test)] but was clear at 90 days when 6 out of 19, (31.6%) CYP3A5 expresser liver recipients had BPAR compared with 19 out of 171, (11.1%) non-expresser recipients, (p=0.012, Chi-square test).

At the 365 day, there was a trend towards higher incidence of BPAR in the expresser group, although this did not reach statistical significance [7 (36.8%) out of 19 expresser recipients compared with 34 (19.9%) out of 171 non-expresser recipients (p=0.088, Chi-square test).

Figure 4.18 below demonstrates the differences in BPAR between patients transplanted with livers from CYP3A5 expressers and non-expressers at each of the time points.

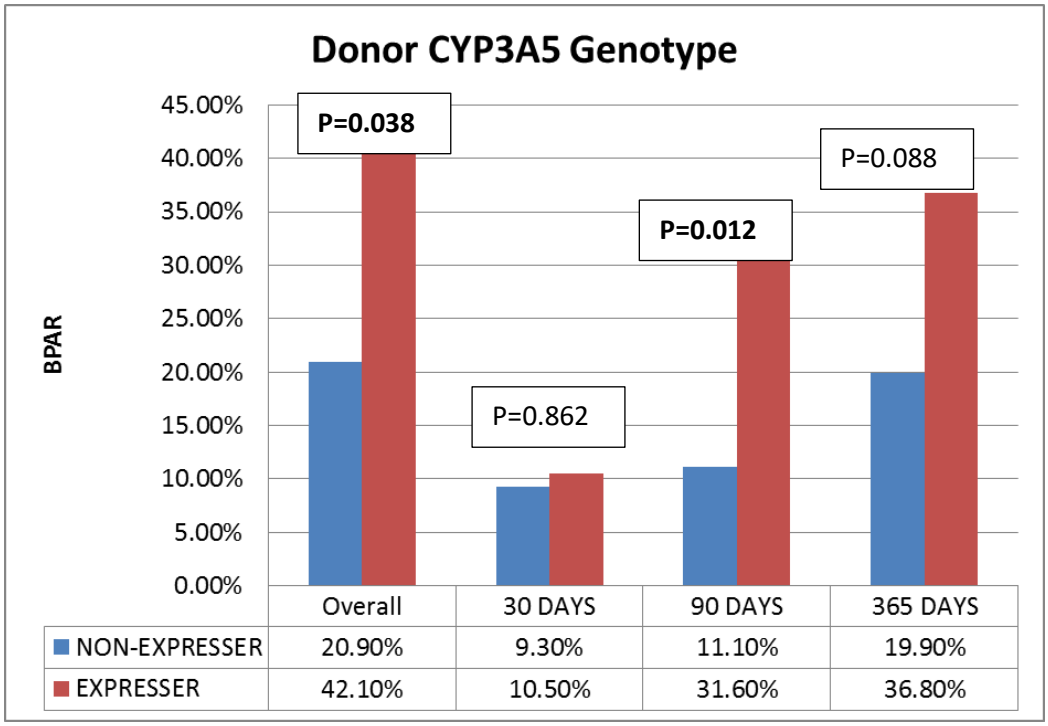
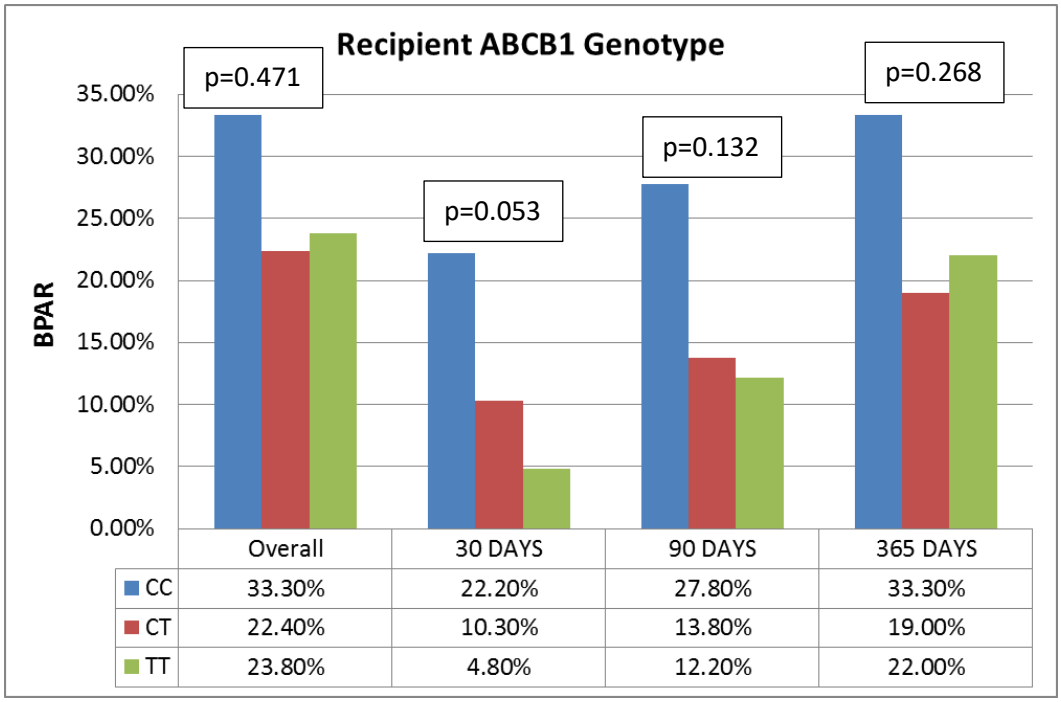


Figure 4.18 Biopsy proven acute rejection rate relative to donor CYP3A5 expression over the entire study period and at 30, 90 and 365 days.

4.3.38 ABCB1 Recipient Genotype and Acute Rejection

There was no correlation between any of the three possible recipient genotypes of ABCB1 C3435T (CC, CT, TT) and the overall incidence of BPAR [12 (33.3%) out of 36 CC genotype recipients, 13 (22.4%) out of 58 CT genotype recipients and 10 (23.8%) out of 32 TT genotype recipients (p=0.471, Chi-square test).

At 30 days post-transplant, 8 (22.2%) of 36 patients with the CC genotype compared with 6 (10.3%) out of 58 patients with the CT genotype and 2 (4.8%) out of 42 patients with the TT genotype respectively had at least one episode of acute rejection, although this not reach statistical difference (p=0.053, Chi-square test). The incidence of BPAR was comparable at the 90 day and 365 day time points.



	Overall	30 DAYS	90 DAYS	365 DAYS
CC	33.30%	22.20%	27.80%	33.30%
CT	22.40%	10.30%	13.80%	19.00%
TT	23.80%	4.80%	12.20%	22.00%

Figure 4.19 Biopsy proven acute rejection rate relative to recipient ABCB1 genotype over the entire study period and at 30, 90 and 365 days.

4.3.39 Donor ABCB1 Genotype and Acute Rejection

Similarly to the recipient genotype of ABCB1, there were no significant differences in BPAR between the recipients of CC, CT or TT donor genotype livers [10 (27.0%) out of 37 patients for the CC genotype, 21 (21.6%) out of 97 patients for the CT genotype and 10 (23.8%) out of 42 patients for the TT genotype (p=0.802, Chi-square test)].

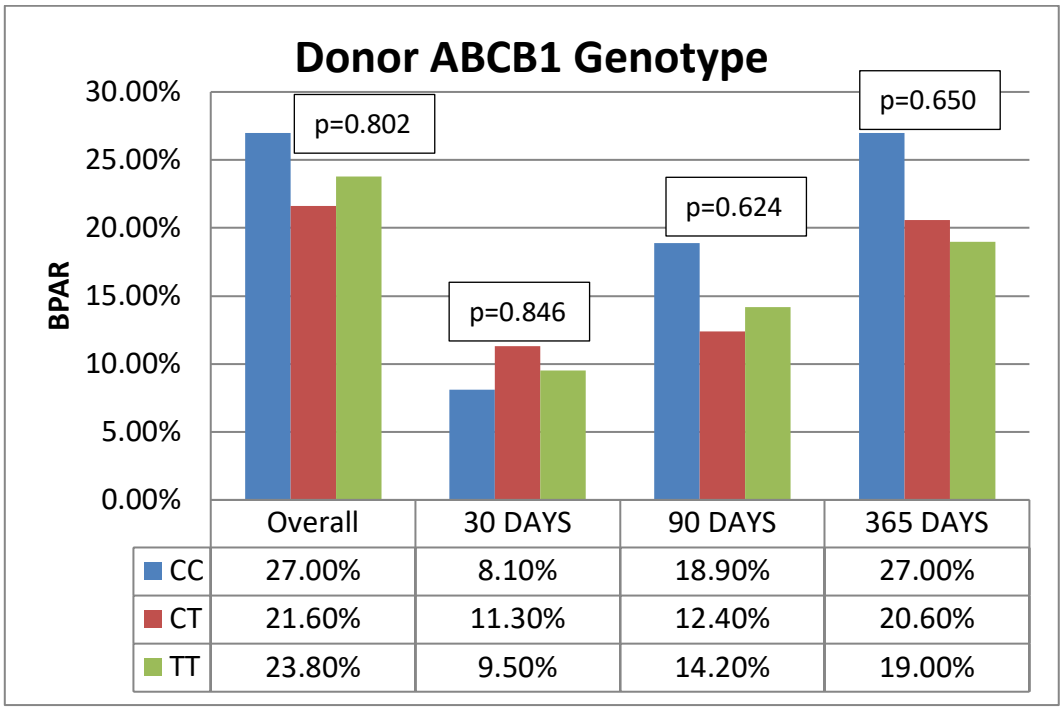


Figure 4.20 Biopsy proven acute rejection rate relative to donor ABCB1 genotype over the entire study period and at 30, 90 and 365 days.

*4.3.40 CYP3A4*22 Recipient Genotype in Relation to Acute Rejection*

Due to the low allele frequency of the T allele of CYP3A4*22, patients were placed into 2 groups for analysis: CC wild type and those who express either one or both T alleles (CT/TT).

Overall, there were no significant differences in acute rejection between the two recipient genotypes [33 (26.0%) out of 127 patients with the recipient CC genotype compared with 2 (18.2%) out of 11 patients with the CT/TT genotype (p=0.568, Chi-square test)].

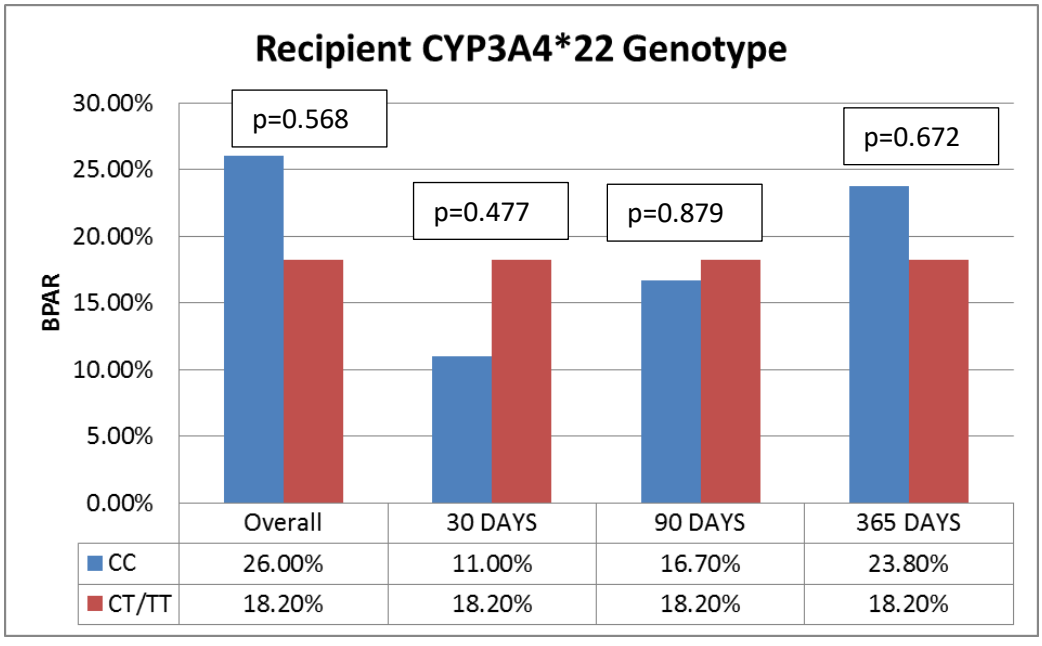


Figure 4.21 Biopsy proven acute rejection rate relative to recipient CYP3A4*22 T allele expression over the entire study period and at 30, 90 and 365 days

*4.3.41 Donor CYP3A4*22 Genotype in Relation to Acute Rejection*

When the donor genotype was analysed, there was no significant difference between the overall and individual time point BPAR incidence according to the 2 genotypes of CYP3A4*22 [39 (24.5%) out of 159 patients with a donor CC genotype compared with 6 (31.6%) out of 19 patients with a CC/CT donor liver genotype (p=0.504, Chi-square test)]. Figure 4.22 shows biopsy proven acute rejection in relation to donor CYP3A4*22 genotype overall and at the 30, 90 and 365 day time points.

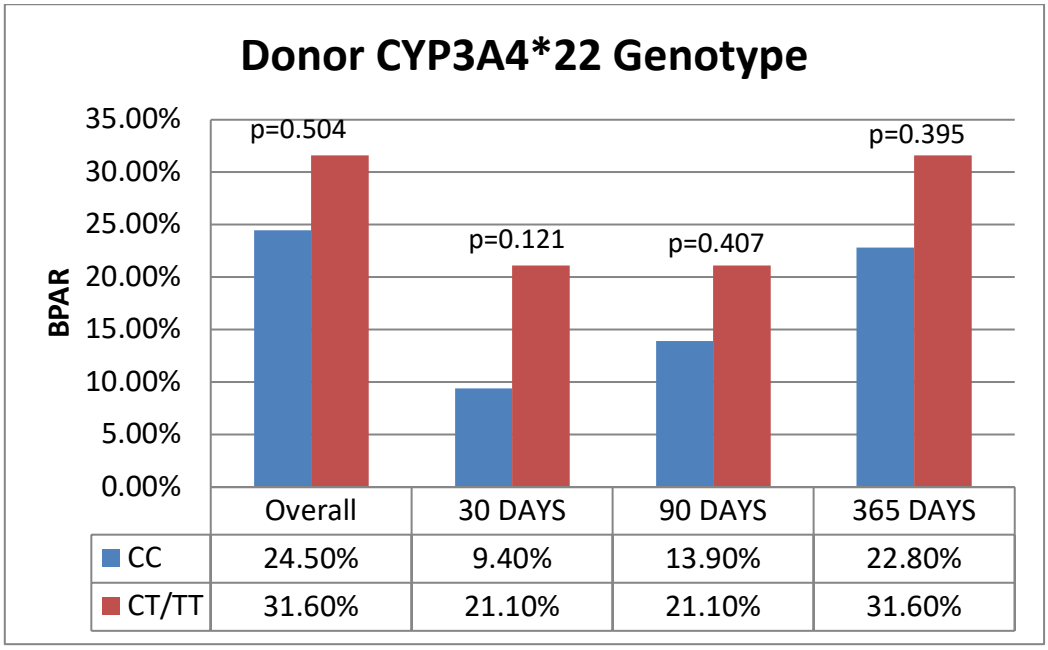


Figure 4.22 Biopsy proven acute rejection rate relative to donor CYP3A4*22 T allele expression over the entire study period and at 30, 90 and 365 days

4.3.42 Cox Regression Multivariate Analysis

Multivariate analysis also showed that donor expression of CYP3A5 was associated with increased biopsy proven acute rejection within the first year following transplant, p=0.027. Interestingly CYP3A4*22 donor T allele expression was also associated with increased biopsy proven acute rejection in multivariate analysis (p=0.006) however this was not seen in a univariate analysis. The T allele frequency is very low in the CYP3A4*22 genotype and a much larger study would be needed to further evaluate whether acute rejection was associated with T allele expression in the donor.

Table 4.4 Cox regression multivariate analysis for acute rejection

Variable	**P value**
CYP3A5 Recipient Genotype	0.347
CYP3A5 Donor Genotype	**0.027**
ABCB1 Recipient Genotype	0.668
ABCB1 Donor Genotype	0.612
CYP3A4*22 Recipient Genotype	0.648
CYP3A4*22 Donor Genotype	**0.006**
Recipient Age	0.689
MELD	0.952
Cold Ischaemic Time	0.121
Donor Type (DBD/DCD)	0.073

4.3.43 Graft Survival

There were 7 episodes of graft loss within the first year following transplantation giving a 1 year graft survival for this cohort of 96.39%. Overall in this study there were 17 episodes of graft loss in the study period, equating to graft survival of 91.2% (on a variable time scale from the date of transplantation).

4.3.44 CYP3A5 Expression and Graft Loss

There was no significant difference found in graft survival with either recipient expression of CYP3A5 or donor expression of CYP3A5 as shown in Figure 4.23 below.

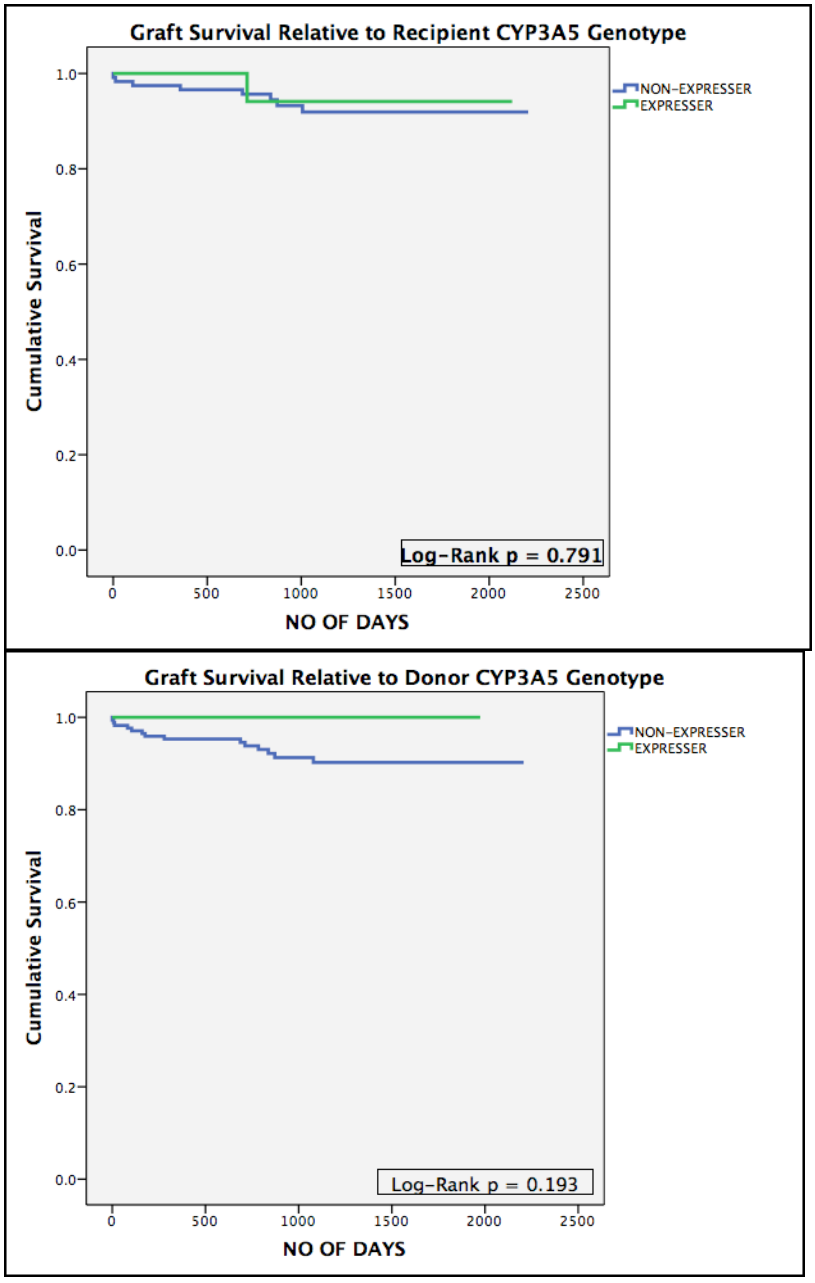


Figure 4.23 Kaplan-Meier plots of graft survival following liver transplantation relative to CYP3A5 expression of both the donor and recipient

A univariate Kaplan-Meier analysis does not demonstrate any significant difference in graft survival between recipient CYP3A5 expression (Log-Rank p = 0.791) and donor CYP3A5 expression (Log-Rank p = 0.193).

4.3.45 Grouped Genotype Analysis of CYP3A5 and Graft Survival

There was similarly no significant difference in graft survival between any of the donor/recipient combination genotypes of CYP3A5.

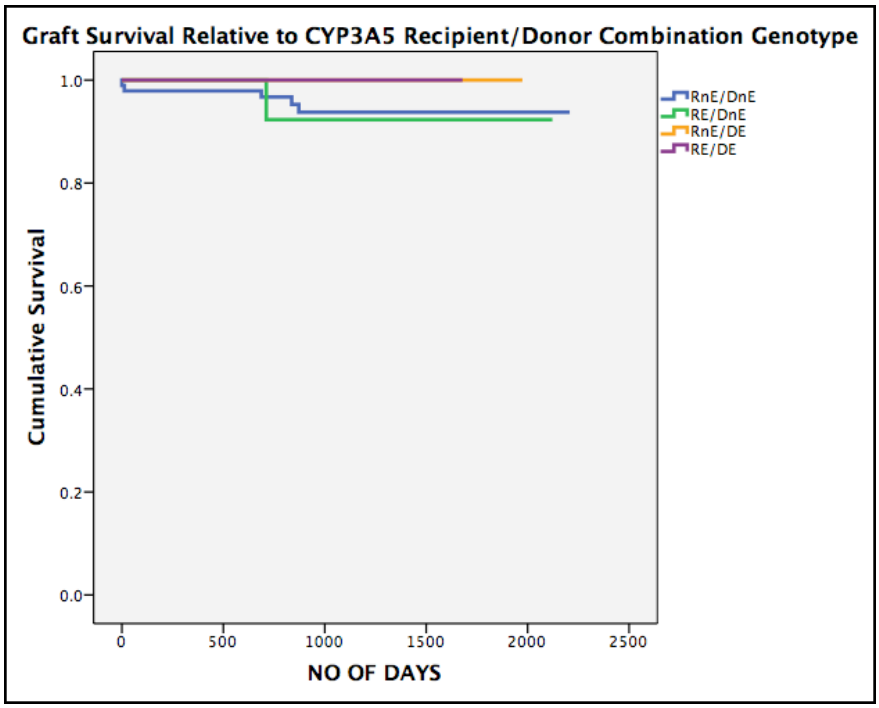


Figure 4.24 Kaplan-Meier plot of graft survival following liver transplantation relative to CYP3A5 donor/recipient grouped genotypes. (RnE = recipient non-expresser, RE = recipient expresser, DnE = donor non-expresser, DE = donor expresser)

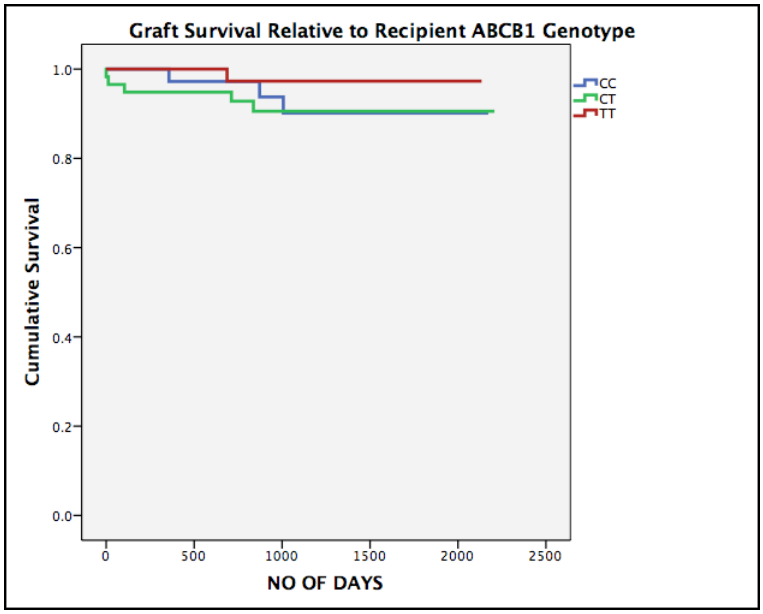
Log rank test	RnE/DnE	RE/DnE	RnE/DE	RE/DE
RnE/DnE		0.828	0.380	0.720
RE/DnE	0.828		0.337	0.695
RnE/DE	0.380	0.337		x
RE/DE	0.720	0.695	x	

4.3.46 Graft Loss Relative to ABCB1 Genotype

There was no significant difference in graft survival between any of the 3 different genotypes of ABCB1 for either the recipient or donor genotypes. The tables below show the different p values for the Log-rank test between the three different recipient and donor genotypes shown in the Kaplan-Meier survival curves that follow in Figure 4.25 below.

Recipient Genotypes	CC	CT	TT
CC		0.854	0.308
CT	0.854		0.211
TT	0.308	0.211	

Donor Genotypes	CC	CT	TT
CC		0.576	0.606
CT	0.576		0.215
TT	0.606	0.215	



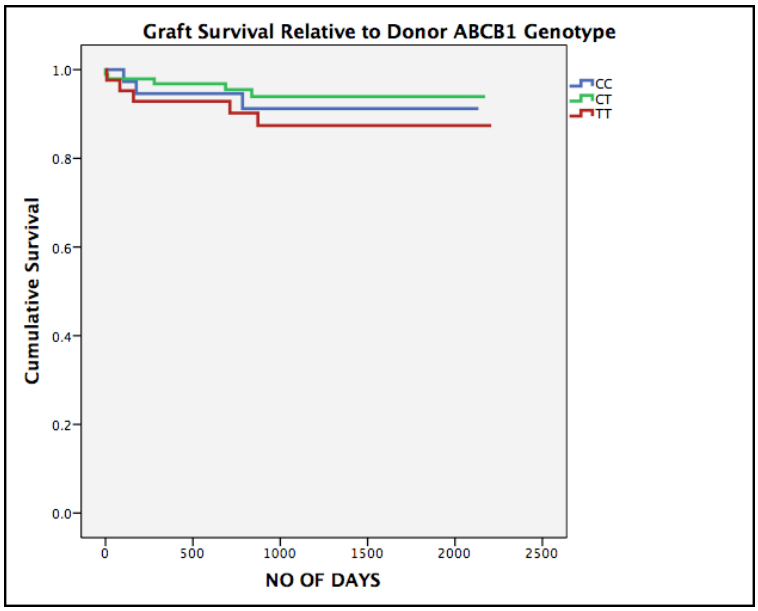


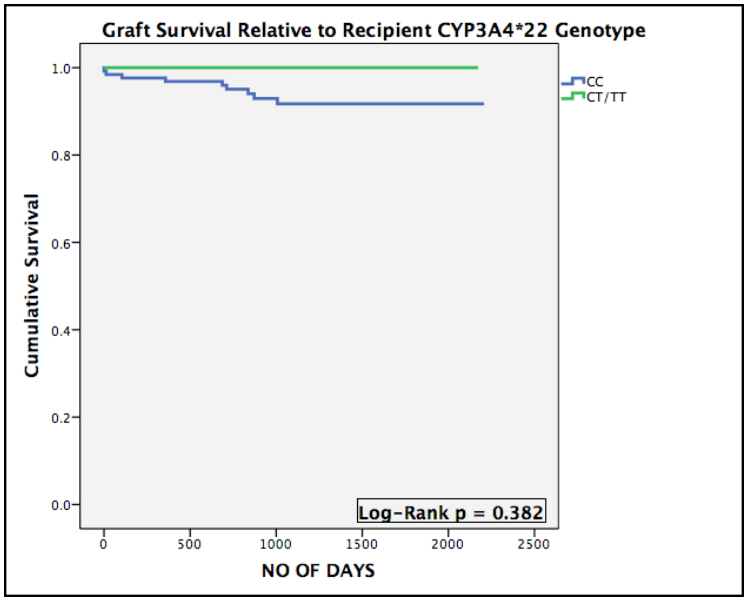
Figure 4.25 Graft survival following liver transplantation relative to ABCB1 genotype for both donor and recipient.

A univariate Kaplan-Meier analysis did not show any significant difference in graft survival between the 3 different genotypes of ABCB1 for either the recipient or donor.

*4.3.47 Graft Survival Relative to CYP3A4*22 Genotype*

There was no significant difference in graft survival due to either the recipient or donor genotype of CYP3A4*22 on univariate Kaplan-Meier curves with Log-rank analysis as shown in Figure 4.26 below.

This study did not find that any of the different genotypes of the 3 SNPs had a significant impact on graft survival on univariate analysis.



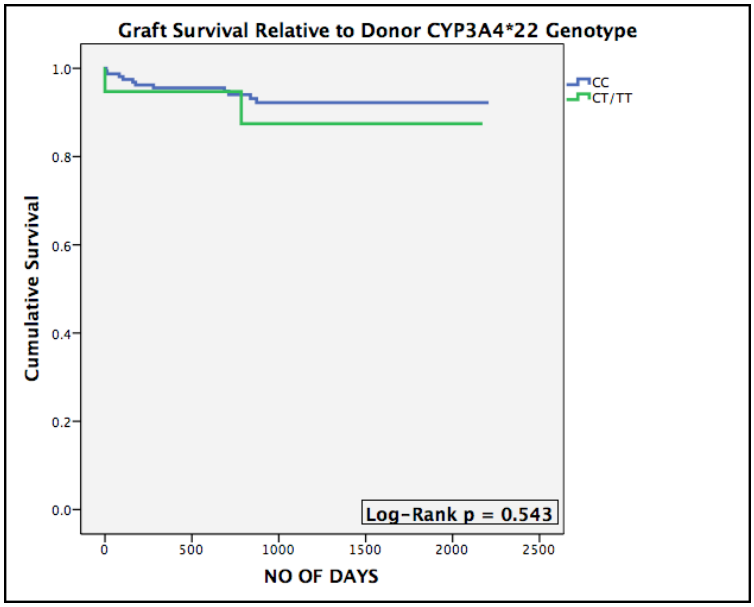


Figure 4.26 Graft survival following liver transplantation relative to CYP3A4*22 T allele expression of both donor and recipient

A univariate Kaplan-Meier analysis does not demonstrate any significant difference in graft survival between recipient CYP3A4*22 expression (Log-Rank p = 0.382) and donor CYP3A4*22 expression (Log-Rank p = 0.543).

4.3.48 Cox Regression Multivariate Analysis

Multivariate analysis did not demonstrate any significant difference in graft loss between any of the genotypes of the 3 SNPs in this study. This was the same for both the recipient and donor genotypes. There were no variables in the analysis that significantly increased graft loss.

Table 4.5 Cox regression multivariate analysis graft survival

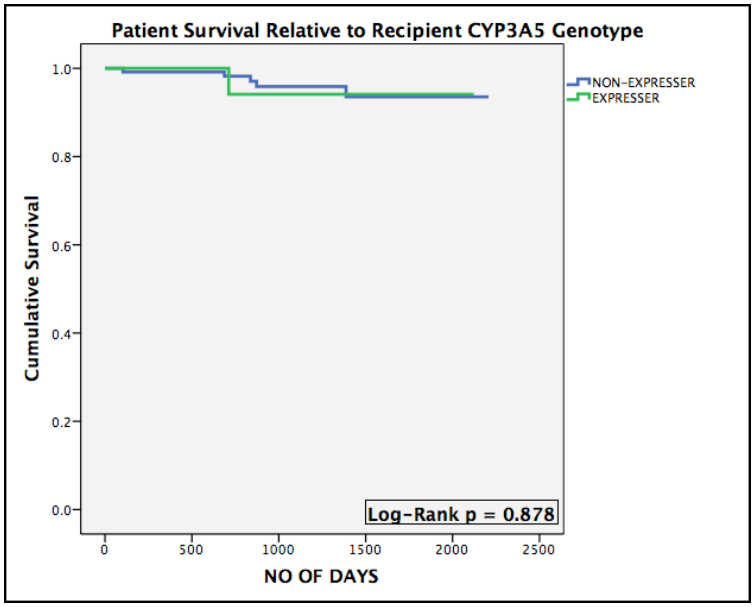
Variable	**p value**
CYP3A5 Recipient Genotype	0.983
CYP3A5 Donor Genotype	0.990
ABCB1 Recipient Genotype	0.466
ABCB1 Donor Genotype	0.467
CYP3A4*22 Recipient Genotype	0.995
CYP3A4*22 Donor Genotype	0.993
Recipient Age	0.382
Recipient Gender	0.572
Donor Age	0.530
MELD	0.384
Cold Ischaemic Time	0.809
BPAR	0.976
Donor Type (DBD/DCD)	0.254

4.3.49 Patient Survival

There were 3 patient deaths within the first 12 months of transplantation giving a 1 year patient survival rate of 98.45%. Overall there were 9 deaths during this study period giving an overall patient survival rate of 95.4% (on a varying time scale from the point of transplantation).

4.3.50 Patient Survival Relative to CYP3A5 Genotype

There was no significant difference in patient survival relative to either the recipient or donor genotype of CYP3A5 as shown in Figure 4.27 below.



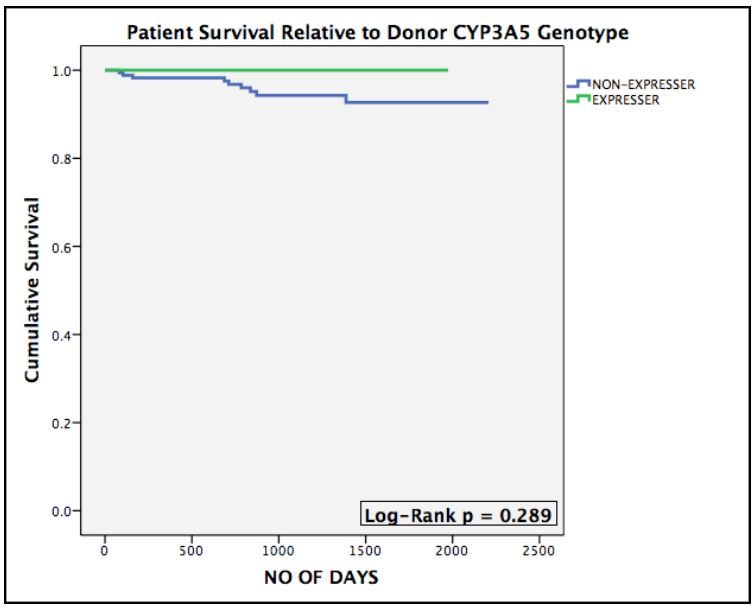


Figure 4.27 Patient survival following liver transplantation relative to CYP3A5 genotype of both donor and recipient

A univariate Kaplan-Meier analysis does not demonstrate any significant difference in patient survival between recipient CYP3A5 expression (Log-Rank p = 0.878) and donor CYP3A5 expression (Log-Rank p = 0.289)

4.3.51 Grouped Genotype Analysis of CYP3A5 and Patient Survival

There was no significant difference seen between any of the CYP3A5 expresser/non-expresser combinations of donor or recipient genotype and patient survival as shown in Figure 4.28 below.

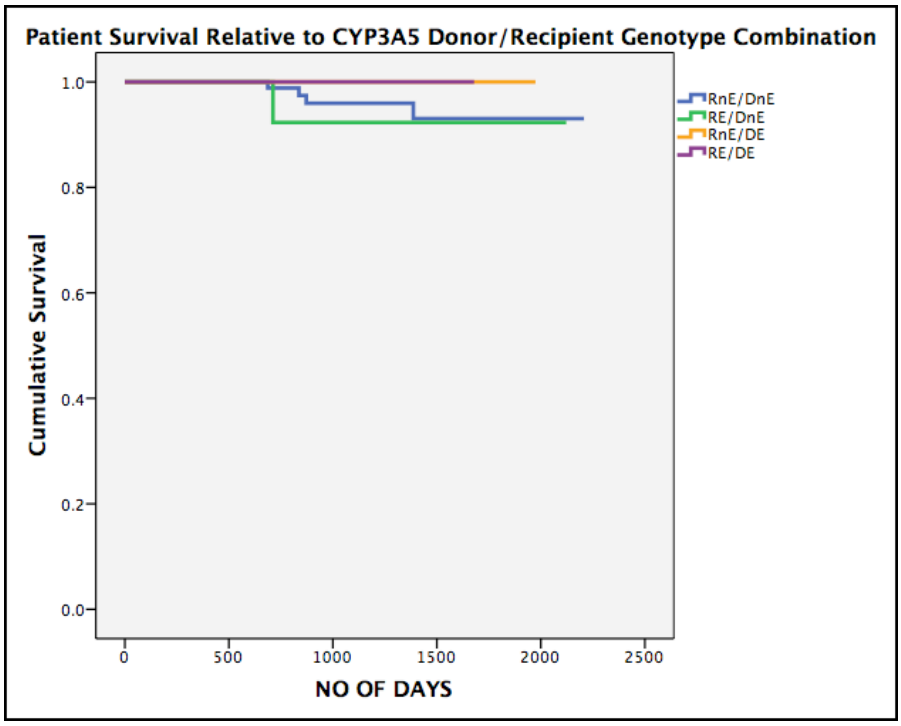


Figure 4.28 Patient survival following liver transplantation relative to combined CYP3A5 genotype of both donor and recipient. (RnE = recipient non-expresser, RE = recipient expresser, DnE = donor non-expresser, DE = donor expresser)

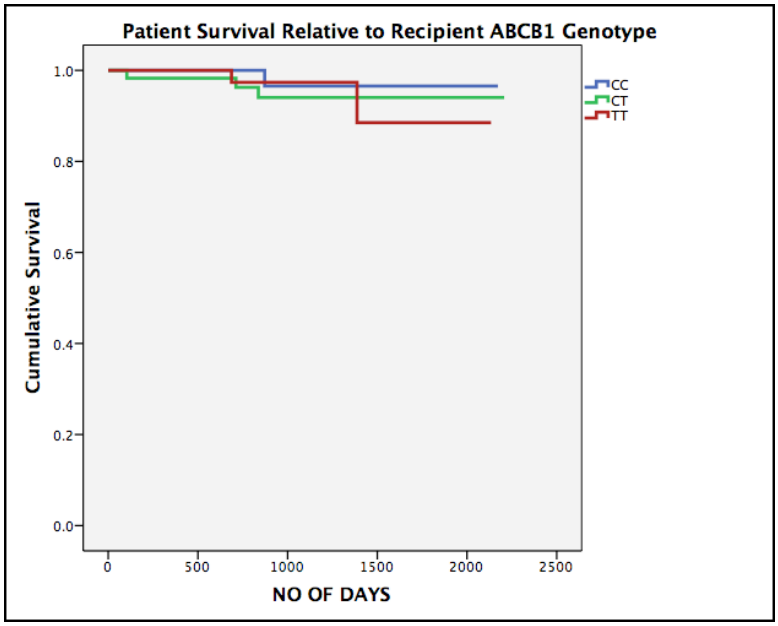
Log rank	RnE/DnE	RE/DnE	RnE/DE	RE/DE
RnE/DnE	-	0.685	0.440	0.706
RE/DnE	0.685	-	0.337	0.695
RnE/DE	0.440	0.337	-	-
RE/DE	0.706	0.695		

4.3.52 Patient Survival Relative to Recipient and Donor ABCB1

There was no significant difference in patient survival relative to the recipient genotype of ABCB1 or the donor genotype of ABCB1. The tables below show the different p values for the Log-rank test between the three different recipient and donor genotypes shown in the Kaplan-Meier survival curves that follow. (Figure 4.29)

Recipient Genotypes	CC	CT	TT
CC	-	0.521	0.458
CT	0.521	-	0.976
TT	0.458	0.976	-

Donor Genotypes	CC	CT	TT
CC	-	0.561	0.530
CT	0.561	-	0.176
TT	0.530	0.176	-



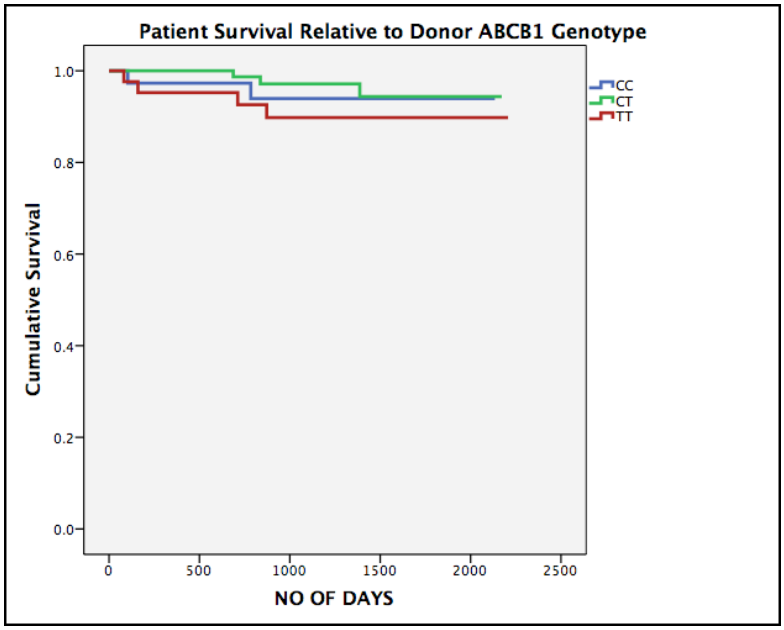
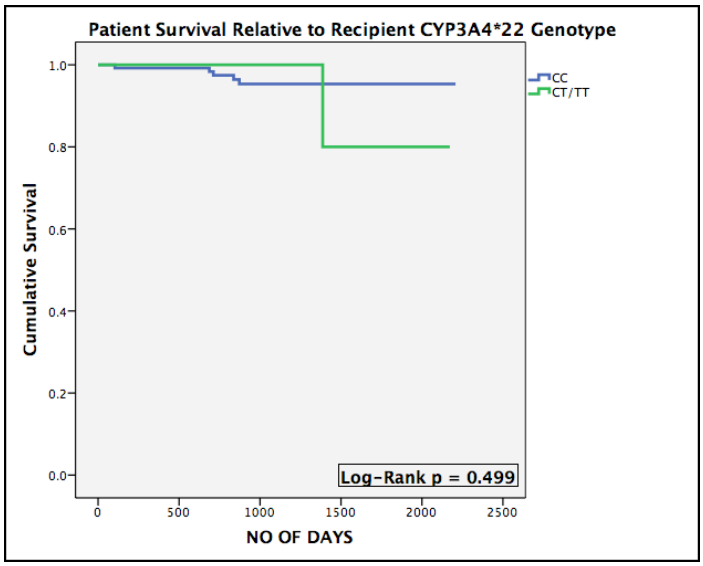


Figure 4.29 Patient survival following liver transplantation relative to ABCB1 genotype of both the donor and the recipient.

A univariate Kaplan-Meier analysis did not show any significant difference in patient survival between the 3 different genotypes of ABCB1 for either the recipient or donor.

*4.3.53 Recipient and Donor CYP3A4*22 and Patient Survival*

There was no significant difference in patient survival due to either the recipient or donor genotype of CYP3A4*22 on univariate Kaplan-Meier curves with Log-rank analysis as shown in Figure 4.30 below.



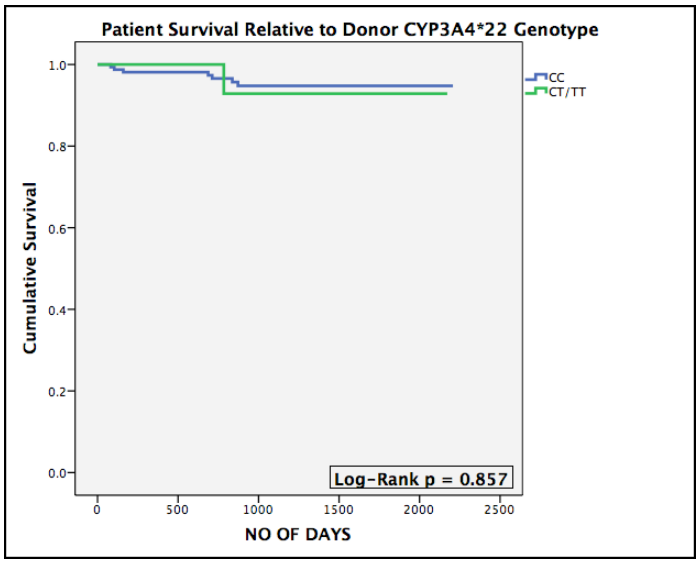


Figure 4.30 Patient survival following liver transplantation relative to CYP3A4*22 T allele expression.

A univariate Kaplan-Meier analysis does not demonstrate any significant difference in patient survival between recipient CYP3A*22 expression (Log-Rank p = 0.499) and donor CYP3A5 expression (Log-Rank p = 0.857).

This study did not find that any of the different genotypes of the 3 SNPs had a significant impact on patient survival on univariate analysis.

4.3.54 Cox Regression Multivariate Analysis for Patient Survival

Cox regression survival analysis did not reveal any significant difference in patient survival for any of the genotypes of the 3 SNPs. None of the variables in the analysis demonstrated a significant impact on patient survival as shown below.

Table 4.6 Cox regression analysis for patient survival following liver transplantation

Variable	**p value**
CYP3A5 Recipient Genotype	0.958
CYP3A5 Donor Genotype	0.376
ABCB1 Recipient Genotype	0.559
ABCB1 Donor Genotype	0.413
CYP3A4*22 Recipient Genotype	0.883
CYP3A4*22 Donor Genotype	0.900
Recipient Age	0.245
Recipient Gender	0.541
Donor Age	0.249
Donor Type (DBD/DCD)	0.185
MELD	0.985
BPAR	0.131
Diabetes After Transplantation	0.129
Myocardial Infarction	0.107
Cerebrovascular Accident	0.686

Many factors affect patient survival and with a relatively short follow up time and few events, further larger studies are much better placed to evaluate post liver transplant survival.

This study has found no evidence that variation in expression of the genotype of CYP3A5, ABCB1 or CYP3A4*22 affects graft or patient survival when taking into account the genotype of both the donor and the recipient of a liver transplant patient.

4.3.55 Power of The Study

Given that there are no significant differences in graft or patient survival in the univariate analysis for donor or recipient and in the multivariate analysis, factors that are known to be independent risk factors for graft loss such as donor age, did not reveal a significant result, the power of this study is worth considering. Given that this is a retrospective analysis, a prospective power calculation is not possible however it is feasible to calculate, retrospectively, the power of the study. Some statistical purists consider this a meaningless exercise however it can inform in a very broad sense whether the study was large enough to detect a true difference.

A retrospective power calculation was undertaken for the recipient and donor CYP3A5 expressers for graft and patient survival.

Table 4.7 Retrospective power calculation for graft and patient survival relative to CYP3A5 expression with assumed alpha value of 0.05.

	Graft Survival Power	Patient Survival Power
Donor CYP3A5	2.5%	0.9%
Recipient CYP3A5	2.8%	7.2%

The standard minimum power for a study is 80% therefore we should consider this study underpowered to detect a true difference in graft or patient survival relative to different genotypes. A power calculation was not carried out for the other chapters or different SNPs for graft and patient survival as it is highly likely they are all equally underpowered to detect these differences, given the results are similar to the liver recipients and their CYP3A5 genotype.

4.4 Chapter Summary

The data presented in this chapter have shown that CYP3A5 expression has the most significant impact on tacrolimus metabolism requiring significantly higher doses compared with patients in whom CYP3A5 expression is absent.

As CYP3A5 is made primarily in liver tissue, the donor genotype of CYP3A5 had a greater influence than that of the recipient. In addition to the higher dose requirements, it also took longer for both the donor and recipient expressers of CYP3A5 to reach a therapeutic level compared with non-expressers. Biopsy proven acute rejection was significantly higher in those patients who received a liver transplant from a donor who was a CYP3A5 expresser in both univariate and multivariate analyses. Recipient expression of CYP3A5 did not influence acute rejection. Both graft and patient survival was unaffected by CYP3A5 expression from either the donor or the recipient.

The 3 different genotypes of ABCB1 appear to play little role in the metabolism and pharmacodynamics of tacrolimus. There was no significant difference in tacrolimus dose requirements or trough levels between any of the 3 possible genotypes of ABCB1 for either the donor or the recipient genotype. ABCB1 expression did not seem to have any impact on the time taken to reach a therapeutic level nor did it have any influence on biopsy proven acute rejection, all the results were comparable between the different genotypes for both donor and recipient. Patient and graft survival were unaffected by ABCB1 genotype. These findings for ABCB1 are similar to those found in the more extensively studied renal transplant population in the current literature.

To accurately ascertain the impact of T allele expression of CYP3A4*22 in relation to tacrolimus metabolism and pharmacodynamics is somewhat difficult due to the infrequency of which a T allele is expressed. This study showed that donor T allele expression of CYP3A4*22 showed a trend towards lower tacrolimus dose requirements although there was less of an impact seen on the trough levels. The lower dose requirement is more subtle than the difference in the increased dose requirement seen in donor CYP3A5 expressers. This would be in keeping with previously published literature on renal transplantation which reports an approximate 50% reduction in tacrolimus dose requirement for individuals that express a T allele of CYP3A4*22 [180]. There was no increase in acute rejection on univariate analysis of T allele expressers however on multivariate analysis this became significant if the donor was a T allele expresser. Further studies with larger patient numbers are required to determine if this phenomenon is reproduced in other cohorts of liver transplant patients. CYP3A4*22 expression in either the donor or recipient had no impact on graft or patient survival.

The influence of CYP3A5 in renal transplantation has been established for some time now however the impact this polymorphism has on tacrolimus in liver transplantation is only just beginning to emerge. It seems that donor expression carries the greater influence than native genotype and it may well

have more clinical implications with acute rejection which has not been seen in studies of renal transplant patients.

Early studies of acute rejection following liver transplantation found that it was a common event arising in up to 65 % of liver transplant patients within the first year, the majority of which occurred in the first 3 months [201]. Another review of 18 studies reported acute rejection rates of between 24 – 80% (mean 49%) [202]. In liver transplantation the clinical impact of acute rejection on graft and patient survival is thought to be minimal as it is easily reversible with steroid boluses in the majority of cases. While there are some more recent studies that suggest acute cellular rejection in liver transplant patients may, in fact, negatively impact graft and patient survival [203] the majority of the current evidence does not support this.

This study found that CYP3A5 expression in donor livers was associated with significantly higher rates of acute rejection, however this appeared to happen late after transplantation rather than within the first 3 months as one might expect. Acute rejection which occurs late has been shown to negatively impact on graft and patient survival [204, 205]. There could be several reasons for late rejection but a favoured opinion is that it occurs due to changes in immunosuppression, either the stopping of steroids, the reduction of CNIs or both. Other independent risk factors that have been

reported are a young age, female gender and an autoimmune pathology prior to liver transplantation [206]. A detailed multivariate analysis is out with the remit of this study however these factors should be borne in mind when interpreting the results of increased late acute rejection in the patients whose donor livers are CYP3A5 expressers.

Chapter V

Pharmacogenomics and Kidney & SPK Transplantation

5 PHARMACOGENOMICS AND KIDNEY & SPK TRANSPLANTATION

5.1 Background

In renal transplantation, careful HLA matching alongside a robust immunosuppressant regimen is required to minimise the risk of organ rejection. Calcineurin inhibitors (CNI) have been the backbone of immunosuppression for renal transplant patients and currently Tacrolimus is the most commonly used agent.

Given the narrow therapeutic index of Tacrolimus, therapeutic drug monitoring (TDM) reduces the risk of a patient developing tacrolimus toxicity or being inadequately immunosuppressed. However, there is considerable variability between individual patients as well as within an individual recipient, making a standard dosing protocol somewhat problematic, some individuals requiring significantly higher doses in order to achieve the pre-determined therapeutic levels of tacrolimus. This phenomenon was noted more frequently in black patients prompting investigation into a genetic basis for the increased dose requirements [100]. Several genetic polymorphisms including single nucleotide polymorphism of

the cytochrome CYP3A5, ABCB1 and CYP3A4*22 genes have been reported to influence tacrolimus pharmacokinetics [100] [124] [105, 207].

This chapter will examine the effect of the expression of SNPs of CYP3A5 (6898 A>G), ABCB1 (3435C>T) and CYP3A4*22 (15389 C>T) on tacrolimus dose requirements and trough levels in a cohort of Scottish renal transplant patients with particular emphasis on clinical impact in terms of acute rejection, delayed graft function, renal function and in graft and patient survival. A smaller cohort of simultaneous pancreas-kidney (SPK) patients has also been genotyped for these 3 SNPs and the correlations with tacrolimus dose requirements, trough levels and clinical end points explored.

5.2 Chapter Methodology

185 renal transplants and 32 SPK transplants with stored frozen DNA samples available for genotyping were included in this study. DNA samples were genotyped for the CYP3A5 A6986G transition (rs776746), the ABCB1 C3435T transition (rs1045642) and the CYP3A4*22 C15389T transition (rs35599367) using the Taqman® genotyping assay and real-time polymerase chain reaction (RT-PCR) technique as described in the methods chapter.

Clinical data were collected from a variety of sources including medical case notes, electronic medical records and donor information records. This included gender, age, ethnic group, height, weight, BMI, renal disease diagnosis, medical co-morbidities, blood group, number of transplant, and virology status (HCV/HBV/HIV/CMV). Donor information included gender, age, ethnic group, height, weight, BMI, blood group, donor type (DBD/DCD/LIVE), cause of death, region, co-morbidities, renal function and virology status (HCV/HBV/HIV/CMV). Transplant information included the cold ischaemic time (CIT) and the HLA mismatch.

Tacrolimus dose and trough levels (C_0) were collected at a total of 14 time points: 3 time points in weeks 1-3, 2 time points in the 4^{th} week and then at 3, 6 and 12 months. The time to reach the therapeutic C_0 tacrolimus level was recorded. In addition, renal function (MDRD creatinine) was recorded at each of the time points. Biopsy proven acute rejection episodes, graft survival and patient survival were recorded and significant post operative complications were noted.

5.3 Chapter Results

5.3.1 Renal Transplant Recipient Demographics

59 female renal transplant recipients (31.9%) and 126 male recipients (68.1%) were included in this study. The majority of the recipients were Caucasian with 174 patients (94.1%) of the White – Scottish ethnic group, 5 patients (2.7%) of the White – other ethnic group, 5 patients (2.7%) of the Asian – Indian ethnic group and 1 patient (0.5%) of the Asian – Chinese ethnic group. The mean age of the renal transplant recipients was 47.20±13.42 years (range 18 – 78 years) with a mean height of 1.727±0.105 metres (range 1.49 – 1.93 meters) and a mean weight of 76.90±14.91 kg (range 42.20 – 111.30 kg).

For the majority of the patients this was their first renal transplant, 153 (82.7%). There were 26 patients (14.1%) receiving their second kidney, 5 patients (2.7%) receiving their 3rd kidney transplant and a single patient (0.5%) who received their 4th renal transplant. All patients were transplanted between 30 Jan 2008 and 24 Aug 2012.

5.3.2 Renal Disease

There was considerable variation in the disease processes that led to end stage renal disease. The most common cause of end stage renal disease was IgA nephropathy [38 out of 185 patients (20.5%)] followed by adult polycystic kidney disease [27 patients (14.6%)]. The causes of renal failure in this cohort are detailed in Table 5.1 below.

Table 5.1 Renal disease diagnosis in kidney transplant patients in this cohort

Renal Diagnosis	**Number (n)**	**Percentage (%)**
Uncertain aetiology	14	7.60%
Focal segmental glomerulosclerosis with nephrotic syndrome	6	3.20%
Adult polycystic kidney disease	27	14.60%
Alport's syndrome	2	1.10%
IgA nephropathy	38	20.50%
Alagille (prune belly) syndrome	1	0.50%
Pyelonephritis with vesicoureteric reflux	14	7.60%
Congenital obstructive vesicoureteric reflux	6	3.20%
Glomerulonephritis	10	5.40%
Diabetic glomerulosclerosis/nephropathy	13	7%
Interstitial nephritis	2	1.10%
Crescentric glomerulonephritis	1	0.50%
Pseudomembranous glomerulonephritis	1	0.50%
Hypertensive renal vascular disease	6	3.20%
Recurrent pyelonephritis	5	2.70%
Polyarteritis	2	1.10%
Congenital renal hypoplasia	5	2.70%
Drug induced renal failure	2	1.10%
Laurence Moon Biedl Syndrome	1	0.50%
Haemolytic uraemic syndrome	2	1.10%
Goodpasture's syndrome	2	1.10%
Wegner's granulomatosis	2	1.10%
Familial nephropathy	1	0.50%
Neurogenic bladder and reflux nephropathy	4	2.20%
Microscopic polyangitis	1	0.50%
Chronic pyelonephritis	6	3.20%
ANCA positive glomerulonephritis	2	1.10%
Bilateral hydronephrosis	1	0.50%
Lupus erythematosis	2	1.10%
Membrano-proliferative glomerulonephritis	2	1.10%
Fibrillary glomerulonephritis	1	0.50%
Mesangiocapillary glomerulonephritis	2	1.10%
Medullary cystic disease	1	0.50%
Total	**185**	**100%**

Sixteen patients (8.6%) underwent pre-emptive renal transplantation whilst all other patients were on dialysis [111 patients (60.0%) on haemodialysis, 43 patients (23.2%) on peritoneal dialysis].

5.3.3 HLA Mis-matching

In the United Kingdom kidneys are allocated based on an assigned mismatch level of HLA antigens at the A, B and DR loci (as well as many other clinical factors). The mismatch level is 1-4 with a level 1 mismatch equal to a 000 mismatch for the 3 HLA antigens. Therefore, the best matched kidneys are level 1 and the worst are level 4.

The table below summarises the mismatch levels for the patients in this cohort.

Table 5.2 HLA mis-match level

HLA mismatch level	**Number of patients**	**Percentage**
1	29	15.8%
2	37	20.1%
3	95	51.6%
4	23	12.5%

5.3.4 Donor Demographics

The mean donor age was 48.16±15.31 years. There were 97 female donors (52.4%) and 87 male donors (47.0%). Donors were classified as locally retrieved (by the Scottish Organ Retrieval Team or a live donor) or imported from elsewhere in the UK. Accordingly, there were 96 locally retrieved kidneys (51.9%) and 89 imported kidneys (48.1%). 88 donors (47.6%) donated following brain stem death (DBD), 35 donors (18.9%) donated following circulatory death (DCD) and 62 were live donors (33.5%).

5.3.5 Ischaemic times

The mean cold ischaemic time (CIT) was 09:45±05:55 (hh:mm) [range: 00:44-23:04]. The mean anastomosis time (also known as the second warm ischaemic time [SWIT]) was 00:39±00:14 [ranging from 00:18 to 02:02]

5.3.6 Recipient and donor Virology status

72 (38.9%) recipients had CMV positive virology. All recipients were hepatitis C virus (HCV) negative, human immunodeficiency virus (HIV)

negative and HBV surface antigen negative. There were 89 donors who were CMV positive (48.1%). All donors were HCV, HIV and HBV negative.

5.3.7 CYP3A5 Genotype in Renal Transplant Recipients

149 patients (80.5%) who were of the GG genotype (*3/*3). 36 patients made functional CYP3A5 [30 patients (16.2%) were heterozygotes and 6 patients (3.2%) were wild type homozygotes expressing the AA genotype (*1/*1)].

5.3.8 Tacrolimus Dose Requirements Related to CYP3A5 Genotype

Initially both CYP3A5 expressers (GA/AA, *3/*1 or *1/*1) and non-expressers (GG, *3/*3) were prescribed comparable doses of tacrolimus 6.23±2.167 vs 6.59±2.327 mg (p=0.436, one-way ANOVA). However, after this initial dose, the non-expressers (GG, *3/*3) achieved a therapeutic tacrolimus level straight away (8.60±4.942 µg/L) compared with the CYP3A5 expressers (GA, *3/*1 or AA, *1/*1) who achieved a lower tacrolimus trough level (4.18±2.463 µg/L), p<0.0001, one-way ANOVA. The dose requirements during the 12 months are illustrated in Figure 5.1 below. In order to reach target levels, CYP3A5 expresser group requires a significantly higher dose (8.03±2.50 mg) compared with the non-expresser group (7.08±2.14 mg), p=0.034, one-way

ANOVA as early as the second dose and that as time passes there is a clear difference between the two groups in terms of dose requirement.

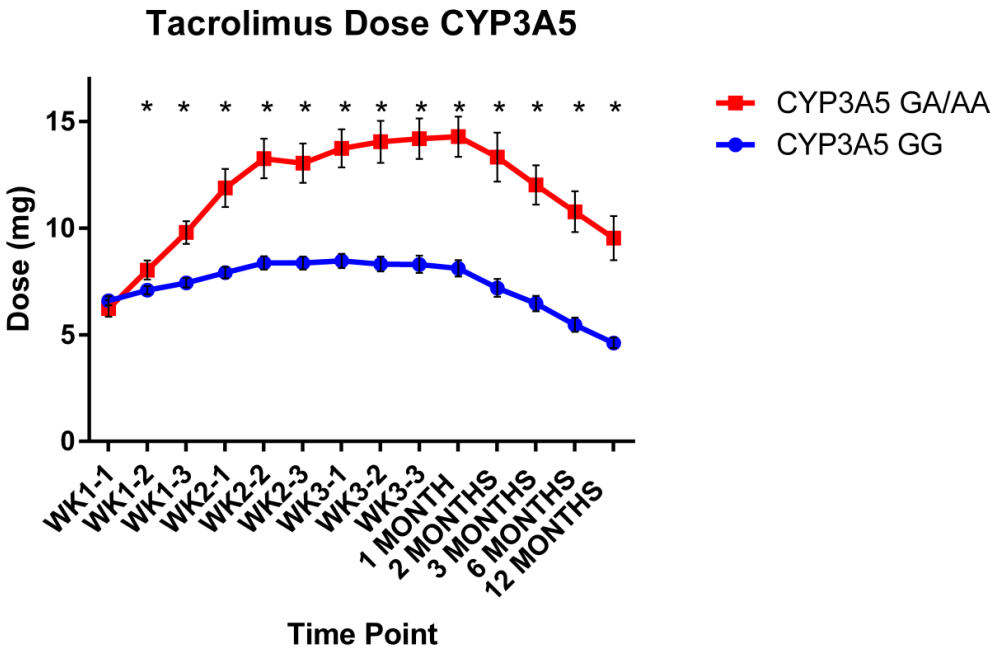


Figure 5.1 Tacrolimus dose requirements following kidney transplantation relative to CYP3A5 expression at each study time point. * denotes a significant result $p<0.05$.

5.3.9 Tacrolimus Levels Relative to CYP3A5 Expression

The tacrolimus trough level in CYP3A5 expressers was significantly lower immediately post transplant and remained sub-therapeutic (despite an increase in the dose) until the start of the 3rd week following transplantation (GG 9.28±3.33 vs GA/AA 7.78±4.61 µg/L, $p=0.061$, one-way ANOVA). The

mean tacrolimus trough levels during the 12 months are shown in Figure 5.2 below.

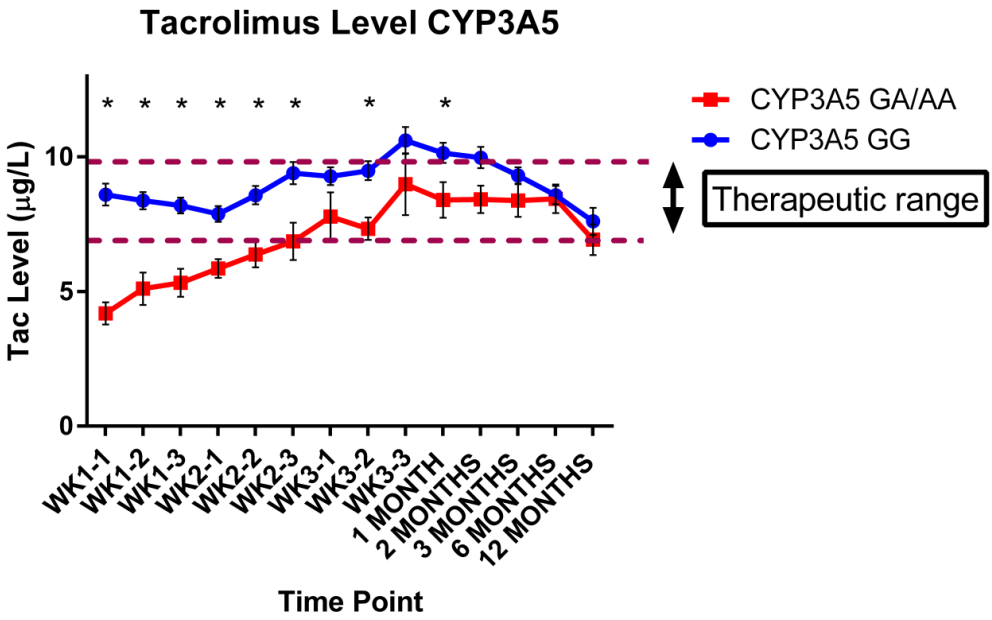


Figure 5.2 Tacrolimus trough concentration (C_0) following kidney transplantation relative to CYP3A5 expression at each study time point. * denotes a significant result p<0.05.

5.3.10 Dose-corrected trough levels Relative to CYP3A5

CYP3A5 expresser patients had significantly lower dose corrected tacrolimus levels compared with non-expressers immediately post-transplant (0.68±0.387 vs 1.39±0.817 µg/L per mg, p <0.0001, one-way ANOVA) and throughout the first 12 months following transplantation. This sustained

difference suggests that individuals who are expressers of CYP3A5 will have increased tacrolimus clearance compared to non-expressers thus having reduced tacrolimus exposure on a mg for mg basis, as shown in Figure 5.3 below.

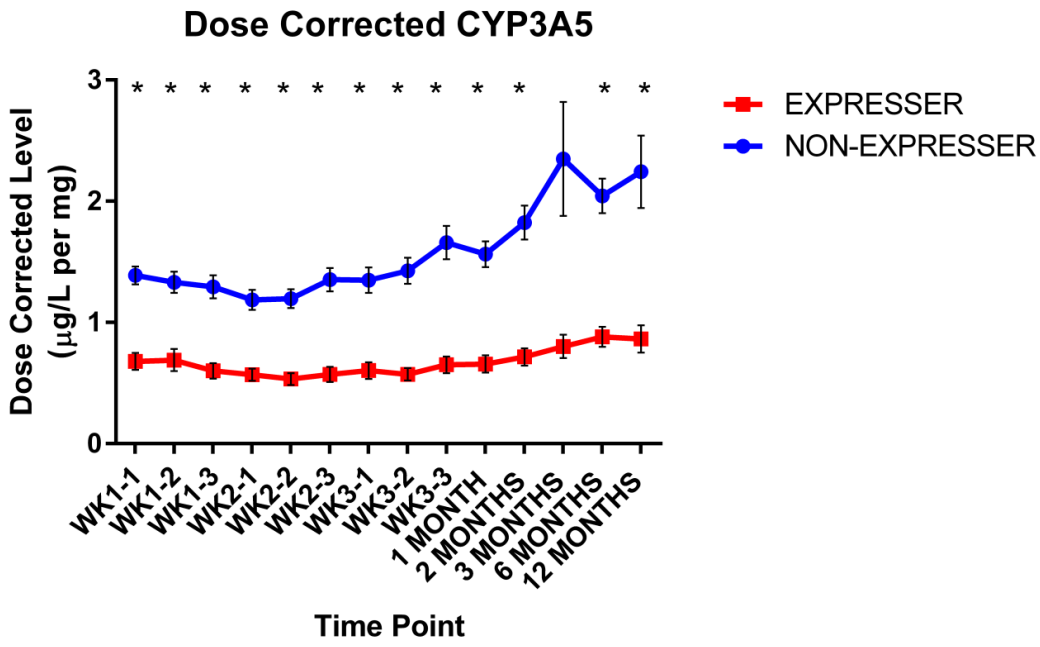


Figure 5.3 Dose corrected tacrolimus trough concentration (C_0/D) relative to CYP3A5 expression in kidney transplant patients. * denotes significant result $p<0.05$

5.3.11 Renal Function Relative to CYP3A5

Given that CYP3A5 expression leads to significantly higher doses to achieve a therapeutic level, it is important to assess if this has a detrimental impact on the renal function.

The creatinine was comparable for both expressers and non-expressers of CYP3A5 at each of the time points. (Figure 5.4)

The initial eGFR differed significantly with a lower initial post transplant eGFR in the CYP3A5 expressers (15.31±11.79 ml/min/1.73m^2) compared with 22.65±17.91 ml/min/1.73m^2 in the non-expressers p=0.023, one-way ANOVA). However, all subsequent measurements remained comparable throughout the duration of the follow-up. The corresponding eGFR at 12 months was comparable between both groups [46.20±15.89 ml/min/1.73m^2 in the expressers and 48.73 ml/min/1.73m^2 in the non-expresser, p=0.520, one-way ANOVA]. (Figure 5.5)

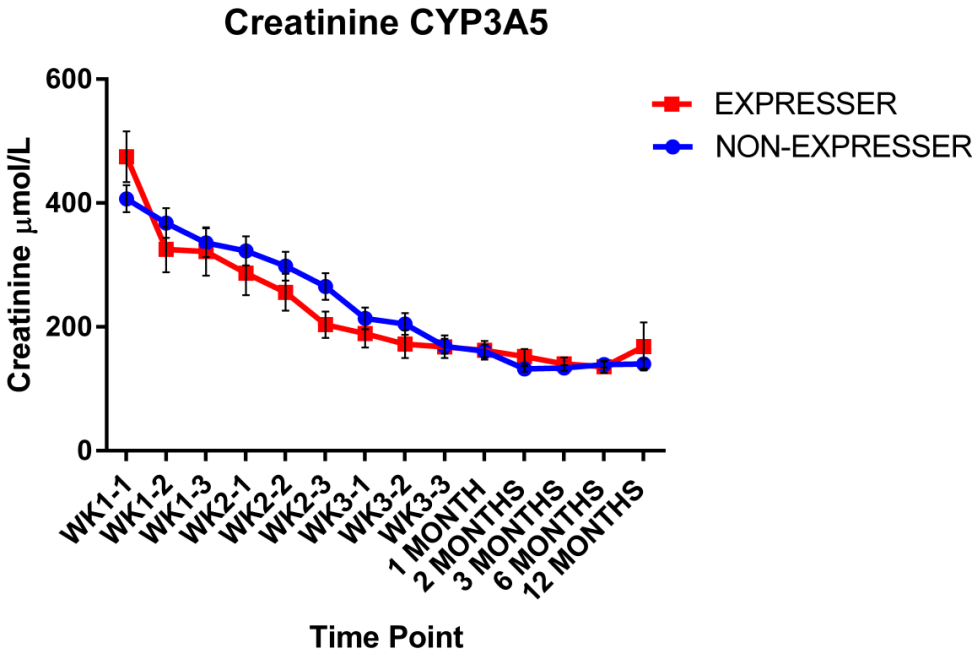


Figure 5.4 Creatinine at each time point relative to CYP3A5 expression following kidney transplantation. * denotes significant result p<0.05

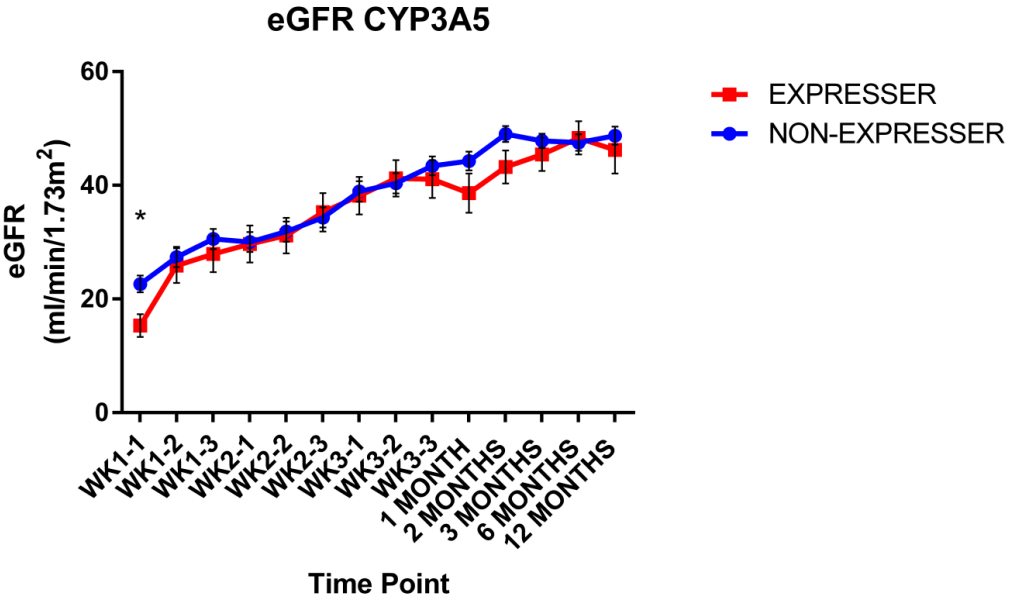


Figure 5.5 eGFR at each time point relative to CYP3A5 expression following kidney transplantation. * denotes significant result p<0.05

5.3.12 ABCB1 Genotype in Relation to Renal Transplant Patients

ABCB1 genotypes were more evenly distributed with 34 patients (18.4%) expressing the CC genotype, 85 patients (45.9%) heterozygotes with a CT genotype and 65 patients (35.1%) who expressed the TT genotype. There was a single patient where the genotype was not identified.

5.3.13 Tacrolimus Dose Requirements in Relation to ABCB1

All transplant recipients were prescribed comparable doses of tacrolimus initially with those of the CC genotype prescribed 6.54±2.51 mg, those of the CT genotype prescribed 6.56±2.40 mg and those patients of the TT genotype prescribed 6.45±2.07 mg. (p=0.990, one-way ANOVA).

There was no significant difference in tacrolimus dose between the three genotypes of ABCB1at any of the time points from the time of transplantation up to the 12 month time point where the tacrolimus dose was comparable between all 3 genotypes [5.23±3.38 mg for the CC genotype, 5.48±2.78 mg for the CT genotype and 5.47±3.91 mg for the TT genotype, p=0.966, one-way ANOVA].

Figure 5.6 below shows the dose requirements for the three different genotypes of ABCB1 C3435T at each of the time points.

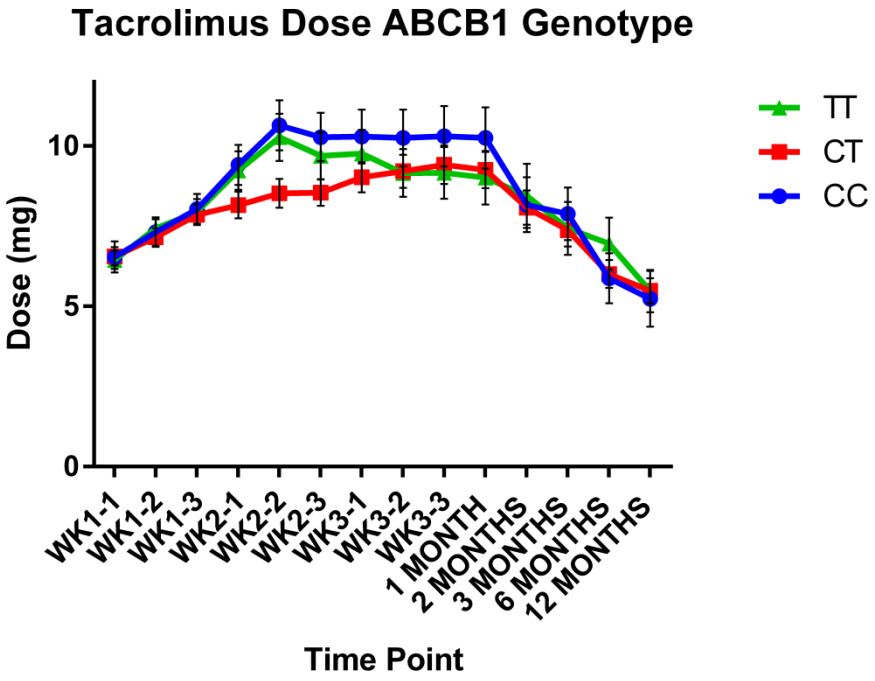


Figure 5.6 Tacrolimus dose requirements relative to ABCB1 genotype following kidney transplantation at each time point. * denotes significant result $p<0.05$

5.3.14 Tacrolimus Trough Levels in Relation to ABCB1

There was no difference between the tacrolimus trough levels for any of the three genotypes of ABCB1 at any time point during follow-up. The initial tacrolimus trough level in recipients of the CC genotype following

transplantation was 7.70±4.33 µg/L, for patients of the CT genotype it was 7.57±4.50 µg/L and for patients of the TT genotype it was 8.00±5.68 µg/L (p=0.958, one-way ANOVA).

At 12 months following transplantation the tacrolimus trough levels remained comparable as shown in Figure 5.7 below. (p=0.237, one-way ANOVA).

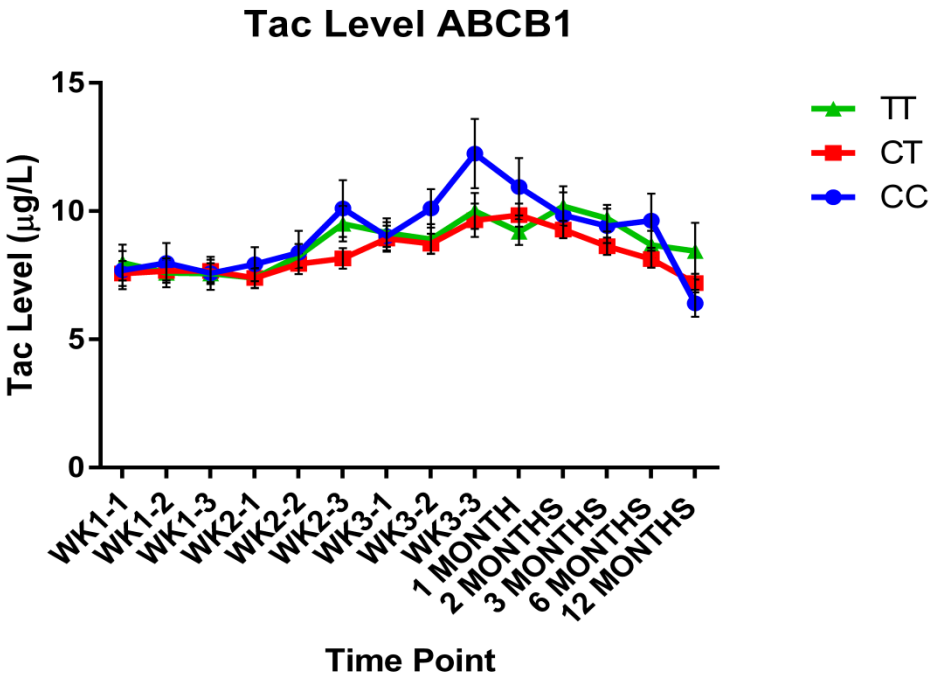


Figure 5.7 Tacrolimus trough concentration (C_0) following kidney transplantation relative to ABCB1 genotype at each time point. * denotes a significant result p<0.05

5.3.15 Dose Corrected Tacrolimus Level in Relation to ABCB1

Given that there was no significant difference in dose or trough level between any of the three genotypes, the dose corrected level of tacrolimus was comparable across the 3 genotypes of ABCB1. Figure 5.8 below shows the dose corrected tacrolimus level relative to ABCB1 genotype for each of the time points in the renal transplant patient cohort.

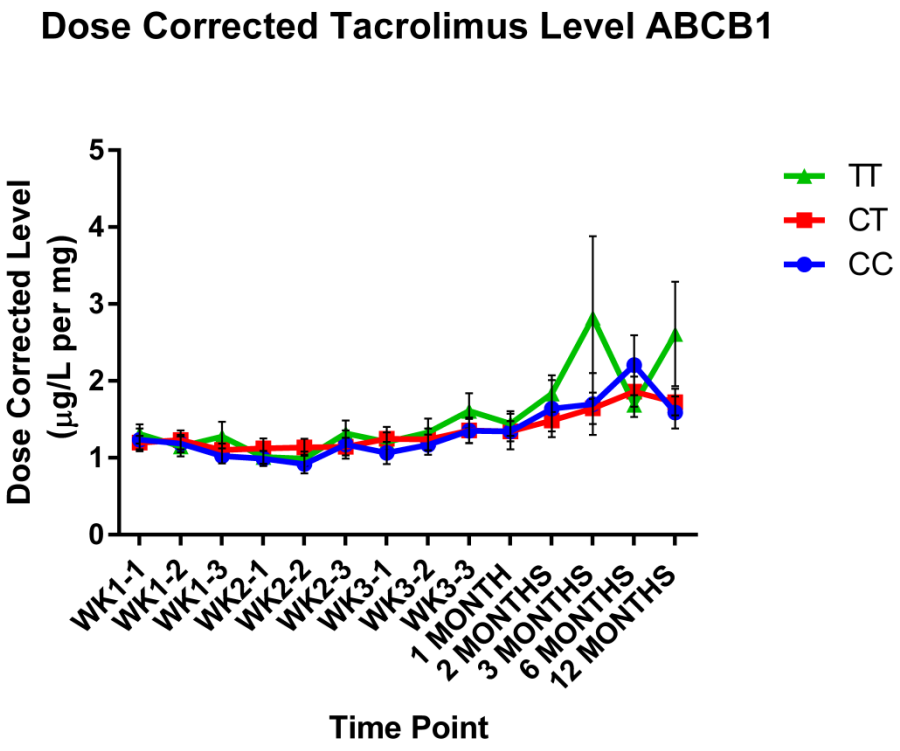


Figure 5.8 Dose corrected tacrolimus trough concentration (C_0/D) following kidney transplantation relative ABCB1 genotype at each time point.
* denotes a significant result $p<0.05$

5.3.16 Renal Function in Relation to ABCB1 Genotype

Given the lack of differences between the genotype in terms of drug exposure, renal function (creatinine) was comparable between these patients at all time points during the study apart from at 12 months where the CC genotype had a higher creatinine. (Figure 5.9)

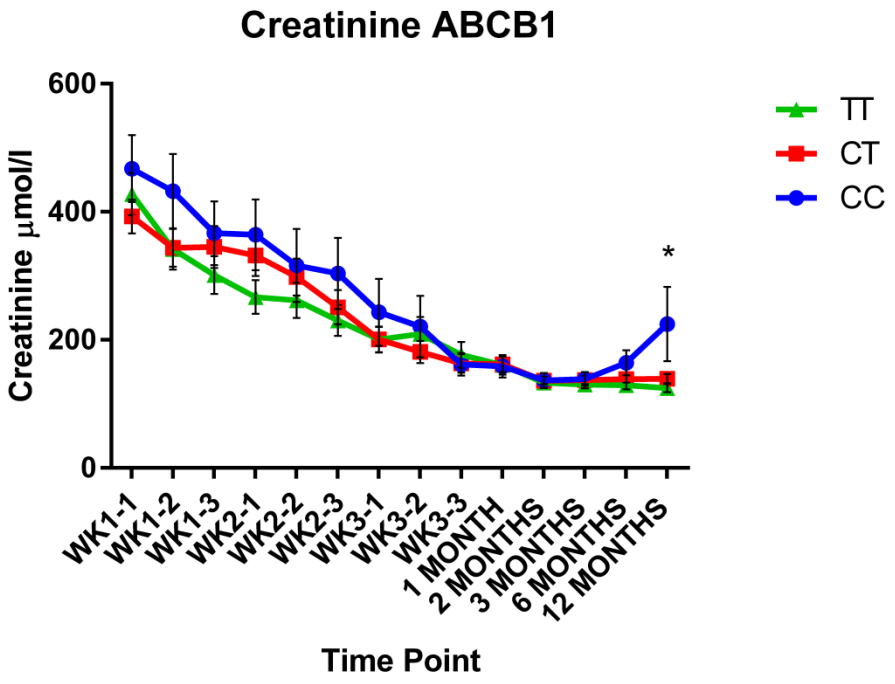


Figure 5.9 Creatinine at each time point following kidney transplantation relative to ABCB1 genotype. * denotes a significant result p<0.05

The only difference noted in eGFR was at 6 and 12 months when the eGFR of the CC genotype patients was lower compared with the other two genotypes [39.83±14.53 ml/min/1.73m^2, vs 47.48±11.38 ml/min/1.73m^2 (CT) and 50.62±11.64 ml/min/1.73m^2 (TT), p=0.030, one-way ANOVA and respectively 40.08±19.84 ml/min/1.73m^2 vs 47.15±11.31 ml/min/1.73m^2 (CT) and 52.73±12.71 ml/min/1.73m^2 (TT) p=0.017, one-way ANOVA]. While the difference in renal function of the CC genotype appeared to be statistically significant, the clinical significance is less certain. The eGFR at each of the time points is shown in Figure 5.10 below.

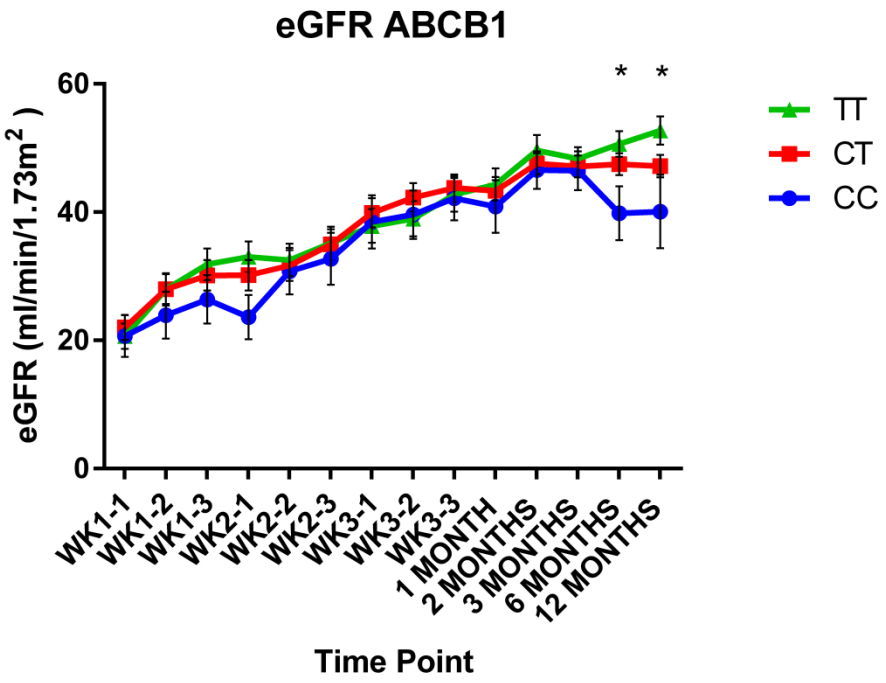


Figure 5.10 eGFR following kidney transplantation at each time point relative to ABCB1 genotype. * denotes a significant result p<0.05

*5.3.17 CYP3A4*22 in Relation to Renal Transplant Patients*

171 patients (92.4%) had the CC genotype for CYP3A4*22, 12 patients (6.5%) were of the CT genotype and a single patient (0.5%) was of the TT genotype. There was one patient in the group where the CYP3A4*22 genotype was not determined. Due to the very low allele frequency of the T allele, those with one copy of the T allele (CT genotype) and both copies of the T allele (TT genotype) were analysed together as a single group and named T allele expressers, [n=13 (7.03%)].

*5.3.18 Dose Requirements in Relation to CYP3A4*22*

The initial dose of tacrolimus was similar for both the T allele expresser group and the non-T allele expresser group (CC genotype) (p=0.403, one-way ANOVA). However, by the end of the second week patients who expressed a T allele tended to have a lower dose requirement although, with the exception of the 3 month time point, this never reached statistical significance (Figure 5.11)

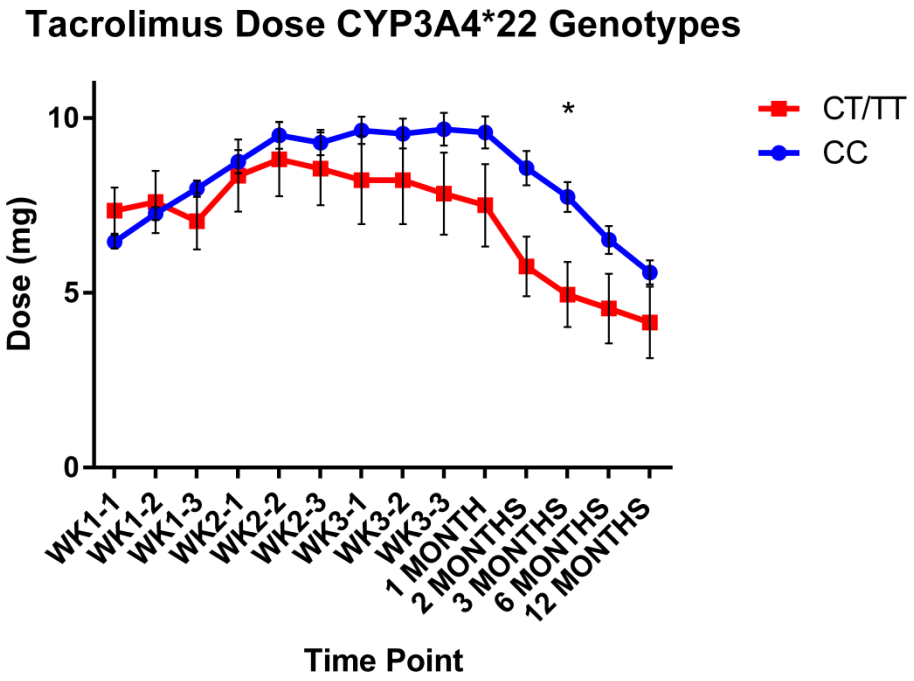


Figure 5.11 Tacrolimus dose requirements following kidney transplantation at each time point relative to CYP3A4*22 T allele expression at each time point. * denotes a significant result p<0.05

*5.3.19 Tacrolimus Trough Levels in Relation to CYP3A4*22*

Despite the slight difference in the dose requirements, the tacrolimus trough levels were comparable at all time points as shown in Figure 5.12 below.

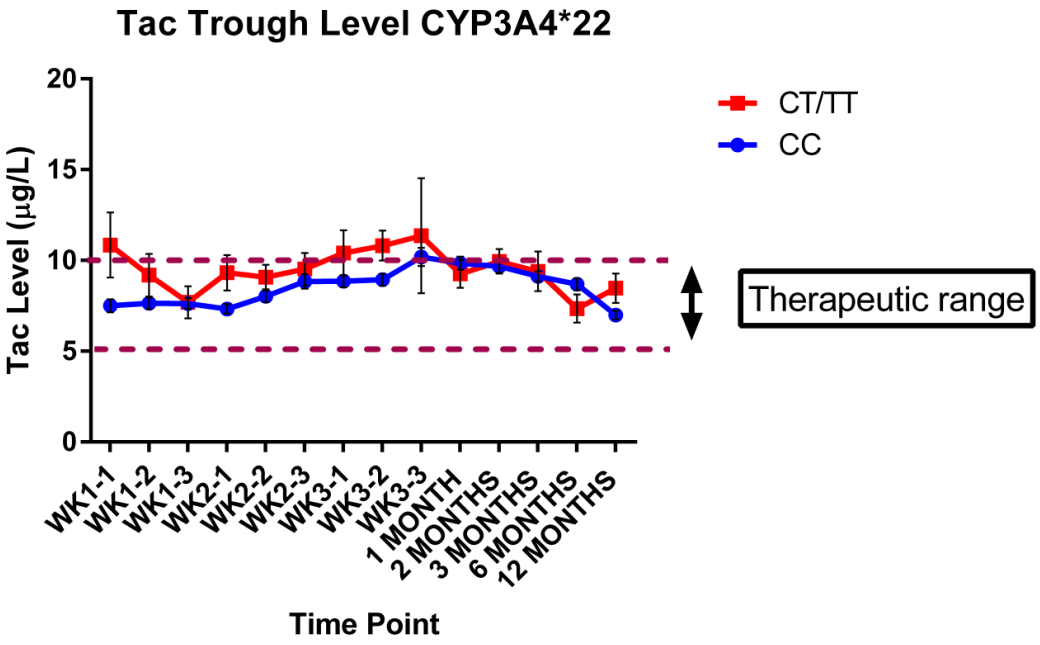


Figure 5.12 Tacrolimus trough concentration (C_0) at each time point following kidney transplantation relative to CYP3A4*22 T allele expression. * denotes a significant result $p<0.05$

*5.3.20 Dose Corrected Tacrolimus Level Relative to CYP3A4*22*

Similarly, the dose corrected tacrolimus levels showed no significant difference between those individuals who expressed a T allele of CYP3A4*22 (CT/TT) and those who did not (CC) ($p=0.471$ one-way ANOVA). The slightly higher dose corrected level seen after transplantation in the CT/TT genotype group was maintained throughout but it was not statistically significant with the exception of the 12 month time point where the dose corrected level in the CC genotype group was 1.63±0.99 µg/L per mg and the CT/TT genotype

was 3.49±3.83µg/L per mg, p<0.0001, one-way ANOVA. Although most of the dose corrected levels for the CYP3A4*22 T allele expressers were slightly higher, there is no obvious reason for the difference at 12 months and therefore, this result should be interpreted with caution. Figure 5.13 below shows the dose corrected tacrolimus level at each of the time points relative to T allele expression of CYP3A4*22.

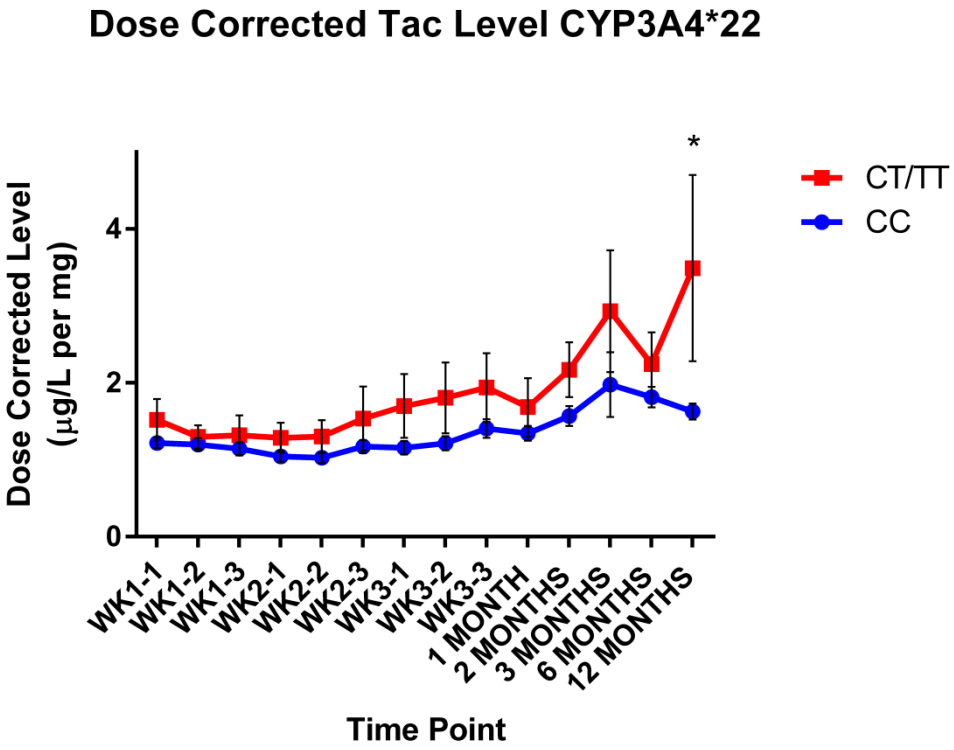


Figure 5.13 Dose corrected tacrolimus trough concentration (C_0/D) at each time point following kidney transplantation relative to CYP3A4*22 T allele expression. * denotes a significant result p<0.05

*5.3.21 Creatinine and eGFR in Relation to CYP3A4*22*

The renal function, measured by the serum creatinine and eGFR, showed comparable results between the CYP3A4*22 T allele expresser group and the non- expresser group at most of the time points. The notable exceptions were at the WK3-1, WK3-2 and WK3-3 time points where the creatinine was significantly higher in the non-expresser group than the T allele expresser group at each of these time points. (WK3-1 CC 210.42±163.79 vs CT/TT 142.78±65.58 µmol/L, p=0.014, WK3-2 CC 197.62±153.09 vs CT/TT 137.20±47.72 µmol/L, p<0.0001 and WK3-3 CC 165.27±94.60 vs CT/TT 139.20±52.78 µmol/L, p<0.0001, one-way ANOVA). The reason for this significant difference at these specific time points is unclear however given the low number of recipients who express a T allele (n=10), this may be a statistical anomaly rather than clinically relevant given that the corresponding eGFR values at the same time points were comparable (WK3-1 CC, 38.69±1.63 vs CT/TT 43.75±16.24 ml/min/1.73m^2, p=0.165, WK3-2 CC, 40.21±16.91 vs CT/TT 46.30±14.78 ml/min/1.73m^2, p=0.190 and WK3-3 CC, 43.06±15.17 vs CT/TT 45.60±15.31 ml/min/1.73m^2, p=0.056, one-way ANOVA.

Figure 5.14 below details the creatinine levels at each of the time points and similarly Figure 5.15 details the eGFR at each of the time points.

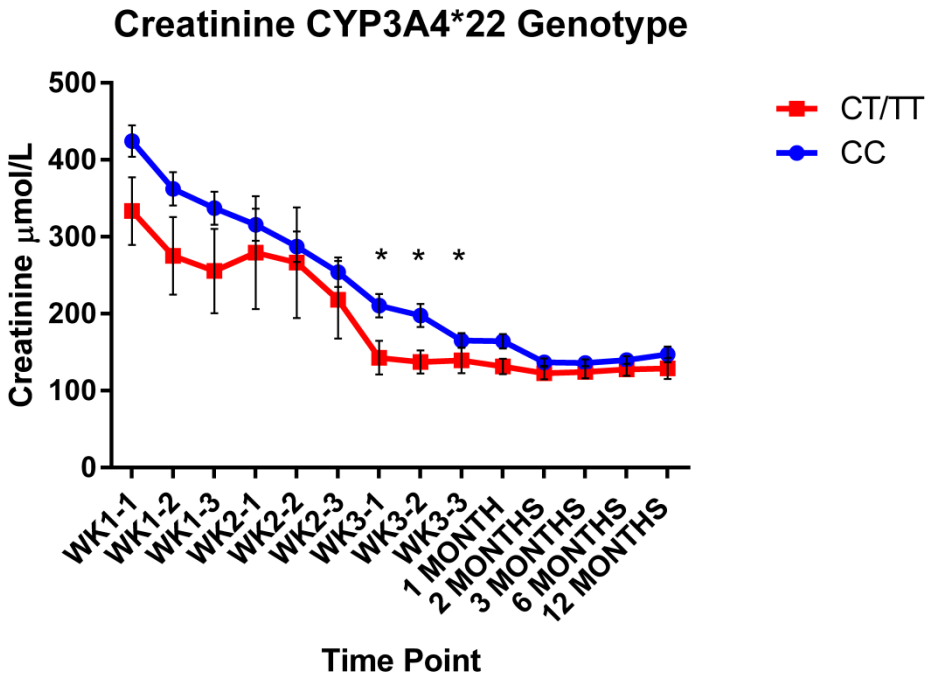


Figure 5.14 Creatinine at each time point following kidney transplantation relative to CYP3A4*22 T allele expression. * denotes a significant result $p<0.05$

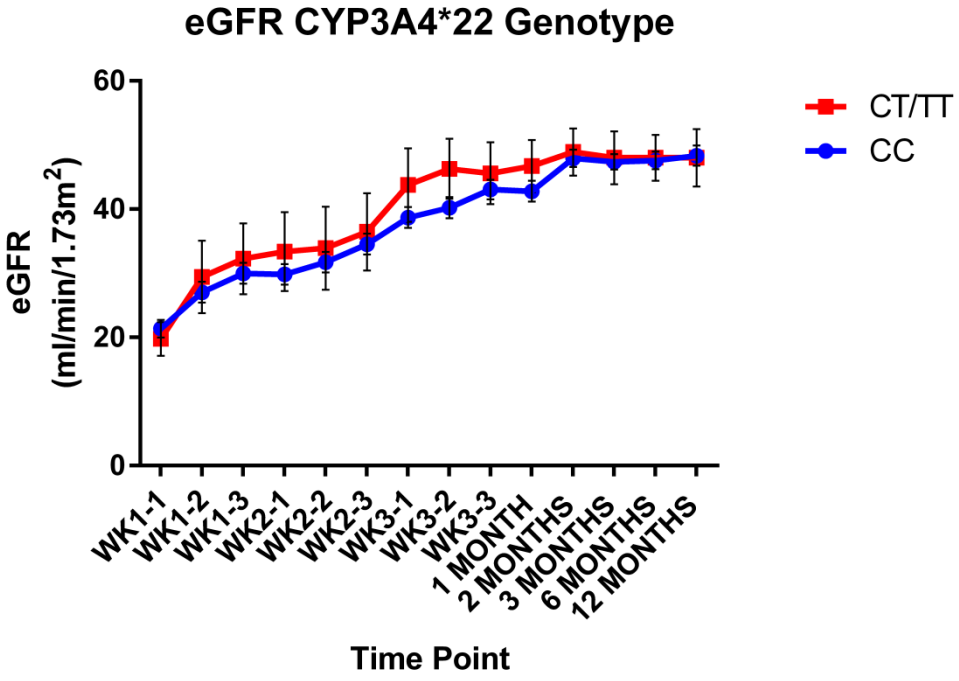


Figure 5.15 eGFR at each time point following kidney transplantation relative to CYP3A4*22 T allele expression. * denotes significant result p<0.05

5.3.22 Time to Reach Therapeutic Tac Levels and Genotype

The time taken to reach a therapeutic tacrolimus trough level does not necessarily in itself represent a detrimental clinical end point but nonetheless a delay in reaching a therapeutic tacrolimus level could pose a significant risk of acute rejection due to sub-therapeutic tacrolimus levels for a prolonged period of time in the immediate post-transplant period.

To that end, the number of days until the first therapeutic trough level of tacrolimus was reached for each patient was recorded with a therapeutic level being 5 μg/L or greater.

5.3.23 CYP3A5 Genotype and Time to Therapeutic Level

Renal transplant recipients of the CYP3A5 GG (*3/*3) genotype took 3.17±2.84 days to reach a therapeutic tacrolimus trough level compared with 7.94±5.26 days of the CYP3A5 GA/AA (*3/*1 or *1/*1) genotype ($p<0.0001$, one-way ANOVA) (Figure 5.16 below).

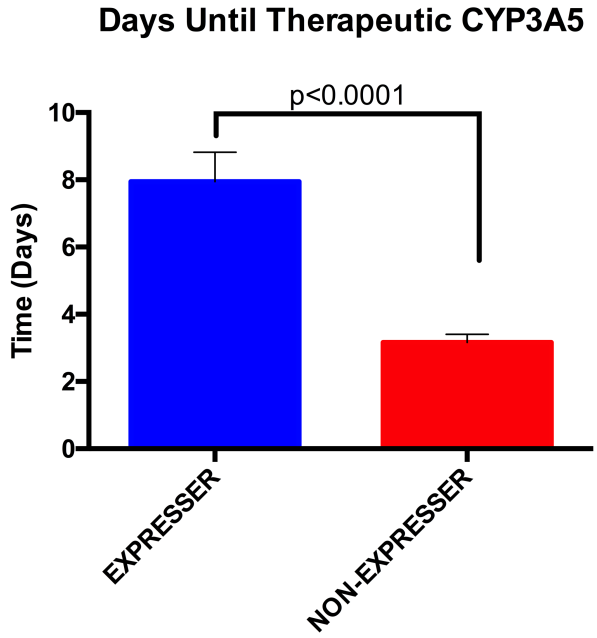


Figure 5.16 Time taken in days following kidney transplantation to reach a therapeutic trough concentration (C_0) relative to CYP3A5 expression.

5.3.24 ABCB1 Genotype and Time to Therapeutic Level

In contrast to CYP3A5 expression, there was no difference in the number of days until a therapeutic level of tacrolimus was reached between any of the 3 different genotypes of ABCB1 (3.88±3.96 days for CC genotype vs 4.23±3.94 days for CT vs 4.13±4.00 for TT genotype, p=0.925, one-way ANOVA) as shown in Figure 5.17 below.

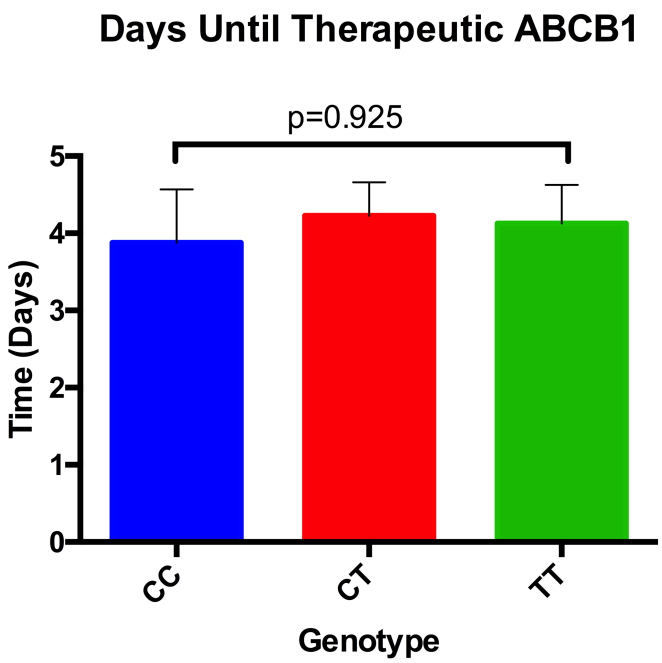


Figure 5.17 Time taken in days following kidney transplantation to reach a therapeutic trough concentration (C_0) relative to ABCB1 genotype.

*5.3.25 CYP3A4*22 Genotype and Time to Therapeutic Level*

Similar to the results of the ABCB1 genotype, there was no difference in the time taken to reach a therapeutic tacrolimus trough level between patients who expressed a T allele of CYP3A4*22 (CC genotype) and those who did not (CT/TT genotype), 4.17±3.99 days for the CC genotype compared with 3.62±3.45 days for CT/TT genotype, p=0.770, one-way ANOVA as shown in Figure 5.18 below.

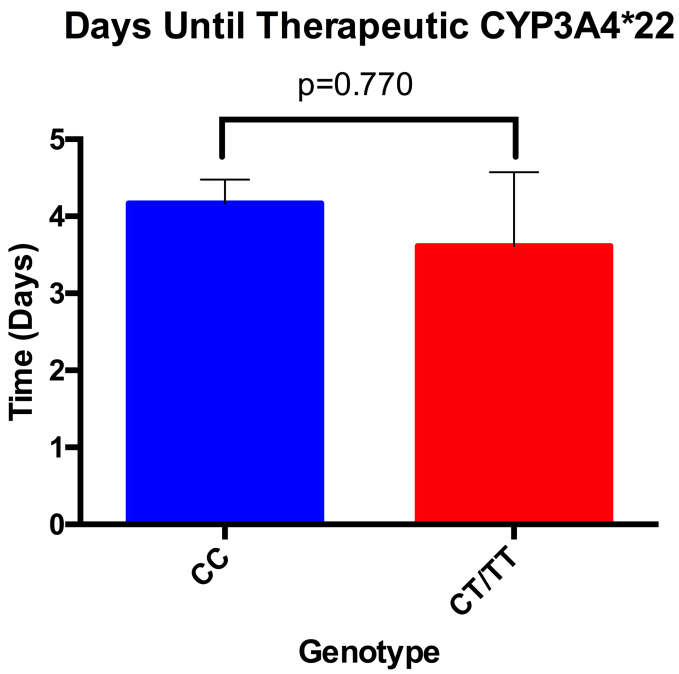


Figure 5.18 Time taken in days following kidney transplantation to reach a therapeutic trough concentration (C_0) relative to CYP3A4*22 T allele expression.

5.3.26 Acute Rejection Relative to Genotype Expression

With CYP3A5 expression resulting in a significantly longer time until a therapeutic tacrolimus level is reached, there is potential that renal transplant patients who express CYP3A5 could be at a higher risk of acute rejection due to under immunosuppression. To investigate whether this was the case, and whether any of the different genotypes of the 3 SNPs had an impact on the incidence of biopsy proven acute rejection (BPAR), data was recorded for each patient on the incidence of biopsy proven acute rejection and at what time this occurred following transplantation. The incidence of BPAR was compared by the different genotypes of each of the 3 SNPs.

5.3.27 Acute Rejection Relative to the CYP3A5 Genotype

The overall rate of BPAR in the entire cohort irrespective of genotype was 27 patients out of 185 giving a rate of 14.6%. Looking specifically at the distribution relative to CYP3A5 genotype, there was no significant difference observed between CYP3A5 non-expressers (22/149 [14.8%]) and the expressers (5/36 [13.9%]), p=0.894, Chi-Square test. Biopsy proven acute rejection within 30 days of transplant was more common in the CYP3A5 non-expresser group (17/149 [11.4%]) compared with the expresser group

(2/36 [5.6%]) but this was not statistically significant, p=0.299, Chi-Square test.

There was no significant difference in the rate of BPAR at 90 days post transplant or at 365 days post transplant either, as is shown in Figure 5.19 below.

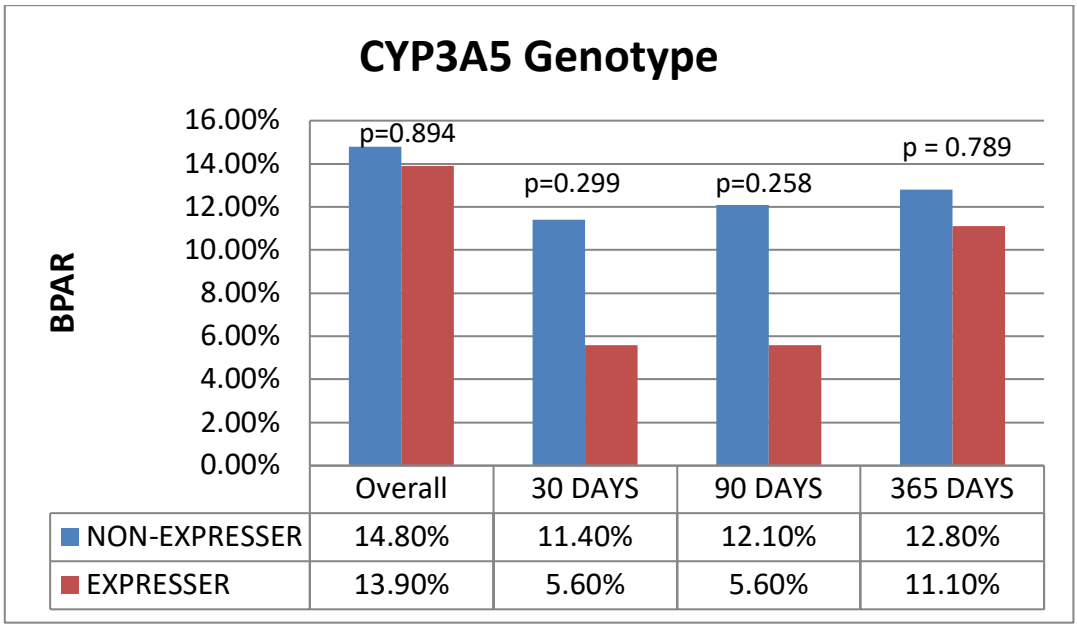
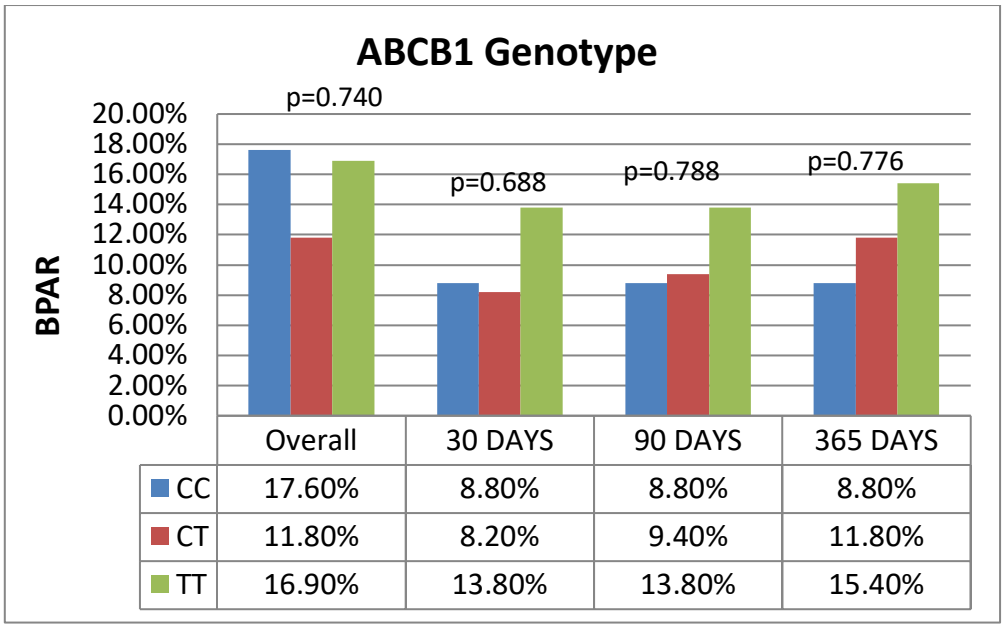


Figure 5.19 Percentage of patients who develop biopsy proven acute rejection following kidney transplantation overall and at 30, 90 and 365 days relative to CYP3A5 expression.

5.3.28 Acute Rejection Relative to ABCB1 Genotype

The distribution of BPAR across the different genotypes of ABCB1 did not show any significant difference overall with 6/34 (17.6%) BPAR in the CC genotype group, 10/85 (11.8%) in the CT genotype group and 11/65 (16.9%) in the TT genotype group, p=0.740. There was also no difference in the incidence of BPAR across the three genotypes of ABCB1 at 30 days, 90 days or 365 days following transplantation as shown in Figure 5.20 below.



	Overall	30 DAYS	90 DAYS	365 DAYS
CC	17.60%	8.80%	8.80%	8.80%
CT	11.80%	8.20%	9.40%	11.80%
TT	16.90%	13.80%	13.80%	15.40%

Figure 5.20 Percentage of patients who develop biopsy proven acute rejection following kidney transplantation overall and at 30, 90 and 365 days relative to ABCB1 genotype.

*5.3.29 Acute Rejection Relative to CYP3A4*22 Genotype*

Biopsy proven acute rejection in relation to CYP3A4*22 T allele expression is somewhat interesting as none of the patients who expressed a T allele (CT or TT genotype) had any episodes of BPAR. Indeed, all of the episodes of BPAR occurred in the CC genotype group with 27/171 in the CC genotype group (15.8%) having an episode of BPAR while 0/13 (0.0%) in the CT/TT group had BPAR. The very small numbers in the T allele expresser group compared to the whole cohort means this figure is not statistically significant with a p value of 0.274, Chi-Square test. The incidence of BPAR overall and at 30 days, 90 days and 365 days post renal transplant are shown in Figure 5.21 below.

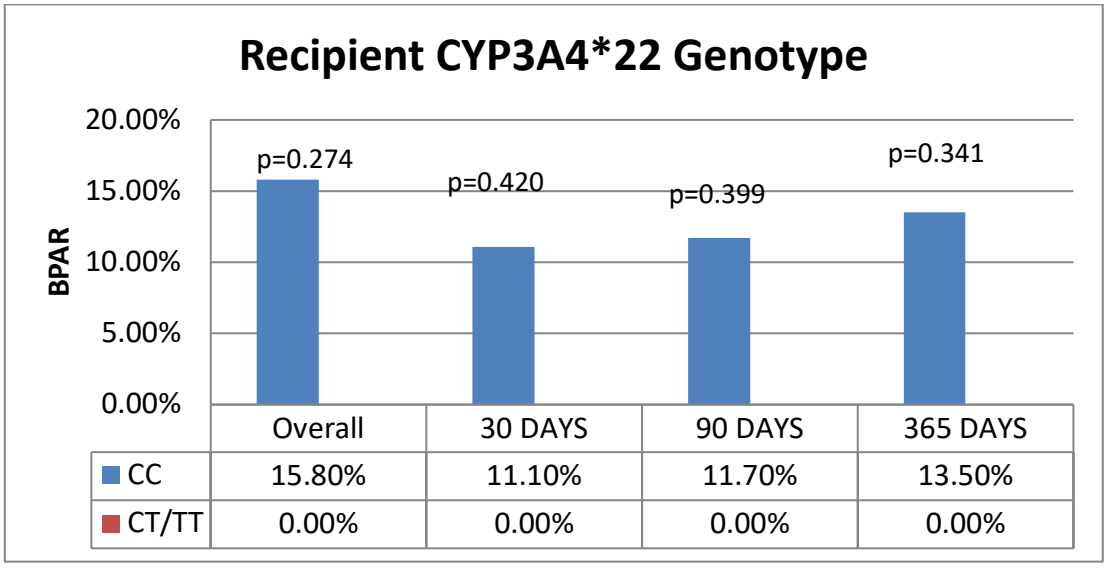


Figure 5.21 Percentage of patients who develop biopsy proven acute rejection following kidney transplantation overall and at 30, 90 and 365 days relative to CYP3A4*22 T allele expression.

5.3.30 Post Operative Complications Relative to Genotype

Given the correlation with trough levels, we evaluated the incidence of some of the complications according to the SNP of CYP3A5, ABCB1 and CYP3A4*22.

5.3.31 Delayed Graft Function

The overall incidence of delayed graft function in this cohort was 33.2%. There was no difference in the rate of DGF in the CYP3A5 genotype group between the non-expressers (49/149, 32.9%) and the expressers (12/35, 34.3%), p=0.874 Chi-Square test. The incidence of DGF was also comparable between the 3 different genotypes of ABCB1 [CC genotype DGF incidence of 13/33 (39.4%), CT genotype incidence 27/85 (31.8%) and the TT genotype incidence 20/65 (30.8%), p=0.417 Chi-Square test]. DGF was also similar across the different genotypes of CYP3A4*22 with a 56/170 (32.9%) incidence in the CC genotype group and 4/13 (30.8%) in the CT/TT group, p=0.358 Chi-Square test.

5.3.32 New Onset Diabetes After Transplantation (NODAT)

The diabetogenic effects of immunosuppressive agents such as prednisolone and tacrolimus are well known therefore some patients will go on to develop diabetes following renal transplantation. New Onset Diabetes After Transplantation (NODAT) is defined as the development of Type II diabetes requiring medical pharmacotherapy with oral antihyperglycaemic medication or insulin after solid organ transplantation, where it was not present beforehand. This definition does not include diabetic patients who were controlled with oral antihyperglycaemic agents prior to transplantation that then went on to require insulin afterwards. There was no difference in the incidence of NODAT between those patients who were CYP3A5 non-expressers (8/144, 5.6%) and those who were expressers of which none developed NODAT, p=0.154 Chi-Square test. There were more patients of the TT genotype of ABCB1 (6/62, 9.7%) who went on to develop NODAT compared with those of the CC genotype (0/32, 0.0%) and those of the CT genotype (2/84, 2.4%), p=0.018 Chi-Square test (Linear-by-Linear Association). There were no patients with the CT/TT genotype of CYP3A4*22 who went on to develop NODAT and 8/166 (4.8%) of the CC genotype who developed NODAT, however this was not significant, p=0.418 Chi-Square test.

5.3.33 Post Operative Infection

Immunosuppression increases the risk of infection in all transplant patients in the perioperative period and in the long term due to reduced T cell activity. The incidence of post-operative bacterial infections, primarily urinary, wound, and respiratory infections (excluding viral infections such as CMV and BK virus) was recorded. The incidence of post-operative infection was evaluated for each genotype of the 3 different SNPs examined in this study. The overall incidence of post operative infection was 29.2% irrespective of genotype. There was no difference in the incidence of post-operative infection between non-expressers of CYP3A5 (44/149, 29.5%) and the expressers of CYP3A5 (10/36, 27.8%), p=0.836, Chi-Square test. Similarly, the rate of post-operative infection between the 3 genotypes of ABCB1 were comparable between CC genotype [10/34 (29.4%)], CT genotype [23/85 (27.1%)] and TT genotype [20/65 (30.8%)], p=0.442, Chi-Square test. There was also no difference in the rate of post-operative infection between the different genotypes of CYP3A4*22 with 50/171 (29.2%) of those with the CC genotype and 4/13 (30.8%) of those with the CT/TT genotype having a post-operative infection, p=0.807 Chi-Square test.

5.3.34 Cardiovascular events

We also examined the correlation between the different SNPs and the incidence of cardiovascular events including myocardial infarction, acute stroke, pulmonary embolus, cardiac arrhythmias, pulmonary oedema and unstable angina. Genotype expression appears to make no difference to the incidence of any of these post- operative complications.

The only correlation identified was a slightly higher incidence of NODAT in patients with the TT genotype of ABCB1. The reason for this difference is not clear and would require further evaluation before it could be determined if it was truly clinically relevant. The post operative complications are detailed fully in table 5.3 below.

Table 5.3 Post transplant complications relative to different genotypes of CYP3A5, ABCB1 and CYP3A4*22. * denotes a significant difference p<0.05

SNP	GENOTYPE	DGF	NODAT	INFECTION	MI	STROKE	ARRHYTHMIA	PE	PULMONARY OEDEMA	UNSTABLE ANGINA
CYP3A5	GG	32.90%	5.60%	29.50%	1.40%	1.40%	2.10%	2.10%	1.40%	1.40%
	GA/AA	34.30%	0.00%	27.80%	2.90%	2.90%	5.90%	0.00%	2.90%	0.00%
ABCB1	CC	39.40%	0.00%	29.40%	3.10%	0.00%	3.20%	0.00%	3.20%	0.00%
	CT	31.80%	2.40%	27.10%	0.00%	1.20%	3.60%	3.60%	1.20%	1.20%
	TT	30.80%	***9.70%**	30.80%	3.20%	3.20%	1.70%	0.00%	1.70%	1.70%
CYP3A4*22	CC	32.90%	4.80%	29.20%	1.80%	1.20%	2.50%	1.80%	1.80%	1.20%
	CT/TT	30.80%	0.00%	30.80%	0.00%	7.70%	7.70%	0.00%	0.00%	0.00%

5.3.35 Graft Survival relative to CYP3A5 genotype

There was no significant difference in graft survival between the CYP3A5 expressers and non-expressers as shown in the Kaplan-Meier graph below, Log-rank p = 0.782.

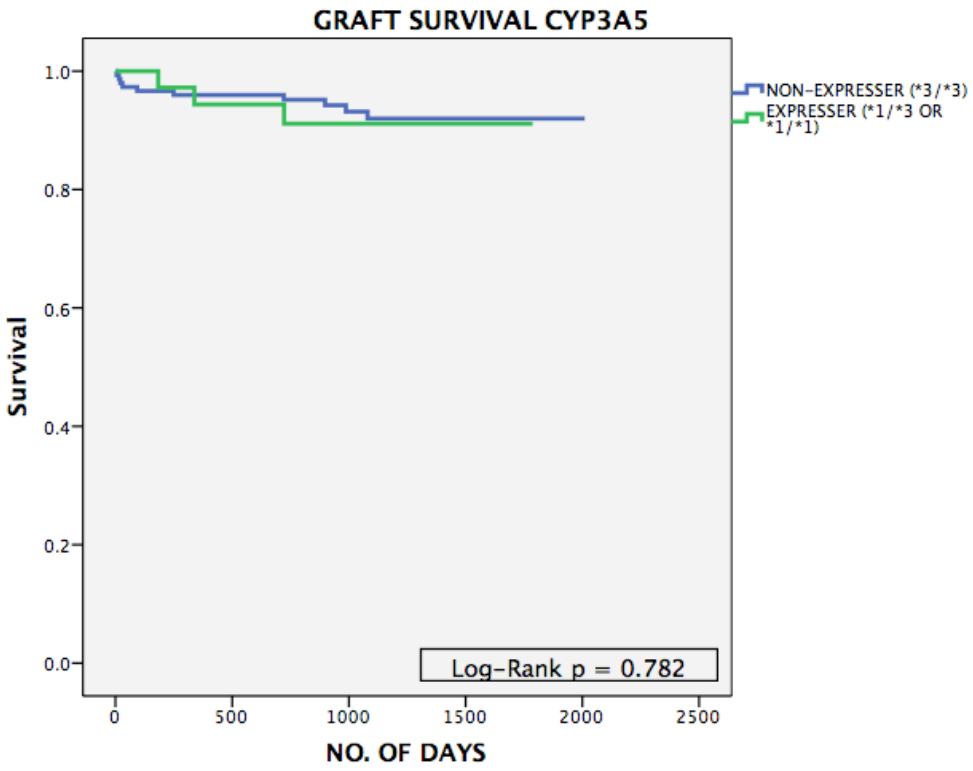


Figure 5.22 Graft survival following kidney transplantation relative to CYP3A5 expression.

5.3.36 Graft survival relative to ABCB1 genotype

There was significantly more graft loss on univariate analysis for the CC genotype of ABCB1 when compared with the CT and the TT genotype.

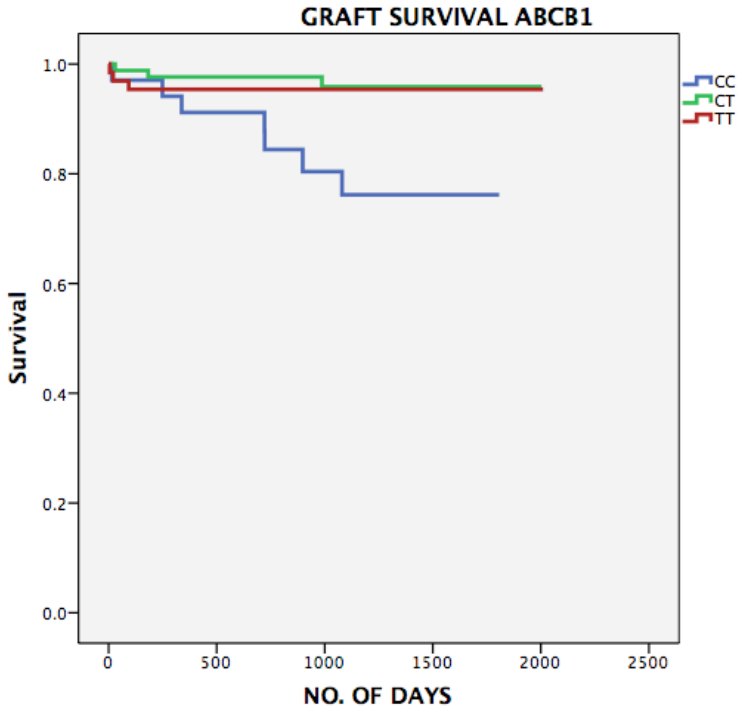


Figure 5.23 Kaplan-Meier plot for graft survival following kidney transplantation relative to ABCB1 genotype

Log Rank Test	**CC**	**CT**	**TT**
CC	-	**p = 0.003**	p **= 0.017**
CT	**p = 0.003**	-	p = 0.702
TT	**p = 0.017**	p = 0.702	-

*5.3.37 Graft Survival Relative to CYP3A4*22 Genotype*

There was no significant difference in graft survival between the T allele expressers of CYP3A4*22 and the non-expressers, Log-rank p = 0.900.

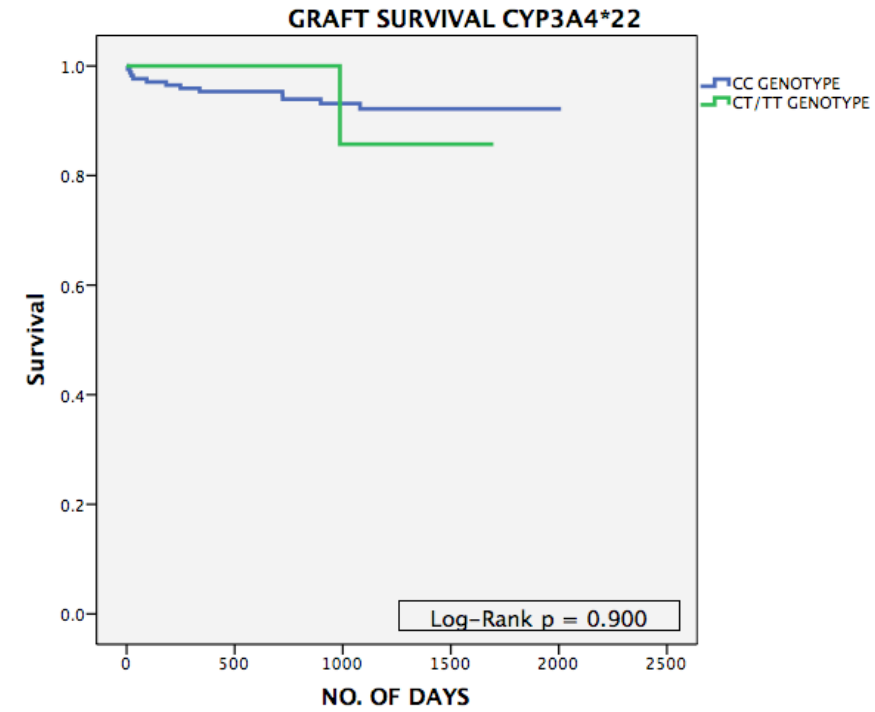


Figure 5.24 Kaplan-Meier plot for graft survival following kidney transplantation relative to CYP3A4*22 T allele expression.

5.3.38 Cox Regression Multivariate Analysis of Graft Survival

Multivariate analysis was carried out and demonstrated no significant difference in graft survival between any of the genotypes of the 3 SNPs. The only 2 significant values in graft survival was a DCD donor, p=0.021, and recipient age which was just statistically significant at p=0.047, Cox-regression.

Table 5.4 Cox regression multivariate analysis graft survival following kidney transplantation.

Variable	**p value**
CYP3A5 Genotype	0.996
ABCB1 Genotype	0.237
CYP3A4*22 Genotype	0.995
Delayed Graft Function	0.400
BPAR	0.179
Recipient Gender	0.390
Recipient Age	**0.047**
Donor Age	0.360
DCD Donor	**0.021**
Mis-match Level	0.154
Cold Ischaemic Time	0.124
Days Until Therapeutic Level	0.378

5.3.39 Patient Survival Relative to CYP3A5 Genotype

There was no significant difference seen in patient survival between the expressers and non-expressers of CYP3A5 as shown in the Kaplan-Meier plot below, Log-rank p = 0.416.

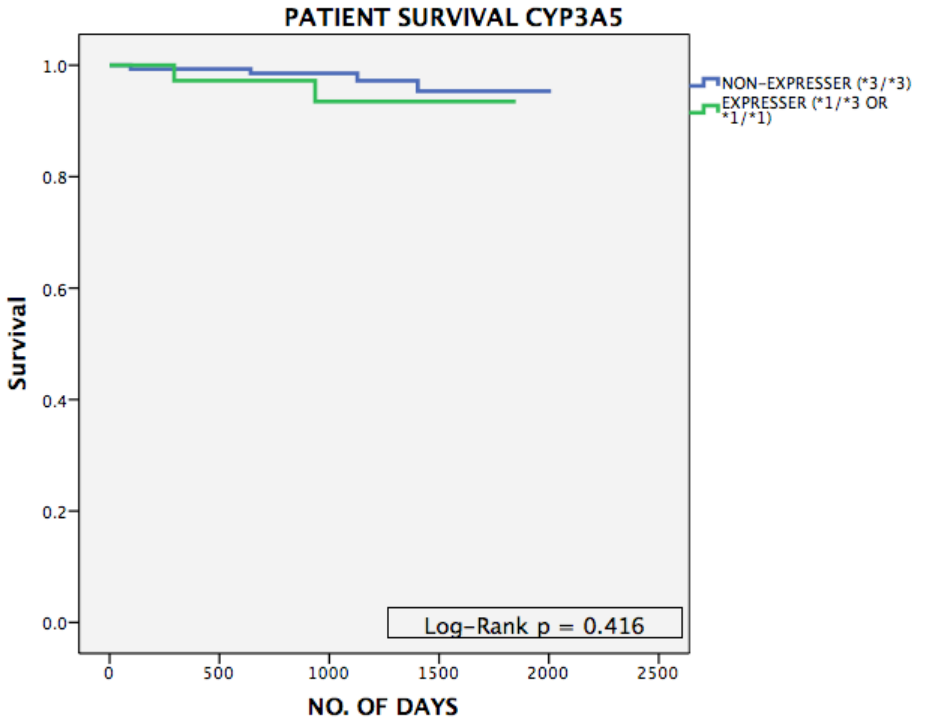


Figure 5.25 Kaplan-Meier plot for patient survival following kidney transplantation relative to CYP3A5 expression

5.3.40 Patient Survival Relative to ABCB1 Genotype

Similarly there was no difference in patient survival between the 3 different genotypes of ABCB1 as shown in the Kaplan-Meier curve below.

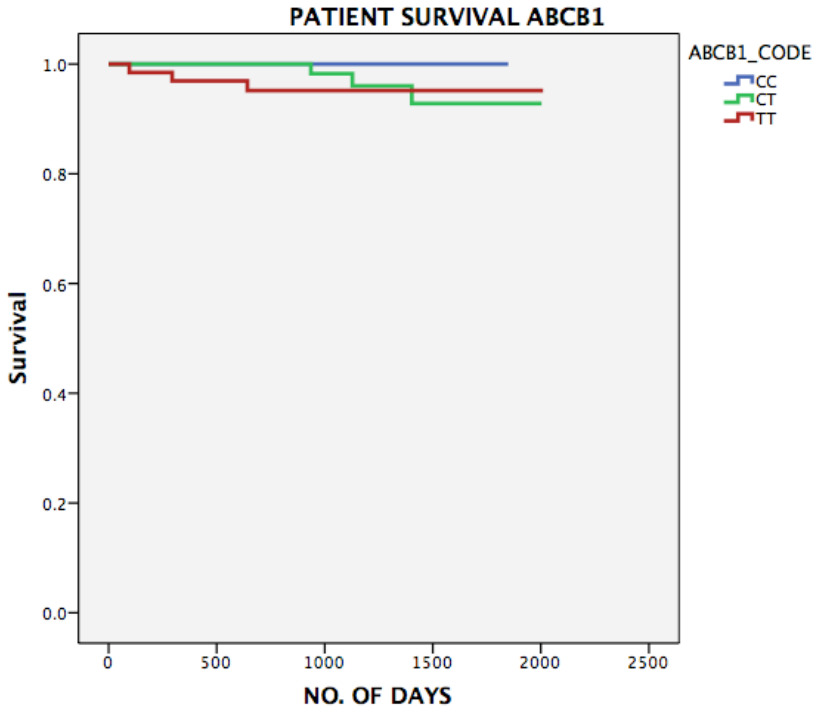


Figure 5.26 Kaplan-Meier plot for patient survival following kidney transplantation relative to ABCB1 genotype.

Log Rank Test	**CC**	**CT**	**TT**
CC	-	p = 0.270	p = 0.209
CT	p = 0.270	-	p = 0.722
TT	p = 0.209	p = 0.722	-

*5.3.41 Patient Survival Relative to CYP3A4*22 Genotype*

There was no significant difference in patient survival between the T allele expressers of CYP3A4*22 and those patients that did not express the T allele as shown in the Kaplan-Meier curve below. Log-rank p = 0.519.

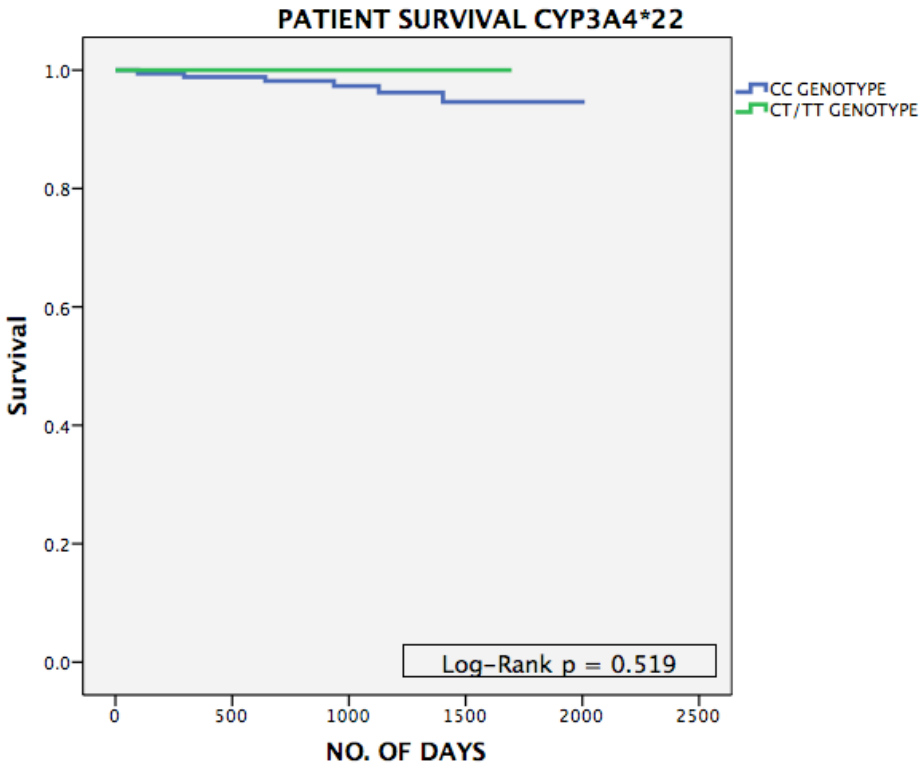


Figure 5.27 Kaplan-Meier plot for patient survival relative to CYP3A4*22 T allele expression.

5.3.42 Cox Regression Multivariate Survival Analysis

There was no significant difference in patient survival between any of the genotypes of the 3 SNPs in the Cox Regression analysis. There was no significant variable that was included in the analysis that significantly affected patient survival. These data should be interpreted within the context of a small sample size over a relatively short follow up period. To best evaluate patient survival in renal transplant patients, a much larger study with a long duration of follow up will reveal more robust results.

Table 5.5 Cox regression multivariate analysis for patient survival following kidney transplantation.

Variable	**p-value**
CYP3A5 Genotype	0.997
ABCB1 Genotype	0.999
CYP3A4*22 Genotype	0.990
Recipient Gender	0.954
Donor Gender	0.992
Recipient Age	0.916
Donor Age	0.927
Diabetes After Transplant	0.908
Myocardial Infarction	0.668
Cerebrovascular Accident	0.532
Malignancy	0.939
Delayed Graft Function	0.893
Graft Loss	0.954
Post Operative Infection	0.956

These results are in keeping with other studies of CYP3A5, ABCB1 and CYP3A4*22 expression in renal transplantation where there has not been any link established between genotype expression of any of these SNPs and graft or patient survival.

5.4 Simultaneous Pancreas-Kidney Patients

While there is now a substantial body of literature examining the role of CYP3A5 A6989G, ABCB1 C3435T and, to a lesser extent the CYP3A4*22 intron 6 polymorphism in renal transplant recipients, there is no published data looking solely at simultaneous pancreas-kidney (SPK) transplant recipients. It may be reasonable to assume that SPK transplant patients are not any different from the kidney transplant patients with regards to these genetic polymorphisms and therefore the data accrued from the kidney studies can be extrapolated to the SPK patients. Nevertheless, some SPK patients do have some important clinical differences to renal transplant recipients, such as the presence of autonomic neuropathy and gastroparesis, which has the potential to impact on the gut absorption and pharmacokinetics of tacrolimus [208].

In order to evaluate the impact of polymorphisms of CYP3A5, ABCB1 and CYP3A4*22 on tacrolimus dose requirements, trough levels and clinical outcome in SPK patients, a cohort of 32 SPK patients were genotyped for the same SNPs of CYP3A5, ABCB1 and CYP3A4*22 as the renal transplant cohort. The tacrolimus dose, trough level, dose-corrected level, creatinine and eGFR were recorded at the same time points within the first year following transplantation. In addition, the HbA1c was recorded at 3 months

and 12 months and the glucose level noted on discharge. Insulin independence was evaluated at discharge, 3 and 12 months. Biopsy proven acute rejection and graft and patient survival were also evaluated.

5.4.1 Patient Demographics

There were 20 male (62.5%) patients and 12 (37.5%) female patients with a mean age of 39.84±6.96 years (range 28 – 55 years). The mean weight of the recipients in this cohort was 69.59±13.55 kg (range 45.5 – 99.0 kg) with a corresponding mean BMI of 24.01±3.54, (range 19.0 – 31.6). All patients in this cohort were of the White-Scottish ethnic group.

5.4.2 Donor Demographics

All donors donated after brain death. There were 11 male donors (34.4%) and 20 female donors (62.5%). The mean donor age was 36.31±13.63 years (range 14 – 57 years). The mean donor BMI was 23.04±3.14 (range 16.50 – 29.73). 15 donors (46.9%) were blood group O, 13 donors (40.6%) blood group A, 3 donors (9.4%) blood group B and a single AB donor (3.1%).

The mean cold ischaemic time for the pancreas was 11:47±02:41 (hh:mm) (range 06:36 – 16:48) and the mean cold ischaemic time for the kidney was 12:19±03:24 (range 06:21 – 19:51).

5.4.3 Genotype Distribution

In the SPK cohort there were 25 patients (78.1%) who had the GG (*3/*3) genotype for CYP3A5. The remaining seven patients (21.9%) had the GA (*3/*1) genotype of CYP3A5 and made functional CYP3A5 protein.
16 patients (50.0%) had the CT genotype for ABCB1, whilst 9 patients (28.1%) were of the TT genotype and seven patients (21.9%) had the CC genotype of ABCB1,
28 patients (87.5%) expressed the CC genotype of CYP3A4*22 and 3 patients (9.4%) had the CT genotype.

5.4.4 Tacrolimus Dose Requirements Relative to CYP3A5

With the caveat of a small number of patients and even smaller number of expressers, there were significant differences in the dose of tacrolimus in the non-expresser group (10.22±3.36 mg) and the expresser group (18.67±5.60

mg0, p<0.0001, one-way ANOVA. There was a statistically significant higher dose requirement at time points WK3-3, 1M, 2M, and 12M.

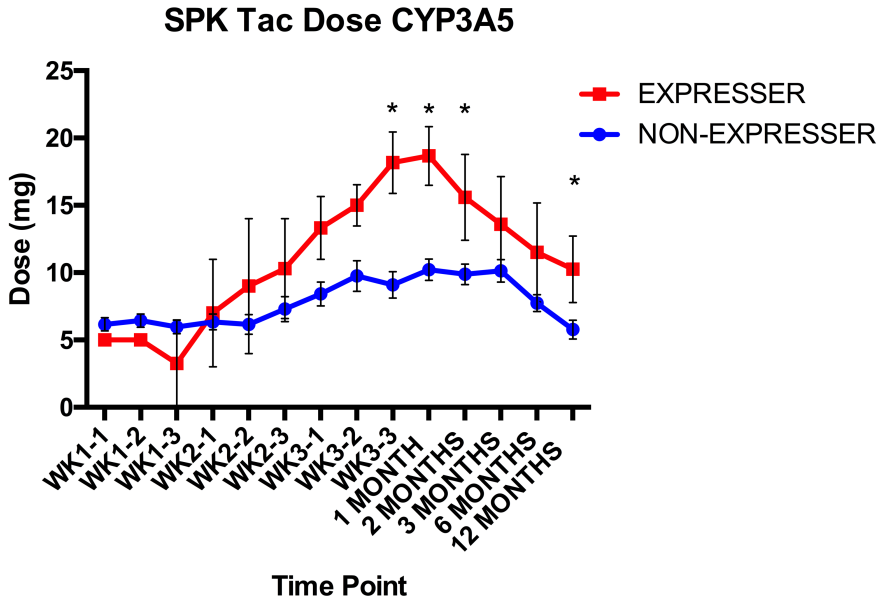


Figure 5.28 Tacrolimus dose requirements following SPK transplantation at each time point relative to CYP3A5 expression. * denotes significant difference p<0.05

5.4.5 Tacrolimus Trough Levels Relative to CYP3A5

The initial tacrolimus trough level was lower in the CYP3A5 expresser group (6.40±2.66 μg/L) compared to the non-expresser group (11.08±4.94 μg/L), p=0.033, one-way ANOVA. From the 2nd time point (WK1-2) there was no significant difference in the tacrolimus trough levels with the exception of the WK2-2 time point and the 6M time point where the trough level were

lower in the CYP3A5 expresser group compared with the non-expresser group as shown in Figure 5.29 below.

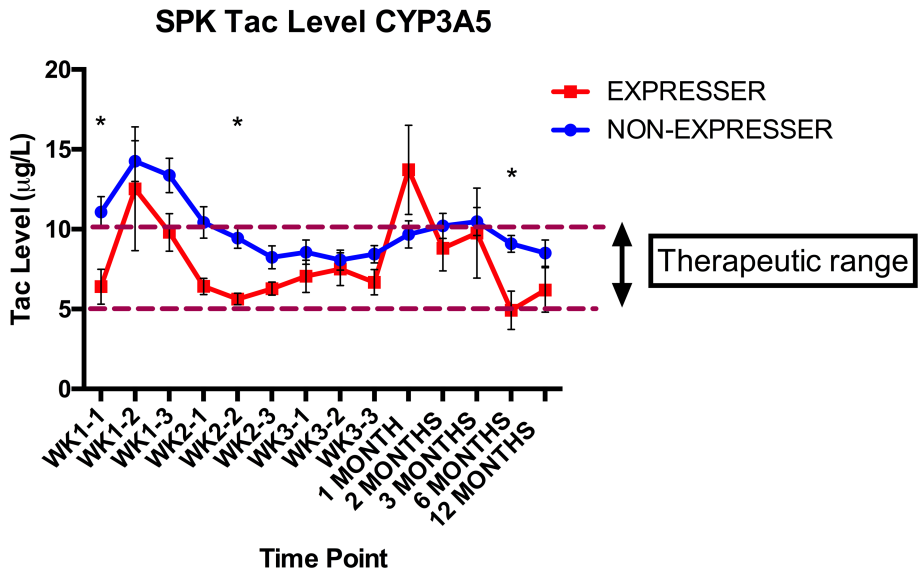


Figure 5.29 Tacrolimus trough concentration (C_0) at each time point following SPK transplantation relative to CYP3A5 expression. * denotes a significant difference $p<0.05$

There appears to be considerable fluctuation within the CYP3A5 expresser group however the non-expresser group appears to have a steadier level, but these results can be affected by the low number of patients in the expresser group.

5.4.6 Dose Corrected Tacrolimus Levels Relative to CYP3A5

The general trend appears to show that the dose-corrected tacrolimus level runs slightly lower in the CYP3A5 expresser group, although very few of these differences are statistically significant, as is shown in Figure 5.30 below.

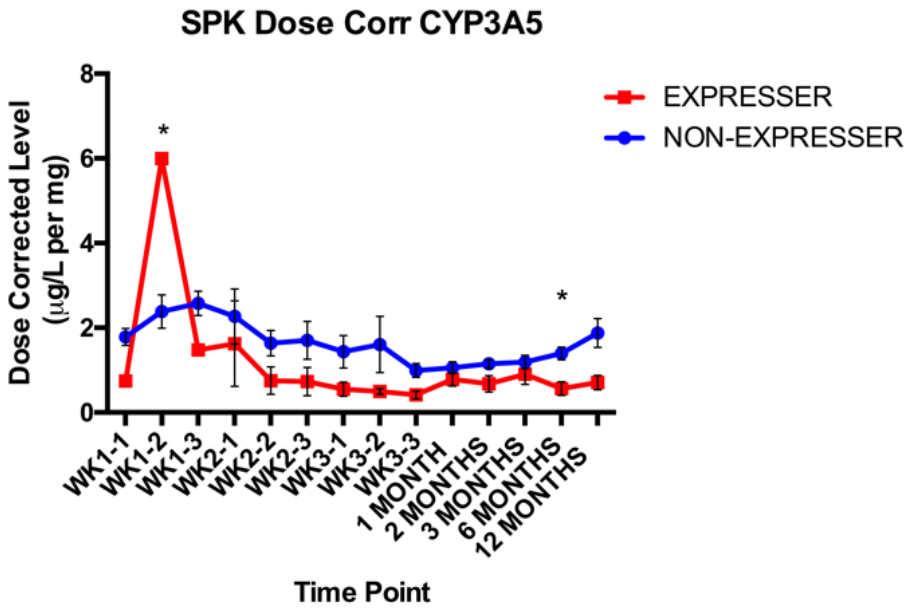


Figure 5.30 Dose corrected tacrolimus trough concentration (C_0/D) at each time point following SPK transplantation relative to CYP3A5 expression. * denotes a significant difference $p<0.05$

5.4.7 Renal Function in SPK Patients Relative to CYP3A5

There is very little difference in renal function between CYP3A5 expressers and non-expressers in SPK patients. The starting creatinine is similar for both groups, (450.04±238.83 μmol/L for non-expressers compared with 438.00±211.61 μmol/L in the CYP3A5 expresser group). The creatinine is comparable at all time points other than WK3-1. Although this is the only time point where there is a statistically significant difference in the creatinine, Figure 5.31 below shows that the trend of slightly higher creatinine in the expresser group disappears in the later months.

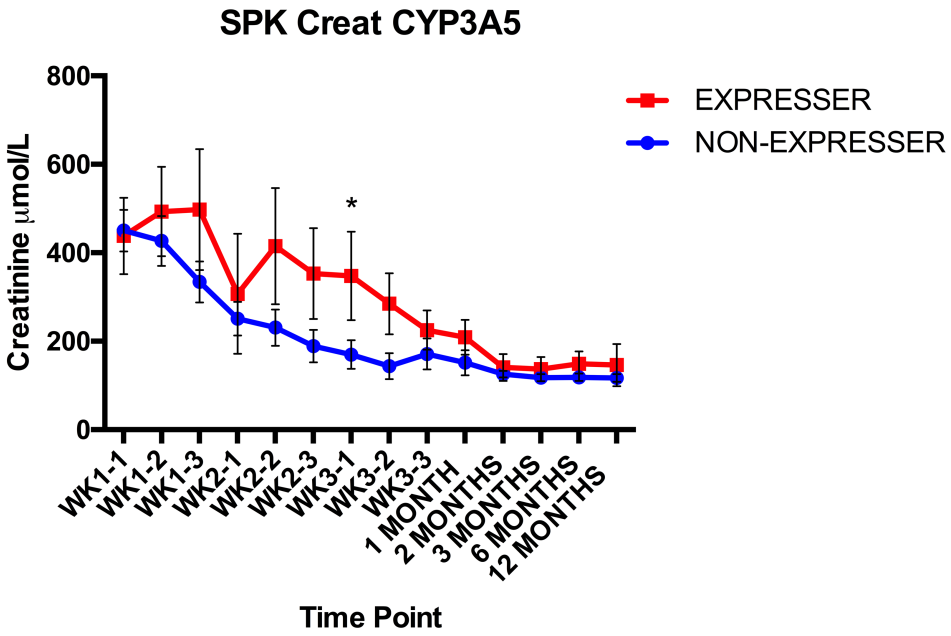


Figure 5.31 Creatinine for each time point following SPK transplantation relative to CYP3A5 expression. *denotes a significant difference p<0.05

The eGFR follows a similar pattern to the creatinine in the CYP3A5 non-expresser and expresser groups as is shown in Figure 5.32 below with no real difference throughout the study.

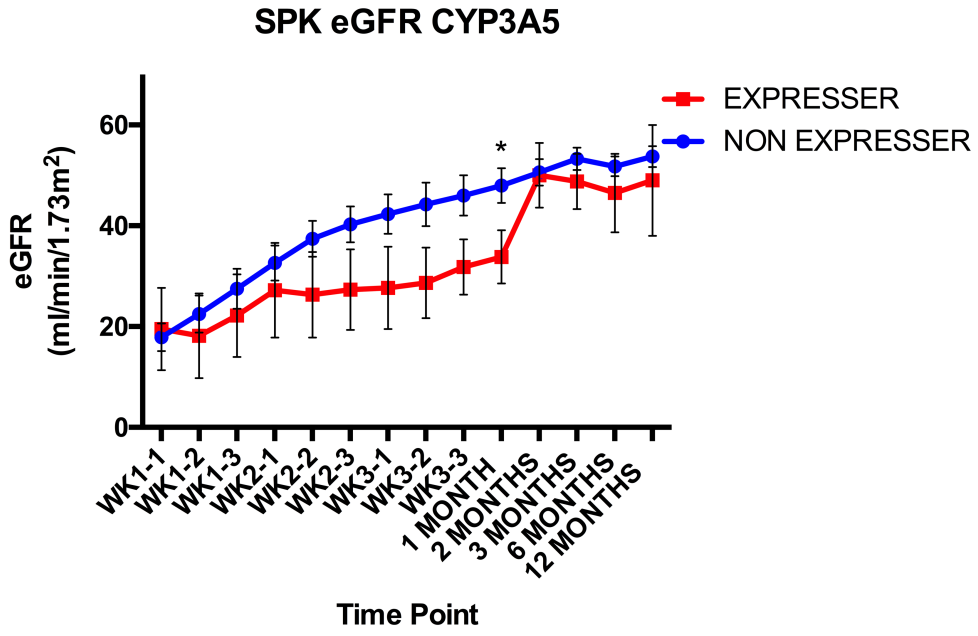


Figure 5.32 eGFR at each time point following SPK transplantation relative to CYP3A5 expression. * denotes a significant difference $p<0.05$

5.4.8 Tacrolimus Dose Requirements Relative to ABCB1

The mean tacrolimus dose was comparable between all 3 genotypes of ABCB1 at all time points up to and including the 12 month time point as shown in Figure 5.33 below.

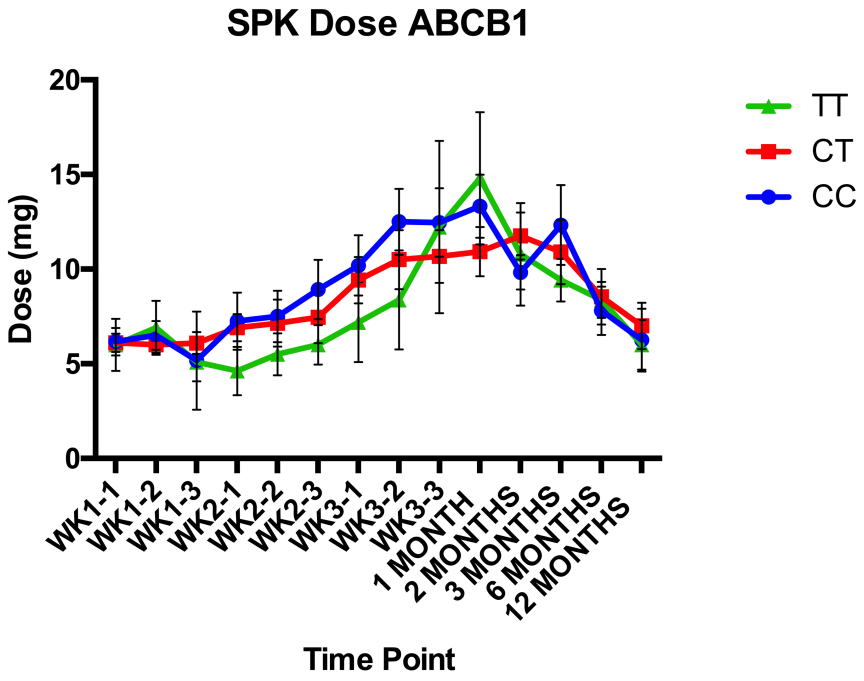


Figure 5.33 Tacrolimus dose requirements at each time point following SPK transplantation relative to ABCB1 genotype. *denotes a significant difference $p<0.05$

5.4.9 Tacrolimus Trough Levels Relative to ABCB1

Similarly, the tacrolimus trough levels were comparable between all three genotypes of ABCB1 at all time points up to and including 12 months as shown in Figure 5.34 below.

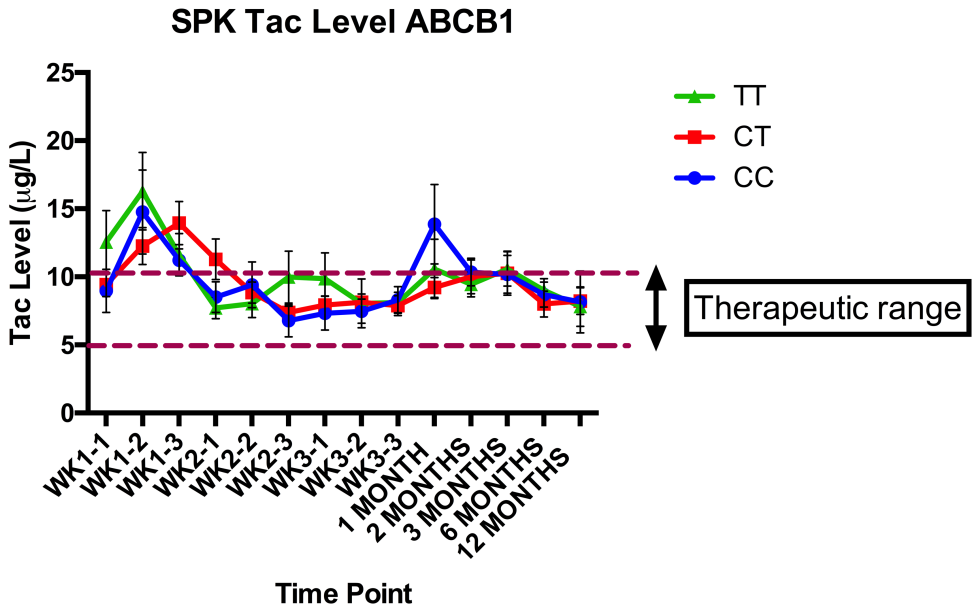


Figure 5.34 Tacrolimus trough concentration (C_0) at each time point following SPK transplantation relative to ABCB1 genotype. * denotes a significant difference

5.4.10 Dose Corrected Tacrolimus Levels Relative to ABCB1

The dose Corrected Tacrolimus Level relative to the ABCB1 genotype was comparable at all time points as shown in Figure 5.35 below.

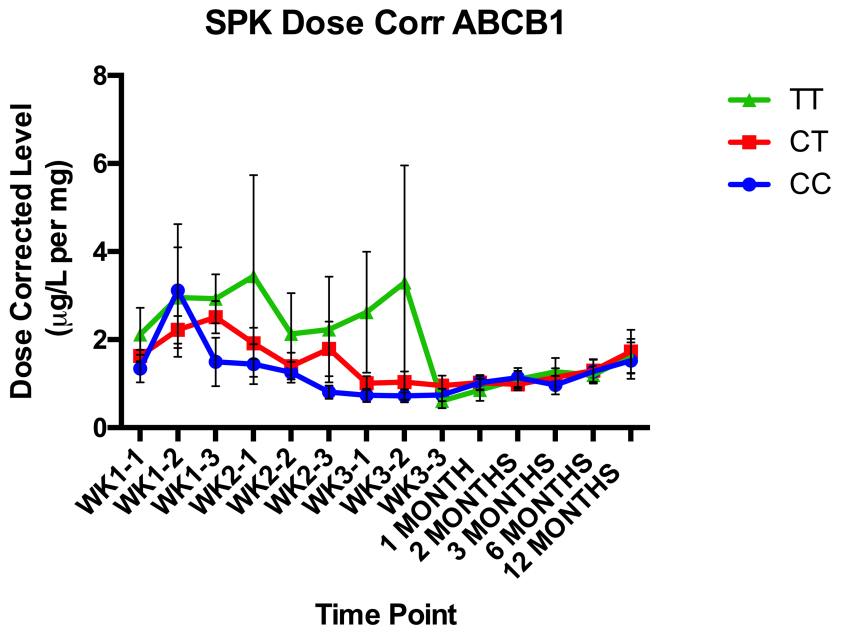


Figure 5.35 Dose corrected tacrolimus trough concentration (C_0/D) at each time point following SPK relative to ABCB1 genotype. * denotes a significant result $p<0.05$

5.4.11 Renal Function in Relative to ABCB1

Immediately after transplantation patients of the CT genotype had a significantly lower creatinine level (331.94±212.93 µmol/L) compared with those of the CC genotype (585.14±193.78 µmol/L) and also the TT genotype

(546.89±196.84 μmol/L), p=0.012, one-way ANOVA. The corresponding eGFR immediately post transplant was significantly higher in the CT genotype (25.38±16.34 ml/min/1.73m^2) when compared with both the CC genotype (8.86±3.44 ml/min/1.73m^2) and the TT genotype (12.67±12.17 ml/min/1.73m^2), p=0.017, one-way ANOVA.

This apparent improved renal function in those SPK patients of the CT genotype of ABCB1 disappears by the 3rd week following transplantation where the results are 49.00±12.19 ml/min/1.73m^2 for the CC genotype, 53.20±10.30 ml/min/1.73m^2 for the CT genotype and 56.25±7.50 ml/min/1.73m^2 for the TT genotype, p=0.611, one-way ANOVA.

The creatinine levels for the different genotypes of ABCB1 are shown in Figure 5.36 below and the corresponding eGFR at each of the time points is shown in Figure 5.37 below.

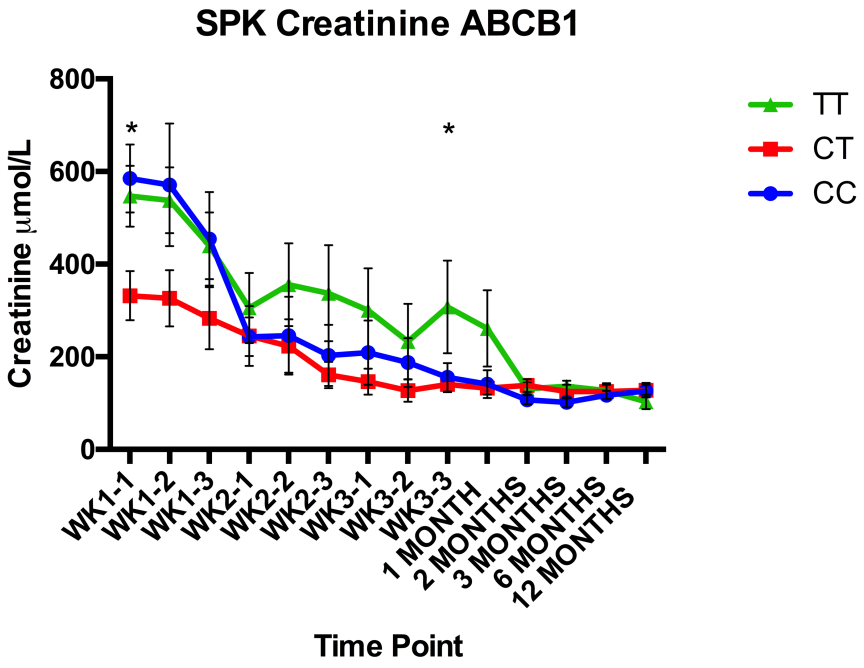


Figure 5.36 Creatinine at each time point following SPK transplantation relative to ABCB1 genotype. * denotes a significant difference p<0.05

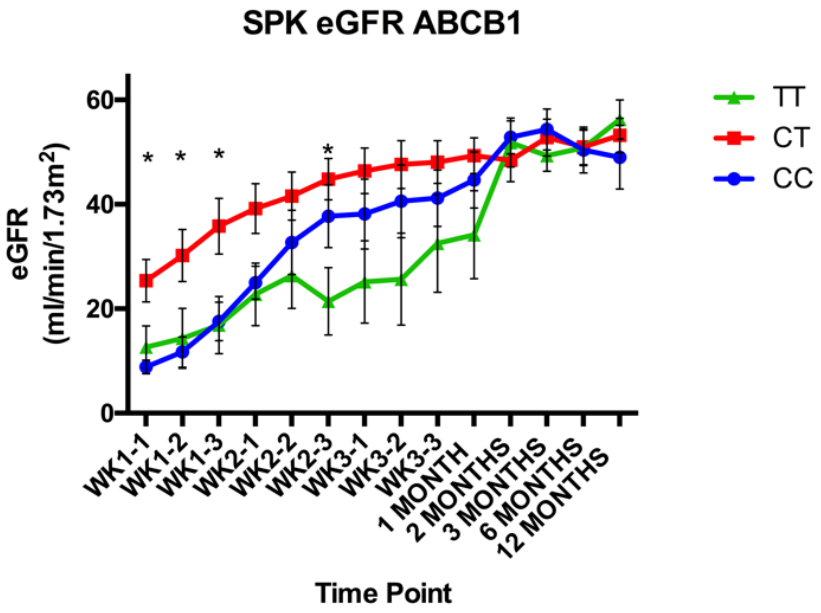


Figure 5.37 eGFR at each time point following SPK transplantation relative to ABCB1 genotype. * denotes a significant difference p<0.05

5.4.12 Delayed Graft Function Across the ABCB1 Genotypes

The reason for the improved renal function in the ABCB1 CT genotype is not immediately apparent based on genotype alone, particularly given that this particular polymorphism does not appear to have any significant impact on tacrolimus dose requirements or trough levels. The fact that the renal function became comparable between the three genotypes after the first few weeks, with those in the CC and TT genotype groups reaching the same renal function as those in the CT group, suggested that perhaps delayed graft function played a role. Delayed graft function was indeed significantly lower in the CT genotype group with only 4/16 patients (25.0%) experiencing delayed graft function compared with 5/7 patients in the CC genotype group (71.4%) and 6/9 patients (66.7%) in the TT genotype group, p=0.045, Chi-Square test.

There was no difference in the pancreatic graft function in relation to ABCB1 genotype. The HbA1c values were similar at 3 months and remained comparable at 12 months with a level of 7.57±4.02% for the CC genotype, 5.80±1.33% for the CT genotype and 5.44±0.44% for the TT genotype, p=0.259, one-way ANOVA.

*5.4.13 Tacrolimus Dose Requirements Relative to CYP3A4*22*

Despite comparable initial tacrolimus doses, the dose requirement for those who expressed a T allele of CYP3A4*22 (CT genotype) tended to be lower after the second week after transplant. The tacrolimus dose was significantly different at WK3-3 (CC 11.18±4.87 mg vs CT 6.00±1.41 mg, p=0.004), 1 month (CC 12.17±4.05 mg vs CT 6.25±2.47 mg, p=0.001) and the 2 month time point (CC 10.59±3.68 mg vs 6.00 mg, p=0.001, one-way ANOVA). The combination of a small cohort size, missing tacrolimus doses and the very low allele frequency of the T allele of CYP3A4*22 makes it difficult to draw firm conclusions regarding this dataset, particularly when, on occasion, there is a single value for mean dose in the CT genotype. Figure 5.38 below shows the dose requirements relative to the CYP3A4*22 genotype.

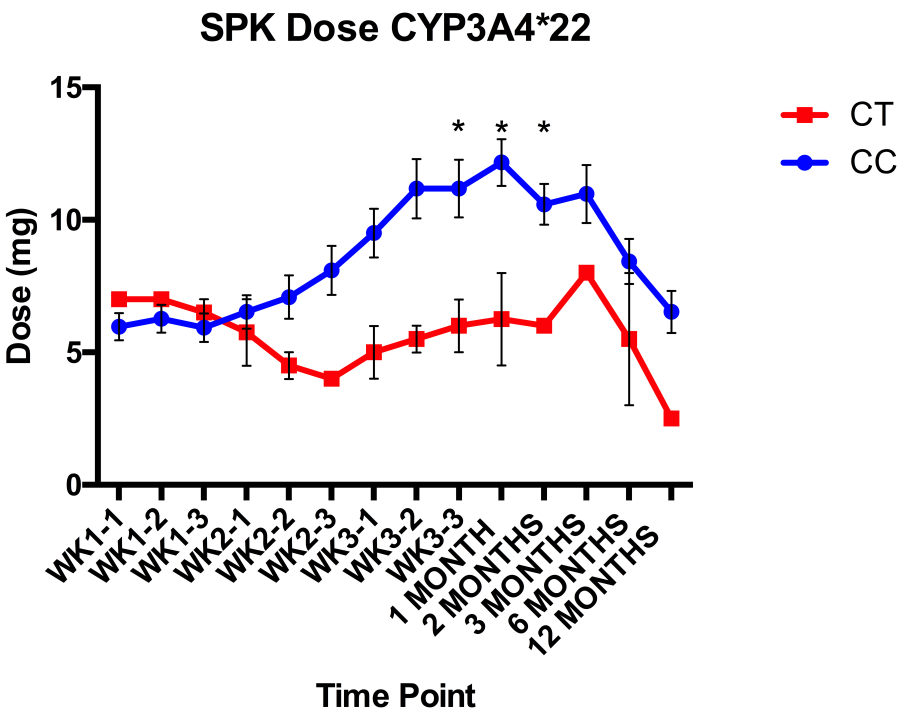


Figure 5.38 SPK Tacrolimus dose requirement at each time point following SPK transplantation relative to CYP3A4*22 T allele expression. * signifies a significant difference p<0.05

*5.4.14 Tacrolimus Trough Level Relative to CYP3A4*22*

The tacrolimus trough levels tended to be slightly higher in the CYP3A4*22 CT genotype group initially (15.20±4.15 μg/L) compared with the CC genotype (9.69±4.84 μg/L) however this was not statistically significant, p=0.184 (Figure 5.39). The low T allele frequency of CYP3A4*22 combined with a small cohort makes interpretation of these results difficult.

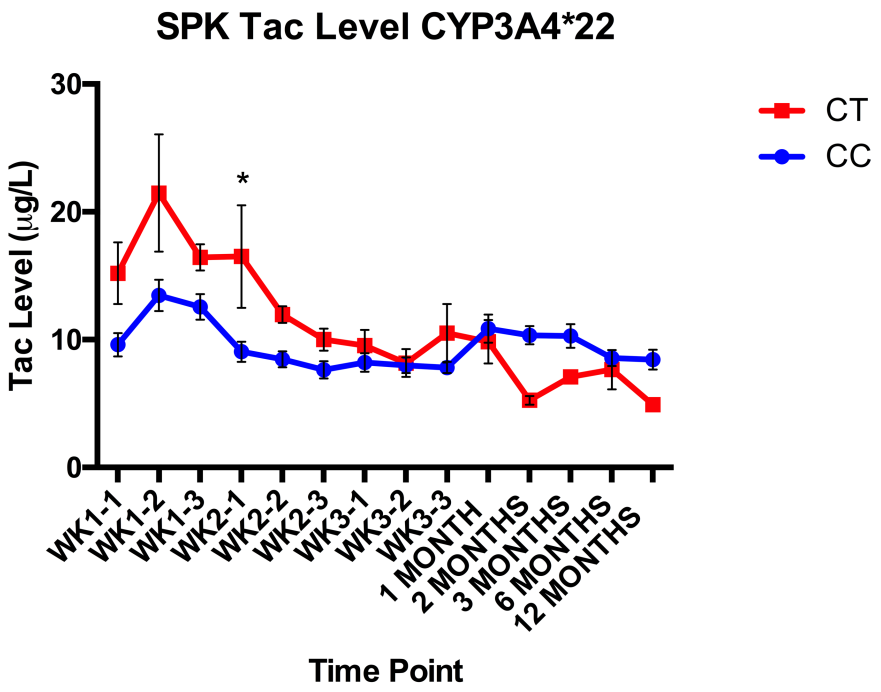


Figure 5.39 Tacrolimus trough concentration (C_0) at each time point following SPK transplantation relative to CYP3A4*22 T allele expression. * denotes a significant difference p<0.05.

*5.4.15 Dose Corrected Tacrolimus Level Relative to CYP3A4*22*

Initially after transplantation there was no difference in the dose corrected tacrolimus level between the CYP3A4*22 CC genotype (1.71±0.87 μg/L per mg) and the CT genotype (1.84±0.17 μg/L per mg), p=0.842. After the first week the CT genotype appeared to have slightly higher tacrolimus dose-corrected level although this only reached statistical significance at WK3-3 (CC 0.74±0.40 μg/L per mg vs CT 2.04±1.36 μg/L per mg, p=0.006, one-way

ANOVA) and at 1 month (CC 0.94±0.372 μg/L per mg vs CT 1.84±1.39 μg/L per mg, p=0.025, one-way ANOVA).

While there is no real significant difference demonstrated between the CC and the CT genotype relative to the dose-corrected tacrolimus trough level, Figure 5.40 below does suggest a trend towards CT genotype having greater tacrolimus exposure.

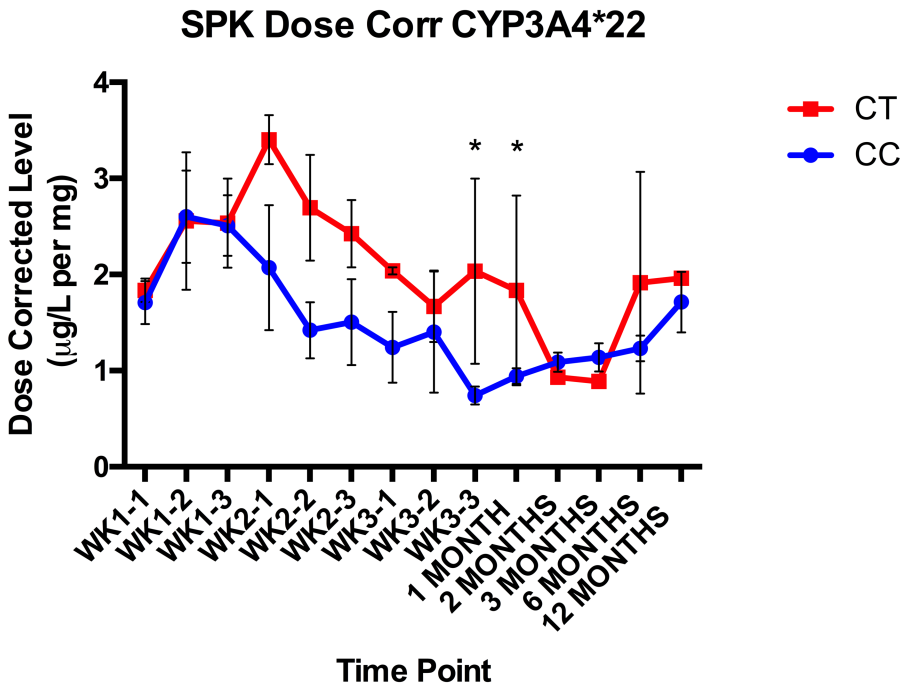


Figure 5.40 Dose corrected tacrolimus trough concentration (C_0/D) at each time point following SPK transplantation relative to CYP3A4*22 T allele expression. * denotes a significant difference p<0.05

*5.4.16 Renal Function in SPK Patients Relative to CYP3A4*22*

There was no significant difference in the creatinine according to the CYP3A4*22 genotype with the exception of the WK3-1 time point where the creatinine in the CT group is significantly higher (322.00±341.27 μmol/L) compared with the CC group (175.04±153.55 μmol/L, p=0.025 one-way ANOVA). Figure 5.41 below shows the creatinine for each genotype at each of the time points.

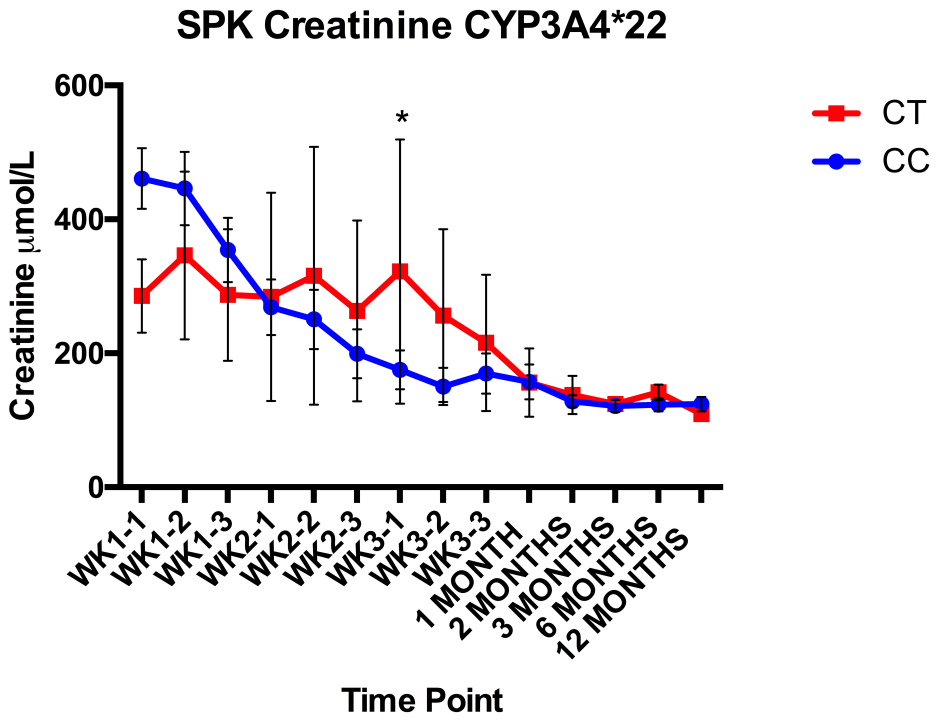


Figure 5.41 Creatinine at each time point following SPK transplantation relative to CYP3A4*22 T allele expression. * denotes a significant result p<0.05.

The eGFR was comparable at all of the time points for both the CC and the CT genotype of CYP3A4*22 up to and including the 12 month time point where the eGFR of the CC genotype was 52.06±10.21 ml/min/1.73m^2 compared with 60.00 ml/min/1.73m^2 of the CT genotype, p=0.595, one-way ANOVA.

The eGFR relative to CYP3A4*22 genotype is shown in Figure 5.42 below.

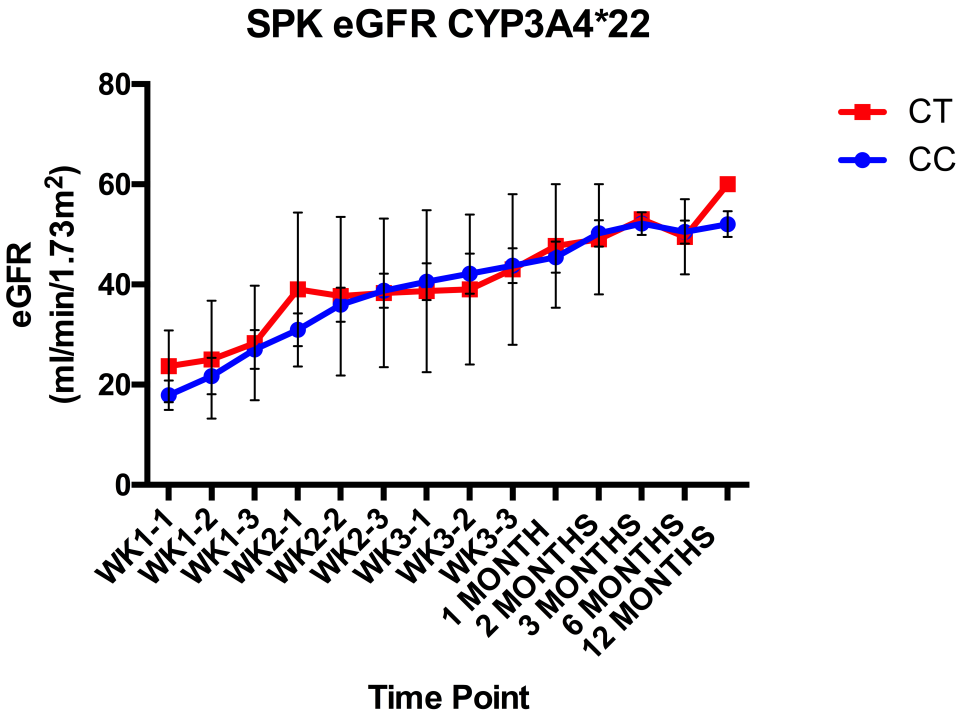


Figure 5.42 eGFR at each time point following SPK transplantation relative to CYP3A4*22 T allele expression. * signifies a significant difference p<0.05

5.4.17 Time Until Therapeutic Tacrolimus Trough Level Reached

There was no significant difference in the number of days taken to reach a therapeutic tacrolimus trough level between the genotypes of any of the 3 SNPs. In the CYP3A5 genotype the non-expresser group had a mean of 3.04±1.43 days until a therapeutic level of tacrolimus was reached compared with 3.00±0.894 days in the expresser group, p=0.950 one-way ANOVA. The time taken to reach a therapeutic tacrolimus level was also comparable across the three genotypes of ABCB1 (CC 2.86±0.69 days, CT 2.94±1.24 days and TT 3.33±1.87 days), p=0.731 one-way ANOVA. Similarly, for the CYP3A4*22 there was also no difference in the time taken to reach a therapeutic tacrolimus level with 3.04±1.35 days for the CC genotype and 3.00±1.73 days for the CT genotype, p=0.999, one-way ANOVA.

5.4.18 Delayed Graft Function

All of the pancreatic grafts had primary function apart from one graft which was removed due to primary non-function. The overall incidence of delayed graft (renal) function in this cohort was 15/32 (46.9%). There was no significant difference in the incidence of delayed graft function (DGF) between the CYP3A5 non-expresser genotype (46.2%) and the CYP3A5

expresser group (50.0%), p=0.608, Chi-Square test. When patients were grouped by ABCB1 genotype there was significantly lower DGF in the CT genotype group (25.0%) compared with the CC genotype group (71.4%) and the TT genotype group (66.7%), p=0.045, Chi-Square test. There was no significant difference in incidence of DGF when patients were grouped by their CYP3A4*22 genotype with 46.4% in the CC genotype group and 33.3% in the CT genotype group, p=0.508, Chi-Square Test. The incidence of DGF is shown in Figure 5.43 below.

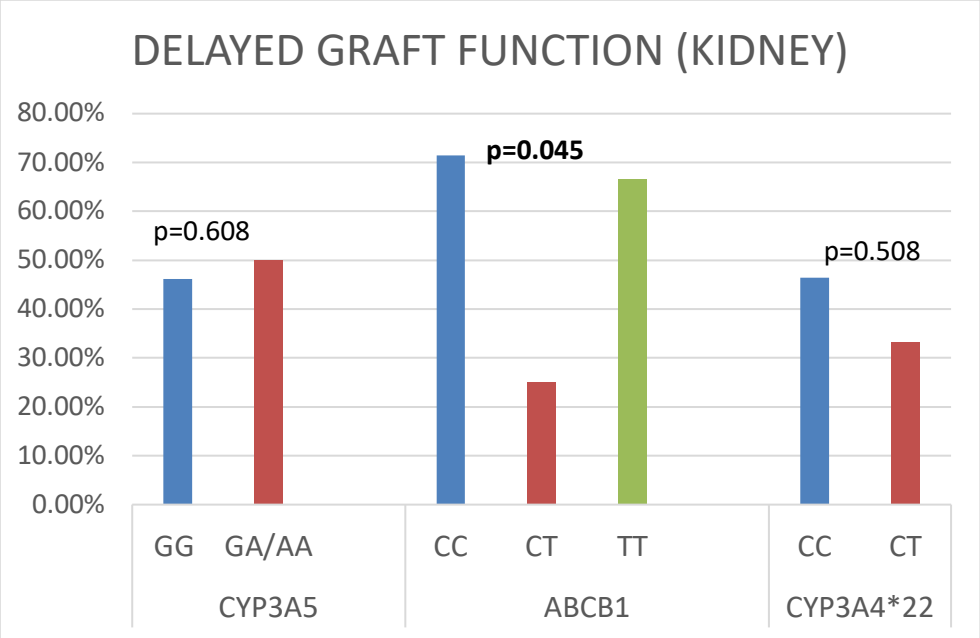


Figure 5.43 Percentage of delayed graft function in the renal allograft following SPK transplantation for each genotype of CYP3A5, ABCB1 and CYP3A4*22.

5.4.19 Biopsy Proven Acute Rejection

The overall incidence of biopsy proven acute rejection (BPAR) in this cohort of patients was 7/32 (21.9%). There was a significantly higher incidence of BPAR in the CYP3A5 expresser group (66.7%) when compared with the CYP3A5 non-expresser group where the incidence was 11.5%, p=0.012, Chi-Square Test. There was no significant difference in the incidence of BPAR in relation to the ABCB1 or the CYP3A4*22 genotypes. The incidence of BPAR for each genotype is shown in Figure 5.44 below.

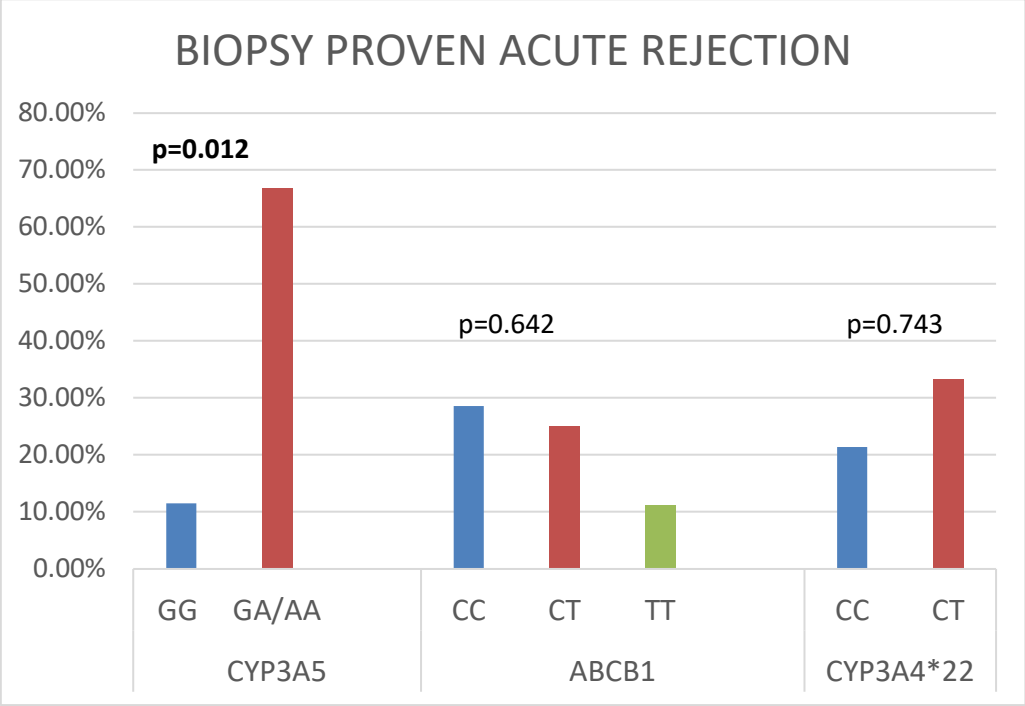


Figure 5.44 Percentage of biopsy proven acute rejection in the renal allograft following SPK transplantation for each genotype of CYP3A5, ABCB1 and CYP3A4*22.

5.4.20 Other Post-Operative Complications

Post operative infection was a very common reported complication following an SPK transplant with 18/32 (56.3%) of patients experience some form of post operative infection. There was no difference in the incidence of post operative infections in relation to the genotypes expressed by the patients for any of the 3 SNPs. The infections were wide ranging and included Klebsiella urinary sepsis, Streptococcal urinary sepsis, pneumonia, intra-abdominal sepsis, wound infections, vancomycin resistant enterococcus diarrhoea, C. difficile infection and duodenal candida.

5.5 Chapter Summary

The data from the renal transplant cohort supports previously reported findings in relation to CYP3A5 expression where those individuals who expressed CYP3A5 required approximately double the dose of tacrolimus in order to achieve a therapeutic level compared with those patients who did not express functional CYP3A5. Consequently, those who were CYP3A5 expressers had initially lower tacrolimus trough levels and also had significantly higher number of days until a therapeutic level was reached compared with the non-expressers. In the renal transplant cohort this did not, however, translate into increased rejection or delayed graft function and

renal function did not differ between the expressers and non-expressers of CYP3A5.

This study did not reveal any significant impact of the different genotypes of ABCB1 on tacrolimus dose requirements, tacrolimus trough levels, renal function or time until a therapeutic trough level was reached in the renal transplant cohort. A patient's ABCB1 genotype also had no significant bearing on renal function, delayed graft function or biopsy proven acute rejection. These findings are in keeping with most of the recent studies that have examined the role of ABCB1 on tacrolimus pharmacokinetics. However some earlier studies had suggested that ABCB1 could influence tacrolimus dose requirements.

This study did show significantly reduced graft survival in patients who carried the ABCB1 CC genotype compared with the CT or TT genotypes. One possible mechanism for this relates to the discrepancy between whole blood concentrations of tacrolimus and the intra-lymphocyte concentration. Approximately 80% of the measured whole blood concentration of tacrolimus relates to concentration within erythrocytes, which are not thought to contribute significantly to the immune response and therefore the concentration within circulating peripheral blood lymphocytes may better reflect the immunosuppressive effect [209]. ABCB1 is present on the

cell membrane of many cells, including lymphocytes, and may play a role in the intracellular concentration of tacrolimus [210]. Indeed *Capron et al* reported higher intra-lymphocyte concentrations of tacrolimus in kidney transplant patients who carry the T allele of 3435 C>T in the ABCB1 gene [211] which may explain the increased graft loss seen in this study. The role of intra lymphocyte tacrolimus concentration in acute and chronic rejection needs further work to determine if the ABCB1 may yet prove significant.

CYP3A4*22 has been less widely studied but published data does suggest that patients require lower doses of tacrolimus if they are either heterozygotes or homozygotes for the T allele of CYP3A4*22. This study has not shown statistically significant differences in the dosing requirements or tacrolimus trough levels in the renal transplant cohort. However, there was a tendency for those with the CYP3A4*22 CT/TT genotype to run slightly lower tacrolimus dose requirements. The very low allele frequency of the T allele means that quite a large sample of patients is required before even a small number of patients of the CT/TT genotype are available to compare and this may be why this study has not shown a convincing impact of T allele expression of CYP3A4*22. Similarly, CYP3A4*22 had no impact on renal function, DGF, BPAR or any other clinical outcome.

Multi-variate analysis in this study did not show any significant difference in graft survival or patient survival relative to any of the different SNPs studied, however it also did not reveal any impact on graft survival of donor age, a factor that has been shown time and time again to be an independent risk factor for graft loss, suggesting that this study may be underpowered for an analysis of graft or patient survival. A larger, prospective, adequately powered study is required for evaluate the impact of these SNPs on graft and patient survival.

The SPK group of patients showed different results from the renal transplant group yet the small number of patients in the cohort makes it difficult to interpret if these differences are genuine or determined by the small sample size. Furthermore, the almost complete absence of any published data in an isolated SPK cohort means there is a paucity of data to compare with the results of this study.

The SPK patients who expressed CYP3A5 had a similar dose requirements trend to the renal transplant cohort, expressers requiring higher doses of tacrolimus. However, this was not as marked as in the renal transplant cohort and was statistically significant at only a few of the time points. It is likely that due to small numbers in the expresser group, the difference is not as convincing as in the renal transplant cohort which had many more

patients. There was no noticeable difference in the time taken to reach a therapeutic tacrolimus level in the SPK group, however those CYP3A5 expresser patients did have a significantly higher incidence of BPAR.

ABCB1 genotype did not appear to influence the dose requirements or trough levels in the SPK cohort much the same as the renal transplant group however there was significantly less DGF in patients who expressed the CT genotype. It is difficult to conceive that this is a genuinely 'protective' genotype and is more likely due to the fact that the majority of patients are of the CT genotype and so those few who have DGF of the CC or the TT genotype will likely form a larger percentage of that group.

The T allele frequency of CYP3A4*22 is so low that in a cohort of only 32 patients, there are instances where only one result is available to represent CT genotype and that makes it almost impossible to draw any conclusions other than to say that the CYP3A4*22 genotype did not appear to significantly affect dose, trough level, renal function or other clinical outcomes in this small cohort of patients. A much larger number of patients is required (both for renal and SPK transplant recipients) to try and better evaluate the influence of this particular SNP on tacrolimus pharmacokinetics.

Chapter VI

Genetic Polymorphisms and Variability with Modified Release Tacrolimus

6 GENETIC POLYMORPHISMS AND VARIABILITY WITH MODIFIED RELEASE TACROLIMUS

6.1 Background

Tacrolimus, although highly effective in preventing allograft rejection, is complicated by considerable variation in pharmacokinetics between individual patients and a narrow therapeutic index which requires close monitoring of the trough levels. Patients exposed to excessive immunosuppression are at risk of neurological and cardiovascular side effects and graft loss due to drug toxicity [212], whilst under-exposure leads to a higher risk of immune mediated rejection and graft loss with an associated increased risk of mortality [213]. Patient variability in the pharmacokinetics of tacrolimus is associated with many different factors, including but not limited to, weight, ethnic group, cytochrome P450 3A5 gene expression and patient adherence.

There have been suggestions that high intra-patient variability may be associated with increased graft loss [49, 214] and although the cause remains poorly understood, patient adherence is thought to play a significant role [215]. Furthermore, current studies suggest that cytochrome P450 3A5

(CYP3A5) expression affects *inter*-patient variability but does not significantly influence tacrolimus variability within individual patients [61, 216].

The two available formulations of tacrolimus [twice-daily (Tac-BD) and modified-release once-daily (Tac-QD)] have slightly different pharmacokinetics. Furthermore, it is perceived that once-daily medications are better adhered to than twice-daily (although the evidence for this is somewhat variable) raising the possibility that medication adherence may play a role in reducing intra-individual variability.

The aim of this study, therefore, was to evaluate if there are any differences in the intra-patient variability before and after conversion from Tac-BD to Tac-QD in renal transplant patients and to examine the impact of tacrolimus variability on graft survival.

The secondary aim is to examine to what extent the different genotypes of CYP3A5, ABCB1 and CYP3A4*22 have on tacrolimus dose requirements and trough levels following conversion to Tac-QD (modified release once daily). It is feasible that given the expression of CYP3A5 in particular is associated with lower exposure of tacrolimus, it could influence tacrolimus variability however there is little evidence currently to evaluate this.

6.2 Chapter Methodology

All renal transplant recipients attending our centre who had been converted from Tac-BD (Prograf®) preparation of tacrolimus to Tac-QD (Advagraf®) preparation between September 2008 and December 2011 were included. Clinical data was collected on 103 renal transplant patients converted from Tac-BD to Tac-QD. Patients were converted at the discretion of the transplant clinician.

To calculate tacrolimus intra-individual variability we used a method previously described by Borra et al [49]. A value of percentage variability is derived for each individual patient, which describes the percentage by which their dose corrected tacrolimus levels vary from their own mean. It is calculated using the formula $\{[(\chi_{mean} - \chi_1) + (\chi_{mean} - \chi_2)..... + (\chi_{mean} - \chi_n)]/n\} / \chi_{mean} * 100$ = intra individual variability (%) where χ_{mean} is the mean dose corrected tacrolimus level (tac C_0/dose) and χ_n are each of the dose corrected tacrolimus levels for that patient. All available dose corrected tacrolimus levels for 12 months prior to conversion (Tac-BD) and 12 months after conversion (Tac-QD) were used and a percentage variability value calculated for each patient before and after conversion.

Patients in the study were categorised as either *high* or *low* variability depending on whether their percentage variability was higher or lower than the median value both before and after conversion to Tac-QD. The overall tacrolimus variability in the 12 months before conversion (Tac-BD) was compared with the tacrolimus variability in the 12 months after conversion to Tac-QD. In addition, 4 separate groups were defined, taking into account the pre and post conversion variability percentage: *Low-low (LL), low-to-high (LH), high-to-low (HL), high-high (HH).* Graft and patient survival as well as the serum creatinine up to 12 months after conversion to Tac-QD were evaluated for these groups. The tacrolimus variability for both preparations was compared between patients converted to Tac-QD within the first 12 months following transplantation and those converted beyond 12 months after transplantation to investigate whether time to conversion from transplant had an impact on tacrolimus variability.

A smaller sub-cohort of these patients who were converted to Tac-QD had stored frozen DNA available (n=43) which was then genotyped as described in the laboratory methodology chapter for CYP3A5, ABCB1 and CYP3A4*22. The tacrolimus dose requirement and the trough level was measured prior to conversion and then at 1, 2, 3, 6 and 12 months after conversion to Tac-QD.

6.3 Chapter Results

6.3.1 Patient demographics

103 patients (67 male and 36 female) were converted from Tac-BD to Tac-QD with a mean time to conversion of 41.50 ± 45.61 months after transplant (range 0 – 155 months). Patient demographic data is shown in Table 6.1 below. Forty-two patients (40.78%) were converted to Tac-QD within the first 12 months of transplantation at a mean time of 4.00 ± 3.20 months (range 0 – 11 months) and 61 patients (59.22%) were converted greater than 12 months after transplantation at a mean time of 67.31 ± 43.21 months (range 13 – 155 months).

Table 6.1 Patients demographics of renal transplant patients converted to once-daily modified release tacrolimus

Patient Demographics	**Values**		
Male:Female	67 (65.0%)	36 (35.0%)	
Transplant 1st:2nd:>2nd	85 (82.5%)	11 (10.7%)	7 (6.8%)
DBD:DCD:Live Donor	69 (67.0%)	10 (9.7%)	24 (23.3%)
Age (mean±SD) (years)	48.96±13.15		
Weight (mean±SD) (kg)	76.65±16.71		
Converted 0-12 months:>12 months	42 (40.8%)	61 (59.2%)	

6.3.2 *Conversion to Tac-QD*

60 patients (58.3%) were converted to Tac-QD on a mg:mg basis, 26 patients (25.3%) were converted to a Tac-QD dose ± 1 mg of the Tac-BD dose and 17 patients (16.5%) were converted to a Tac-QD dose greater than ± 2 mg. The mean dose of Tac-BD prescribed prior to conversion was 6.27±4.81 mg (1-26 mg) and the mean starting dose of Tac-QD was 6.21±4.77 mg (1-27 mg).

6.3.3 *Tacrolimus Variability*

Overall tacrolimus variability was higher for the Tac-BD formulation in the 12 months prior to conversion compared with the Tac-QD formulation in the 12 months following conversion (25.23±14.63% compared to 21.68±12.62%, p=0.043, paired samples t-test) (Figure 6.1 below)

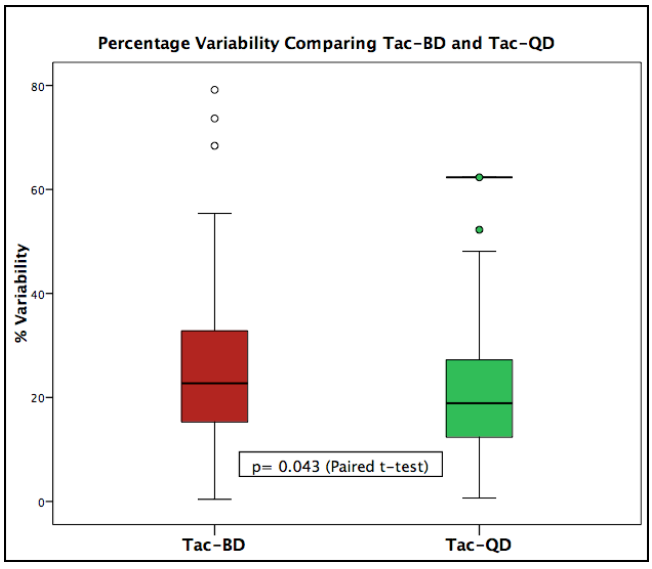


Figure 6.1 Overall tacrolimus variability for both the twice-daily (BD) and once-daily (QD) preparations in this kidney transplant cohort.

There was a significant difference in variability for the Tac-BD formulation up to the point of conversion between patients converted early and those converted beyond 12 months following transplantation, [30.59±16.61% (n=42) versus 21.55±11.89% (n=61), p=0.002, t-test]. Once patients were converted to Tac-QD, intra-patient variability was comparable irrespective of the time of conversion [24.17±12.22% (<12 months) vs 19.97±12.70% (> 12 months), p=0.097 t-test].

There was a significant improvement in intra-patient variability upon conversion from Tac-BD (30.59±16.61%) to Tac-QD (24.17±12.22%) (p=0.038, T-test) in those converted within the first 12 months post-transplant. However, there was no difference between the variability of Tac-BD (21.55±11.89%) and Tac-QD (19.97±12.70%) in patients converted beyond 12 months post transplantation (p=0.447, t-test).

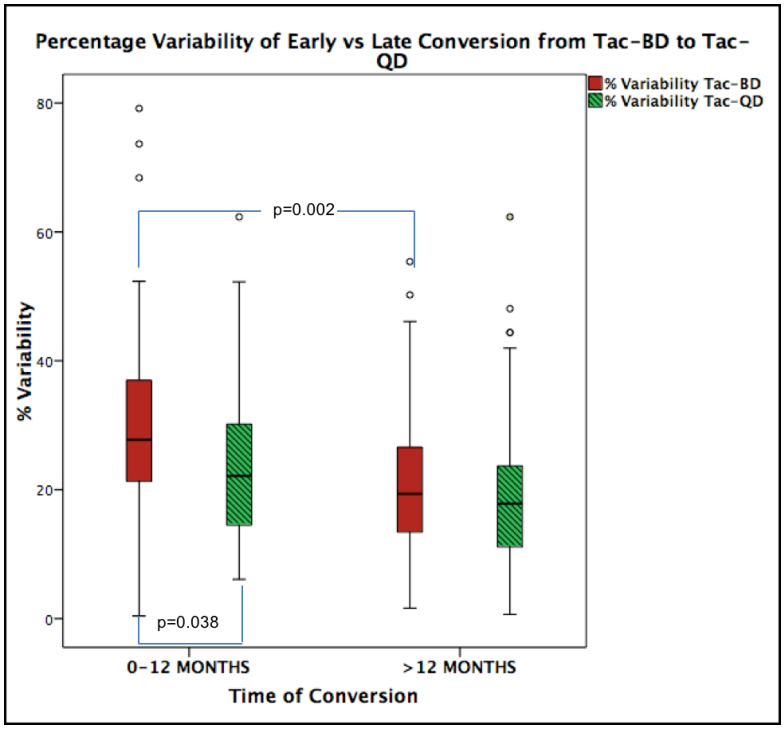


Figure 6.2 Tacrolimus variability for patients converted before and after 12 months demonstrating a significant reduction in variability if converted before 12 months but those converted after 12 months did not have significantly reduced variability between the Tac-BD and Tac-QD preparations.

6.3.4 Variability Change after Conversion to Tac-QD

To determine whether tacrolimus variability had a clinical impact, patients were compared according to the percentage variability before and after conversion: *Low-low (LL), low-to-high (LH), high-to-low (HL), high-high (HH).* In the *LL* group the percentage variability for Tac-BD was 14.92 ± 5.45% and for Tac-QD 11.57 ± 5.02% (n=32). The variability in the *LH* group was 13.72 ± 6.77% for Tac-BD and 29.90 ± 7.83% for Tac-QD (n=19). In the *HL* group the variability was 36.03 ± 14.05% for Tac-BD and 13.12 ± 4.61% for Tac-QD

(n=20). The variability in the *HH* group was 35.64 ± 12.19% for Tac-BD and 32.26 ± 11.86% for Tac-QD (n=32).

Table 6.2 Change in variability in each group for Tac-BD prior to conversion and Tac-QD after conversion

	Tac-BD %Variability	**Tac-QD %Variability**
Low to Low (LL)	14.92 ± 5.45%	11.57 ± 5.02%
Low to High (LH)	13.72 ± 6.77%	29.90 ± 7.83%
High to Low (HL)	36.03 ± 14.05%	13.12 ± 4.61%
High to High (HH)	35.64 ± 12.19%	32.26 ± 11.86%

6.3.5 Acute Rejection

Overall, 21 patients (20.4%) experienced acute rejection episodes but there was no difference between the groups (Chi-square test p=0.953). There were 3 episodes of acute rejection that occurred after conversion to Tac-QD, 1 in the LL group and 2 in the HH group, with no significant difference between the groups (Chi-square test p = 0.487)

6.3.6 Renal Function

There was no significant difference in renal function between the four groups and the serum creatinine levels were comparable when measured prior to conversion and up to 12 months after conversion to Tac-QD (Figure 6.3 below).

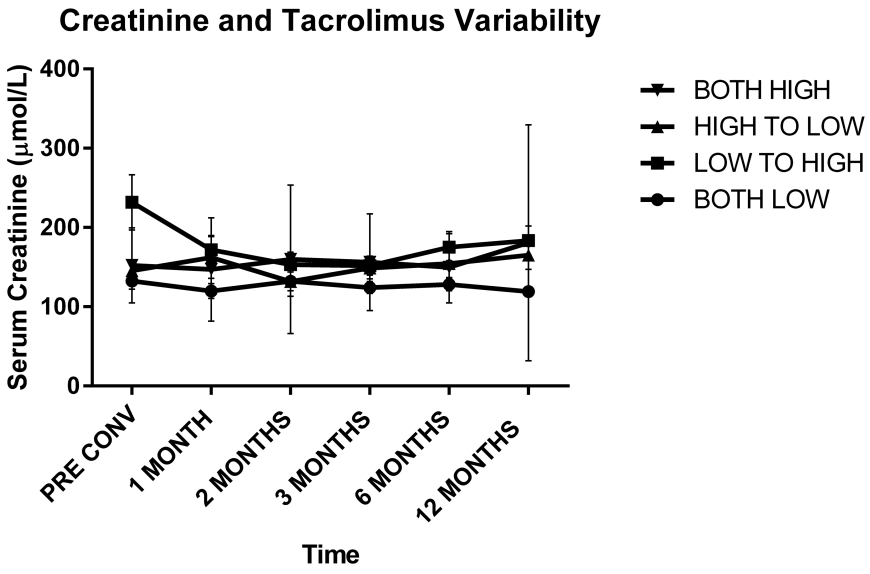
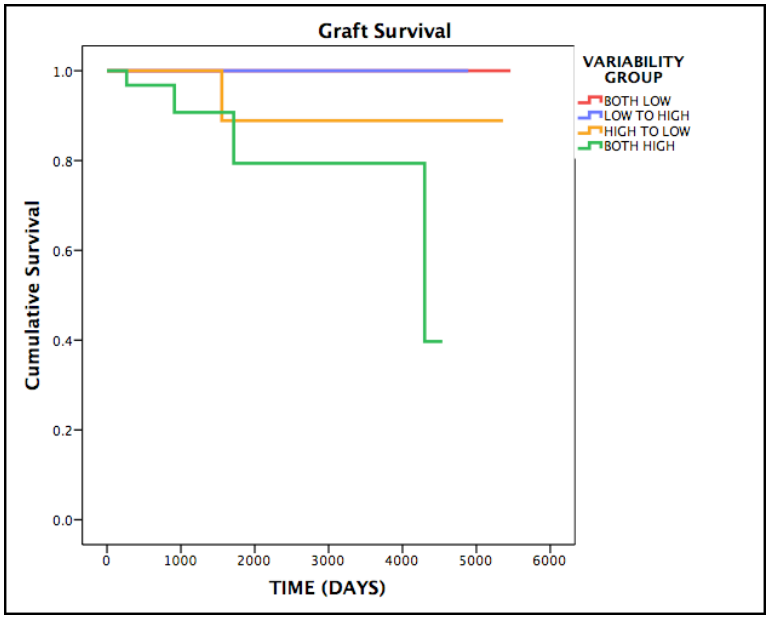


Figure 6.3 Creatinine immediately prior to conversion and at each time point post conversion to Tac-QD for each of the variability groupings with no significant differences between any of the groups.

6.3.7 Graft Survival and Patient Survival

Four patients (12.5%) lost their grafts in the HH group, more compared with all other groups. Kaplan-Meier survival analysis showed a poorer graft survival in the HH (87.5%) group compared with the LL group (100%), (p=0.011 log-rank test) with no difference between the other groups. There were, however, no significant differences in patient survival between any of the groups (Figure 6.4)



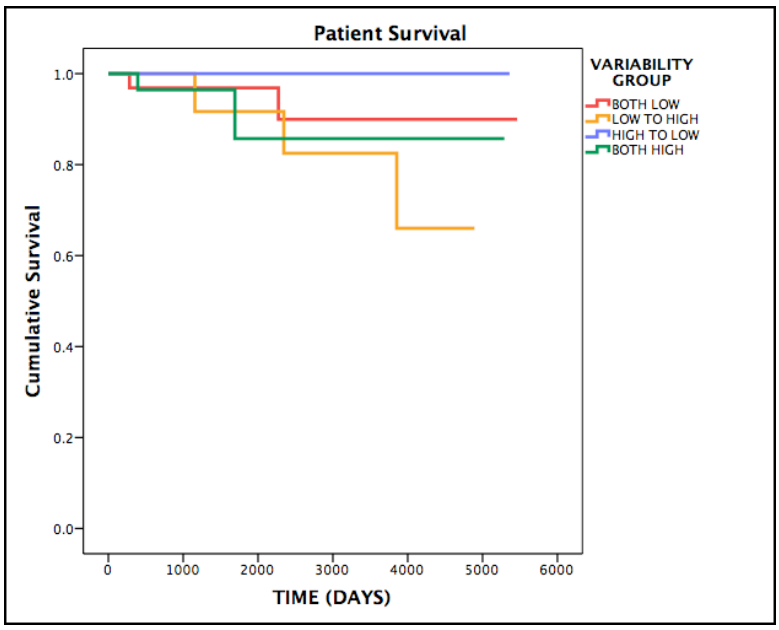


Figure 6.4 Kaplan-Meier survival curves for each variability group following conversion from Tac-BD to Tac-QD. No difference in patient survival is seen however significantly greater graft loss seen in the HH group compared with the LL group, p=0.011 Log-rank test.

6.3.8 Impact of Genetic Polymorphisms on Tac-QD and Variability

43 patients converted from Tac-BD to Tac-QD had stored frozen DNA that was available for genotyping. These patients had their DNA genotyped for polymorphisms of CYP3A5, ABCB1 and CYP3A4*22 as previously described in the laboratory methodology chapter.

6.3.9 CYP3A5

There were 36 patients (83.7%) of the GG (*3/*3) genotype of CYP3A5, 6 patients (14.0%) of the GA (*3/*1) and a single patient (2.3%) of the AA (*1/*1) genotype meaning there were 36 non-expressers of CYP3A5 (83.7%) and 7 expressers (16.3%).

6.3.10 ABCB1

There were 11 patients (25.6%) who expressed the CC genotype of ABCB1, 15 (34.9%) who expressed the CT genotype and 17 (39.5%) who expressed the TT genotype.

*6.3.11 CYP3A4*22*

There were 37 patients (86.0%) who expressed the CC genotype, 5 (11.6%) who expressed the CT genotype and a single patient (2.3%) who expressed the TT genotype of CYP3A4*22. This means there were 6 T allele expressers of CYP3A4*22 (14.0%).

6.3.12 Tacrolimus Variability Relative to Genotype

Tacrolimus variability was comparable between CYP3A5 expressers (24.52±8.43%) and non-expressers (30.80±18.49%) for the Tac-BD preparation, p=0.388, one-way ANOVA and similarly for the Tac-QD preparation where the variability for CYP3A5 expressers was 23.37±6.27% compared with 23.51±11.62% for the non-expressers, p=0.977, one-way ANOVA.

The variability for Tac-BD (Prograf) was significantly higher in patients of the CC genotype of ABCB1 (41.78±19.53%) when compared with the CT genotype (21.52±14.12%), p=0.011, one-way ANOVA. The variability was lower for those of the TT genotype (29.40±14.81%) compared with the CC genotype but it did not reach statistical significance, p=0.171 one-way ANOVA.

For the Tac-QD preparation of tacrolimus the variability did not differ between any of the 3 genotype groups of ABCB1 and was 24.91±13.65% for the CC genotype, 21.28±11.37% for the CT genotype and 24.38±8.59% for the TT genotype, p=0.653, one-way ANOVA for the combined groups.

It is not clear why the CC genotype should have significantly higher variability in the Tac-BD group alone and requires further evaluation in a much larger study before any conclusions can be drawn.

Tacrolimus variability relative to CYP3A4*22 genotype did not show any significant difference between those of the CC genotype (30.43±17.72%), or the CT/TT genotype (25.63±15.05%), p=0.536, one-way ANOVA, for the Tac-BD preparation. There was similarly no difference when patients were converted to the Tac-QD preparation in variability between the CC genotype (23.34±10.46%) or the CT/TT genotype (24.34±14.12%), p=0.838, one-way ANOVA.

6.3.13 Tacrolimus Dose and Trough Levels Relative to Genotype

The smaller cohort of 43 patients who had been converted to Tac-QD and who had their genotype of CYP3A5, ABCB1 and CYP3A4 available had their tacrolimus dose and trough levels measured at various time points up to 12 months after conversion to Tac-QD.

6.3.14 CYP3A5 and Tac-QD

Patients who were expressers of CYP3A5 had a similar 2-fold increase in dose requirement in order to achieve therapeutic levels as patients taking the twice-daily Tac-BD preparation. The increased Tac-QD requirement was sustained throughout the 12 months follow up period after conversion and was highly significant ($p<0.0001$) at every time point, one-way ANOVA. (Figure 6.5 below)

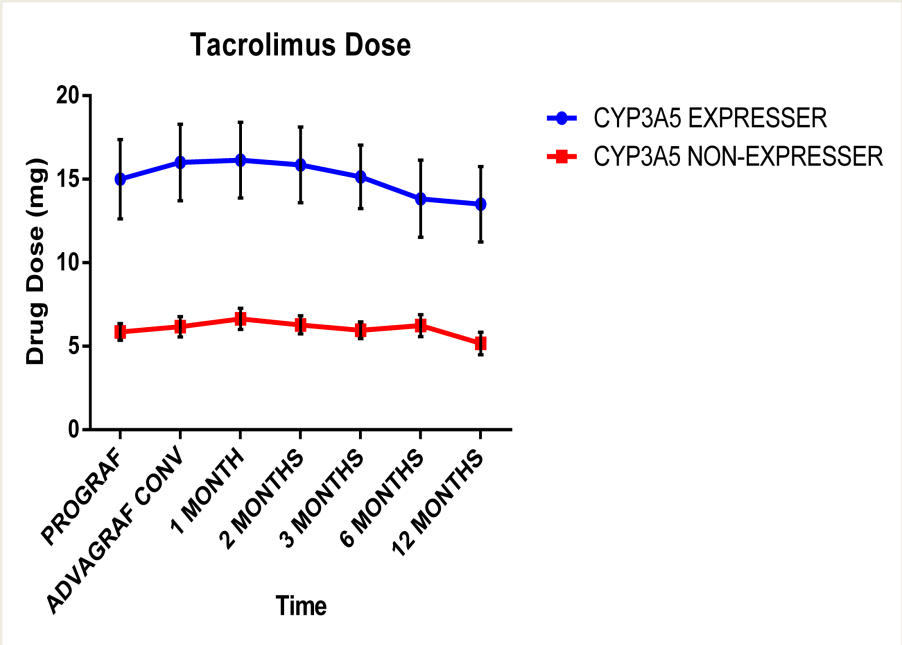


Figure 6.5 Tacrolimus dose requirement at each time point relative to CYP3A5 expression. The dose requirement is significantly higher at each time point for CYP3A5 expressers.

6.3.15 Tacrolimus Trough Levels and CYP3A5 Genotype

The patients in this study were converted at different time points following transplant, therefore many will have been transplanted some time ago and have stable levels. There was no significant difference in tacrolimus trough levels between the expressers and non-expressers of CYP3A5 following conversion to Tac-QD. (Figure 6.6)

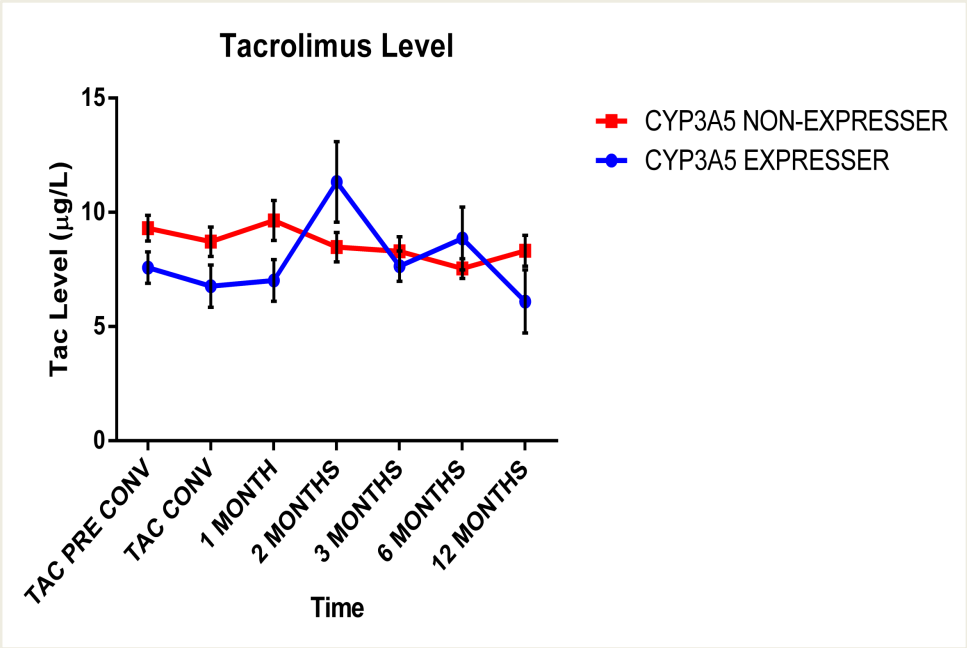


Figure 6.6 Tacrolimus trough concentration (C_0) at each time point relative to CYP3A5 expression which showed no difference.

6.3.16 ABCB1 and Tac-QD

There was no significant difference between the Tac-QD dose requirement at any of the time points between the different genotypes of ABCB1 (Figure 6.7). Correspondingly there was also no significant difference between the tacrolimus trough levels of any of the different genotypes of ABCB1 at any of the time points following conversion to Tac-QD (Figure 6.8).

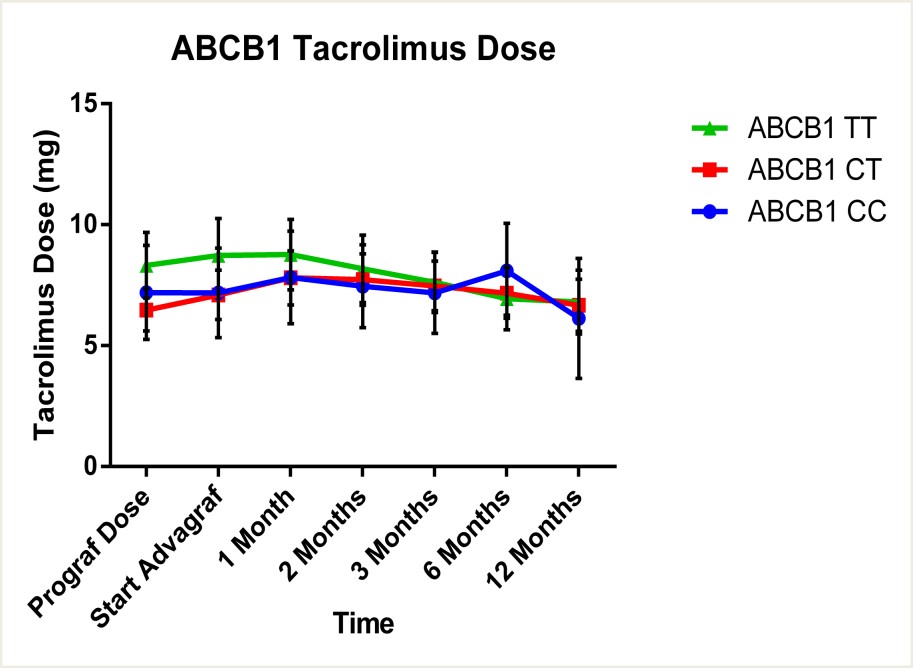


Figure 6.7 Tacrolimus dose requirement at each time point relative to ABCB1 genotype

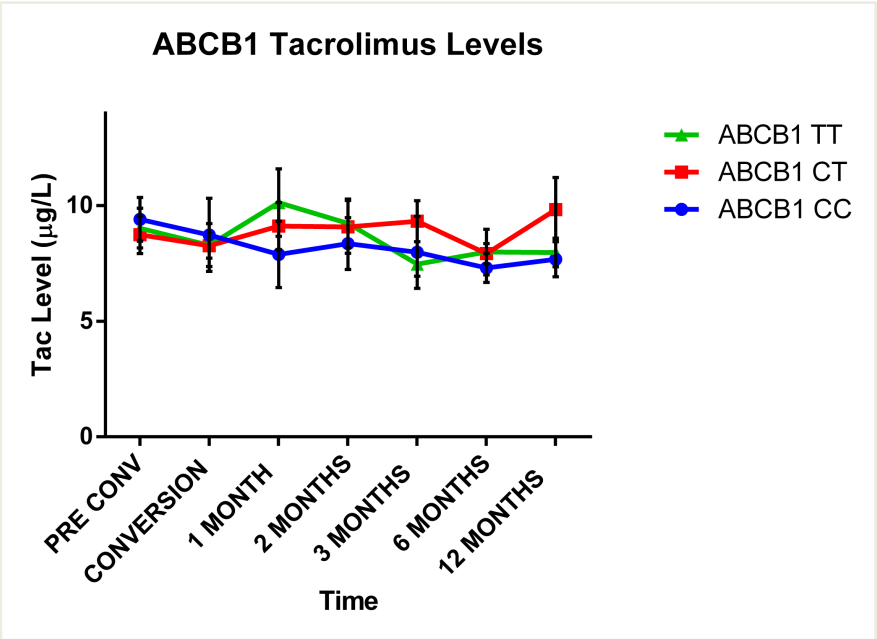


Figure 6.8 Tacrolimus trough concentrations (C_0) at each time point relative to ABCB1 genotype.

*6.3.17 CYP3A4*22 and Tac-QD*

The dose requirement of Tac-QD was slightly lower at each time point for those individuals who expressed a T allele of CYP3A4*22 although it did not reach statistical significance at any of the time points. The trend towards a lower dose requirement for individuals who express a T allele of CYP3A4*22 is appreciable from Figure 6.9 below.

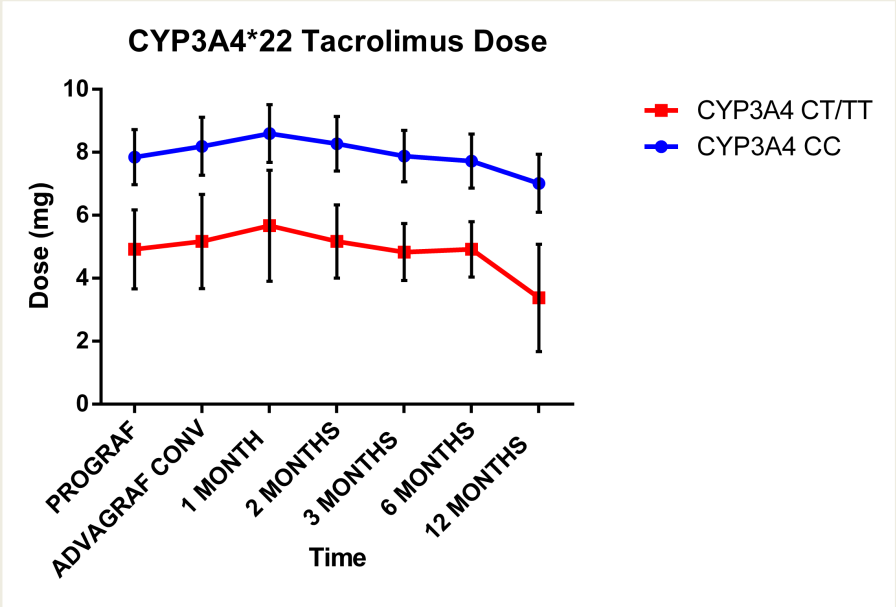


Figure 6.9 Tacrolimus dose requirement at each time point relative to CYP3A4*22 T allele expression.

*6.3.18 Tac Trough Levels and Tac-QD Relative to CYP3A4*22*

There was no difference in the trough levels of tacrolimus following conversion to Tac-QD at any of the time points, similar to the Tac-BD preparation. (Figure 6.10)

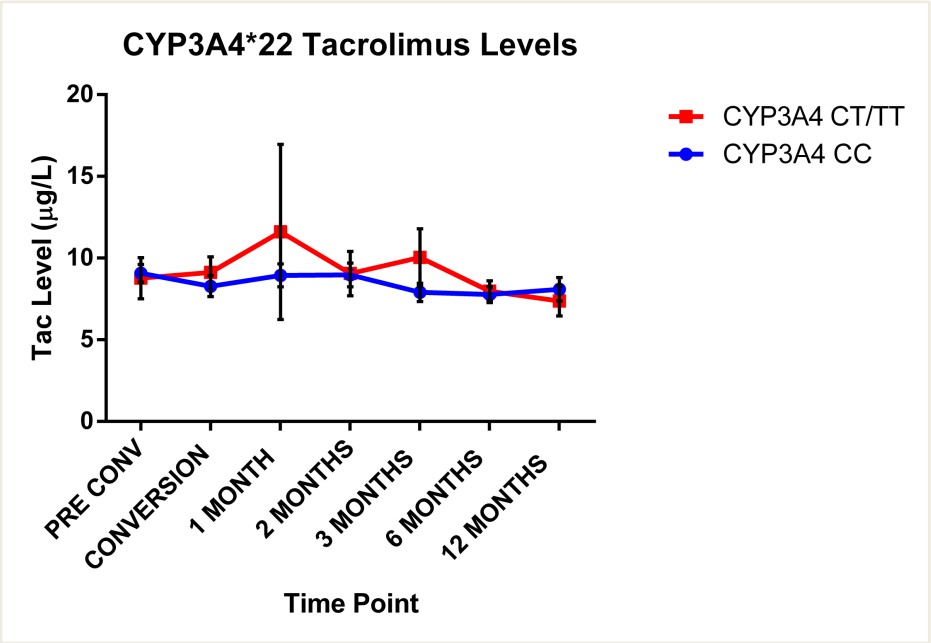


Figure 6.10 Tacrolimus trough concentration (C_0) at each time point relative to CYP3A4*22 T allele expression.

6.4 Chapter Summary

Kidney transplantation is considered the best treatment for end stage renal disease and modern surgical techniques along with advances in immunosuppression have transformed renal transplantation into a routine, cost-effective and highly successful treatment. Despite these successes there remain a proportion of patients who will lose their transplant during the follow up. While undoubtedly the reasons for graft loss are multi-factorial, a study by *Borra et al* has shown that high intra-individual variability of tacrolimus trough concentrations is one of the contributing factors to increased graft loss [49]. Following this report, some

consideration has been given as to whether the once-daily modified release formulation of tacrolimus (Tac-QD or Advagraf®) could result in lower intra-individual variability, given its different pharmacokinetic profile. Wu et al in 2011 suggested that this may be the case [217] whilst Stifft et al [62] presented supportive evidence that once-daily modified release tacrolimus leads to reduced variability although the follow up period was rather short, over the course of 16 weeks.

In this study the variability was calculated before and after conversion to Tac-QD by taking a minimum of 3 trough concentrations in the year prior to conversion and a minimum of 3 trough concentrations in the year following conversion. It is important to note that variability does tend to reduce over time anyway for both the Tac-BD and Tac-QD preparations and thus interpretation of the results can be clouded by this. This may be one reason why different studies have reported that conversion to Tac-QD both does and does not affect variability. The results of this study should be interpreted with that caveat in mind.

In our study, we found that first of all, the twice daily formulation of tacrolimus is associated with a higher intra-individual variability of trough levels however this is only apparent in the first 12 months following transplantation. The exact mechanism for this is unclear however it is generally accepted that much variation in dose and tacrolimus levels will

occur within the first year after transplantation and in particular during the first 3 months. Some of this variation is undoubtedly due to changes in the prescribed dose which are more numerous early after transplantation. Following conversion to a once daily formulation, the intra-patient variability reduced significantly, but only in those patients converted within the first 12 months following transplantation. It may be that during this time the once-daily preparation of tacrolimus leads to a different profile of the area under the curve and therefore reduces some of the variability, but long term there are no differences between the two preparations. A paper by *Shuker et al, Ther Drug Monit, 2015* describes a phenomenon of 'regression to the mean' which may account for the reduction in IPV reported by some other studies [218]. Regression to the mean (RTM) is a statistical phenomenon that can result when the same subjects are sampled repeatedly and can make natural variation appear as if it is real change. This can occur when an unusually large or small value is then followed by a measurement that is closer to the true mean. *Shuker et al* adjusted their IPV values based on a corrected for RTM and reported that 98% of the reduction in the IPV following conversion to Tac-QD was due to RTM rather than a true change. While it is not possible to determine if other studies change in IPV is simply as a result of RTM rather than a true reduction, it must be considered a real possibility, particularly in retrospective and non-randomized studies. We did not correct for RTM in this study and therefore must apply caution in

interpreting the result of a modest reduction in IPV seen in patients converted within the first 12 months after transplant.

Patients who have a high intra-individual variability, irrespective of the tacrolimus formulation, have an increased risk of graft loss. Current evidence has not shown that once-daily tacrolimus started early after transplantation or *de novo* reduces the risk of acute rejection. As this study only shows a significant reduction in intra-patient variability in patients converted to Tac-QD within the first year after transplant, we cannot conclude that there is any significant benefit of early conversion in terms of patient outcome. Our data does suggest that persistently high intra-patient variability, irrespective of the preparation of tacrolimus taken, leads to increased graft loss. One of the questions that needs to be addressed is why some patients have high intra-patient variability. While this question was outside the scope of this study, two factors have been proposed as potential explanations: patient adherence with medication and genetic polymorphisms that may influence tacrolimus pharmacokinetics. Patient adherence is known to influence the outcome of renal transplantation in both adult and paediatric patients [219-221]. Currently, there is limited published data linking non-adherence with increased tacrolimus variability, as an accurate measurement of adherence is difficult to obtain. However

further evaluation of a direct link between adherence and tacrolimus variability would be of value [222].

Genetic polymorphisms of cytochrome P450 are well known to influence the pharmacokinetics and dose requirements of tacrolimus [152, 223]. More recently it has been hypothesised that the expression of CYP3A5 polymorphisms may contribute to the intra-individual variability of tacrolimus, although the supportive evidence is contradictory. *Ro et al* [61, 214, 216] supports a role for CYP3A5 polymorphism in the intra-individual variability of tacrolimus whilst *Spierings et al* [61, 214, 216] and *Pashaee et al* [61, 214, 216] suggest that CYP3A5 polymorphisms have no influence at all on tacrolimus variability. Therefore, the potential impact of genetic polymorphisms on the variability of tacrolimus is yet to be fully ascertained.

This study is limited by the fact that most conversions were at the discretion of the treating physician rather that part of a robust conversion protocol and it is, therefore, conceivable that other patients who were not converted may exhibit a different intra-patient variability profile and have different outcomes that were not captured in this analysis. The non-protocolised conversion strategy utilised in this study is a significant limitation when drawing conclusions about the effectiveness of switching from Tac-BD to Tac-QD as a means of reducing tacrolimus variability. Additionally, our study cohort is not randomized in this retrospective analysis which reduces

the ability to draw firm conclusions from out dataset. While we acknowledge these limitations, this study does provide some preliminary data which adds to the growing evidence that increased tacrolimus variability appears to increase the risk of graft loss in renal transplantation.

Conversion from Tac-BD to Tac-QD appears to be beneficial in reducing intra-patient variability if the patient is converted within the first 12 months however this effect is absent in patients converted after the first year. Increased variability, for both twice-daily and once-daily preparations of tacrolimus has a detrimental effect on graft function and further prospective randomized studies are required to investigate this phenomenon robustly and the correlations with adherence and/or genetic polymorphisms.

Chapter VII

Discussion

7 DISCUSSION

Scotland's multi-organ transplant unit in Edinburgh presented a good opportunity to study the impact of genetic polymorphisms on tacrolimus metabolism and pharmacodynamics across 3 different abdominal organ transplants and to evaluate any differences. The Generation Scotland Bio Resource allowed for a large sample of the population to be genotyped for the genetic polymorphisms of interest in this study and to determine their distribution in a healthy Scottish population. By bringing these two elements together, it was possible to build a picture of how these drug metabolism genetic polymorphisms impact on organ transplantation in a Scottish population and to what extent the distribution of these genetic polymorphisms in the transplant patients are reflected in the healthy Scottish population.

The studies in this thesis add to the existing published data, primarily in renal transplantation, on the impact of SNPs of CYP3A5, ABCB1 and CYP3A4*22 on tacrolimus drug metabolism and support the findings that CYP3A5 expression, in particular, is associated with increased tacrolimus dose requirements and initially reduced trough levels, resulting in a longer

time to reach a steady state blood level post-transplant [119, 123, 125-128, 130, 152, 163].

This study also adds to the emerging data on liver transplant patients demonstrating that donor liver CYP3A5 expression leads to an increased tacrolimus dose requirement to achieve therapeutic levels but also, crucially, results in increased acute rejection of liver allografts and a longer time to reach a therapeutic trough level. These findings re in keeping with more recent publications [224, 225].

It is thought that in Caucasians genetic polymorphisms, primarily of CYP3A5 and CYP3A4, account for about 20% of the variation in tacrolimus dose and trough levels. While there is good evidence to support the influence of genetic polymorphisms on tacrolimus pharmacokinetics, there are many other factors which can influence the tacrolimus dose requirements and trough levels. These factors are often difficult to adjust for and include concomitant medication, variations in haematocrit level and weight, patient adherence and whether the medication is taken alongside food.
Tacrolimus trough concentrations can vary significantly from person to person (inter-patient variability) and also within individual patients between repeated measurements of the trough level (intra-patient variability). Inter-patient variability is likely influenced by the expressed genotype of CYP3A5

and CYP3A4, alongside some newer polymorphisms such as POR*28 [226]. Intra-patient variability is possibly more significant, however, as evidence is now emerging that patients with high variability tacrolimus trough levels have poorer long-term graft survival compared with those with stable levels. This study also explored whether a change from a twice-daily preparation of tacrolimus to once-daily tacrolimus in renal transplant recipients would reduce the intra-patient variability and found an improvement if conversion happened within the first 12 months following transplantation with little effect beyond that. Additionally, it was found that patients who had high intra-patient variability for both twice-daily and once-daily tacrolimus had significantly greater graft loss when compared with patients with low intra-patient variability. This study also found that in a small sub-set of patients, where genomic DNA was available for genotyping, SNPs of CYP3A5, CYP3A4*22 and ABCB1 did not appear to significantly affect tacrolimus variability.

7.1 Distribution of Polymorphisms in a Scottish Population

The first question set out in the introduction of this thesis sought to answer how the SNPs of CYP3A5, ABCB1 and CYP3A4*22 were distributed in a Scottish population and how they compared with other similar Caucasian populations as well as noting differences to other ethnic groups.

This chapter explored the distribution of the three SNPs of interest in this study (CYP3A5, ABCB1 and CYP3A4*22) in a large control group of the Scottish population, with more than 5000 subjects tested. This allowed a confident description of the distribution of these polymorphisms in the Scottish population, a fairly homogeneous ethnic population previously not genotyped for these SNPs. This analysis allowed a direct comparison with the cohort of transplant patients.

This study demonstrates a similar expression of CYP3A5 in White – Scottish subjects as reported in other studies with Caucasian subjects in the UK (86.6%) [152] or Europe [124, 227, 228]. World-wide the GG (*3/*3) genotype of CYP3A5s seen in 75 – 90% Caucasians.

The distribution of ABCB1 was also found to be similar to previously studied populations and in this instance there was another Scottish cohort of inflammatory bowel disease patients to compare against [229]. In this paper a control group of 370 individuals, the distribution of the different genotypes of ABCB1 C3435T was CC 22.2%, CT 51.3% and TT 26.5%, similar to that seen in the Generation Scotland subjects in this thesis (CC 20.6%, CT 49.4%, TT 30.0%). Other studies have described the distribution of the ABCB1 C3435T genotype in Caucasian subjects and have reported similar results [104, 124, 227, 230, 231].

The T allele of CYP3A4*22 has a very low allele frequency in Caucasians, as was the case with this study, and almost completely absent in other non-Caucasian ethnic groups [105].

Examining a large section of the healthy Scottish population and establishing the distribution of the different genotypes of CYP3A5, ABCB1 and CYP3A4*22 gave a robust control group for the next stage of the study where different cohorts of transplant recipients (as well as organ donors) were genotyped for the same SNPs. This study has shown a consistent distribution of the 3 different SNPs across the different organ transplant groups, compared with the sample from the healthy Scottish blood donors.

7.2 Genomics and Liver Transplantation

The impact of the investigated SNPs on liver transplantation has been scarcely examined to date and the next question in the thesis was to explore if the CYP3A5, ABCB1 and CYP3A4*22 polymorphisms had the same effect on tacrolimus pharmacodynamics in liver transplant patients as they had been shown to have in many renal transplant cohorts.

Given that CYP3A5 is a cytochrome p450 enzyme expressed predominantly in liver tissue, it was important to take the donor's genotype into consideration, as we hypothesised that the donor genotype rather than recipient's own genotype plays the prime role in metabolising tacrolimus.

This study did show that the donor expression of CYP3A5 had a significant impact on the tacrolimus dose requirements of liver transplant patients. Significantly higher doses were required to achieve the same therapeutic level as those with livers from CYP3A5 non-expressers. Donor expression had a greater influence than recipient genotype, although recipient expression had a cumulative effect leading to highest tacrolimus dose requirements. In contrast, non-expression of CYP3A5 required the lowest doses.

The CYP3A4*22 genotype has not been extensively studied in liver transplantation. This study showed a trend toward lower tacrolimus dose requirements, particularly in the CYP3A4*22 T allele expressers in the liver donors. This finding again supported the idea that the donor genotype played a more significant role in determining the cytochrome P450 activity than the recipient genotype alone.

There was no significant influence of the C3435T polymorphism of ABCB1 from either the donor or recipient in terms of tacrolimus pharmacodynamics or clinical outcome in this study.

The interaction of these genetic polymorphisms with tacrolimus dose requirements and exposure is of scientific interest and furthers the understanding of drug metabolism and pharmacokinetics, however the translation into clinical relevance for solid organ transplant recipients has been difficult to establish. Theoretically patients who have either a higher or lower exposure to tacrolimus could be at risk of adverse clinical events related to either toxicity or inadequate immunosuppression however these links have not been well established in current literature.

This study did, however, find that only donor expression of CYP3A5 resulted in increased acute cellular rejection in liver transplant patients compared

with the non-expressers. Whilst this could be anticipated given that the donor CYP3A5 expression results in significantly reduced tacrolimus exposure, this is at odds with the results in renal transplantation where there was no increase in acute rejection.

One possible explanation to consider is that the tacrolimus level measured in these studies represents the whole blood concentration trough level which does not necessarily reflect the intracellular concentration of tacrolimus within the hepatocytes. Given that liver donors who express CYP3A5 will metabolise and clear tacrolimus more extensively than the CYP3A5 non-expressers, it is conceivable that the tacrolimus concentration within the liver itself is lower than the whole blood concentration, falsely reassuring the clinician that the tacrolimus levels are adequate. In a renal transplant scenario however, if the recipient is a CYP3A5 expresser, they will need a higher dose to reach a therapeutic level but the whole blood trough levels are likely to be representative of the intra-parenchymal concentration of tacrolimus in the kidney.

If the intrahepatic concentration of tacrolimus were (hypothetically) lower the next thing to consider is whether this would actually increase the risk of rejection within the graft. T cell activity is suppressed by calcineurin inhibitors, and therefore circulating T cells should be adequately suppressed

with a therapeutic whole blood trough level of tacrolimus. It seems theoretically possible, however, that T cells within the liver graft could be more active if the environment within the liver itself were more favourable, with a lower concentration of tacrolimus. While this is speculative in the context of this study, a paper by *Capron et al* in 2007 examined the correlation between hepatic tissue concentration of tacrolimus post liver transplant and acute cellular rejection [232]. They found that the tissue concentration had a more accurate association with acute rejection than the systemic concentration and derived a cut off value of less than 30 pg/mg which was highly sensitive and specific for clinically relevant acute rejection.

Although this study did not evaluate cytochrome P450 genetic polymorphism expression, it revealed that the systemic concentration and the intrahepatic concentration of tacrolimus are not necessarily equivalent and that the intrahepatic concentration appears to be more closely associated with acute rejection. This hypothesis requires further evaluation in future studies.

7.3 Genomics and Renal Transplantation

The role of polymorphisms of genes related to drug metabolism of tacrolimus are most widely described in renal transplantation, where the three SNPs evaluated here are most widely published. The influence of CYP3A5, in particular, which increases the tacrolimus dose requirements approximately two-fold to achieve a therapeutic trough level, is widely established in the current literature and has led to the pursuit of other possible genetic polymorphisms which may also influence tacrolimus pharmacokinetics.

The next question this thesis sought to address was whether the distribution of the genetic polymorphisms in a Scottish renal transplant cohort was similar to those described in other renal transplant studies and whether the influence of these polymorphisms on tacrolimus pharmacodynamics and clinical outcome was similar to those described previously.

Since the first publication of preliminary studies around 15 years ago there has been a plethora of evidence in renal transplantation exploring the relationship between genetic polymorphisms related to drug metabolism enzymes and tacrolimus [111, 154, 162, 209].

In this study, we found that CYP3A5 expression results in an approximately two-fold increase in the dose requirement to achieve a therapeutic level, similar to previously published data. In contrast, the ABCB1 C3435T SNP had no discernible impact on tacrolimus dose requirements.

The results of the CYP3A4*22 assay in renal transplant recipients in this study supported recently published works by *Elens et al* where those who expressed the T – variant allele had a lower tacrolimus dose requirement and greater tacrolimus exposure. This reduction in dose requirement is more subtle than the higher dose required by the CYP3A5 expressers and represents around a 50% reduction from the CC wild type homozygotes of CYP3A4*22.

This study did not find any significant impact on clinical outcomes such as renal function, acute rejection, graft or patient survival in the renal transplant cohort that was related to the genetic polymorphisms of CYP3A5 and CYP3A4*22. However, there appeared to be significantly greater graft loss associated with patients of the CC genotype of ABCB1. There have been reports of different genotypes of ABCB1 conferring an increased risk of renal allograft loss, however the reports have not been consistent. A large study by *Moore et al* in 2012 demonstrated increased graft loss in patients who

received a kidney from a donor, rather than recipient of the CC genotype [233]. A smaller study by *Woillard et al* in 2010 found that, in fact, the TT genotype of the donor was associated with increased graft loss however patients were taking ciclosporin instead of tacrolimus [234]. In our study, it was the recipients only who were genotyped and while the CC genotype of renal transplant patients had greater graft loss, this was only found in univariate analysis.

7.4 Genomics and SPK Transplantation

There is little data about tacrolimus pharmacogenomics in an SPK population and the next question we set to investigate was whether or not SPK patients behaved in the same way as the much better established renal transplant population relative to SNP expression and tacrolimus pharmacodynamics.

While it may seem likely that SPK patients would have similar findings to renal transplant patients, SPK patients could metabolise tacrolimus differently due to a high incidence of autonomic neuropathy and, in particular, gastroparesis and gut dysmotility.

This study found a trend towards an association between CYP3A5 expression and higher doses of tacrolimus for SPK patients, however the results were less clear cut than the kidney alone cohort. One of the potential explanations is the considerably smaller number of patients in the SPK cohort. Similarly, there were no clear significant differences seen in the CYP3A4*22 group, again likely due to small numbers as well as the very low allele frequency of the T-variant allele of this polymorphism.

In the case of SPK transplantation, it seemed plausible that the ABCB1 polymorphism of the 3435 C>T transition may play a greater role than seen in either renal or liver transplantation. The reason for this relates to the gut dysmotility seen in SPK patients, as the distribution of p-glycoprotein varies along the length of the GI tract and these influence changes in the function of the gut, how much tacrolimus is absorbed and crucially, where exactly in the GI tract the tacrolimus is absorbed.

In this cohort of SPK patients, however, there was no difference seen between any of the genotypes of the ABCB1 polymorphism and tacrolimus exposure. However, we did not investigate the gut expression of the polymorphism, which would require duodenal biopsies to test this hypothesis.

7.5 Pharmacogenomics and Abdominal Solid Organ Transplantation

The studies undertaken have shown that CYP3A5 expression has the greatest impact on tacrolimus dose requirements and early trough levels and that patients who are CYP3A5 expressers have significantly lower tacrolimus exposure compared to non-expressers on a comparable dose. This influence was seen in the renal transplant cohort as well as the liver transplant cohort. In the liver cohort, the donor genotype has the greatest significance. A similar trend was seen in the SPK patients however the results were not statistically significant, most likely due to a smaller sample size, and therefore this requires further evaluation.

ABCB1 appeared to have very little, if any, impact on the tacrolimus dosing requirements and tacrolimus exposure. It seems to be a polymorphism included in a lot of studies due to the fact that some early papers appeared to suggest an effect. Subsequent studies have not supported those results and the findings in this thesis would strengthen the conclusion that the effect on tacrolimus dose requirements in any abdominal solid organ transplant is negligible. It is, however, worth noting that CYP3A5 expression reduces tacrolimus exposure so significantly that it may mask the smaller effect of

other polymorphisms. ABCB1 and CYP3A4*22 certainly have a smaller impact and an analysis looking only at CYP3A5 non-expressers to tease out the influence of other SNPs may be relevant.

The effect of the T allele expression of CYP3A4*22 results in lower tacrolimus dose requirements and a greater systemic exposure to tacrolimus compared with those patients of the CC wild-type genotype. This effect is seen in renal and liver transplant patients and suggested in the SPK patients but the lower numbers compounded with a very low allele frequency mean the results do not reach statistical significance at most time points.

The clinical impact, in terms of significant graft or patient level events, related to different drug metabolising genetic polymorphisms has been minimal. The only clinical outcome which has been influenced by expression of a particular genetic polymorphism is an increased acute cellular rejection in liver transplant patients where the donor was a CYP3A5 expresser.

7.6 Tailored Dosing of Tacrolimus Based on Genotype

The current clinical practice in determining the initial dose of tacrolimus is based on the weight of the patient, and the dose is then adjusted based on

therapeutic drug monitoring trough levels. This method, while crude, is fairly reliable for most transplant recipients and will lead to effective immunosuppression in a timely fashion minimising the risk of acute rejection. There will be, however, in a Caucasian population 15 – 20% of individuals who will express at least one copy of the CYP3A5 A (*1) allele, making functional CYP3A5. For these patients, a weight based dose calculation alone may be inadequate, exposing them to a risk of under-immunosuppression in the first week or so after transplantation [103]. It therefore seems sensible to consider individualising the dose of tacrolimus based on the CYP3A5 genotype. There is little evidence either from this study or elsewhere in the literature to justify using ABCB1 genotype in determining the optimal dose of tacrolimus and while T allele expression of CYP3A4*22 may well have an influence on dose requirements, the allele frequency is so low that it is unlikely to have a practical implication in any one transplant unit.

In 2010, *Thervet et al*, published a randomized controlled trial where patients undergoing renal transplantation were randomized to receive standard dose tacrolimus according to weight or a dose based on CYP3A5 expression. They found that at day 10 the patients in the CYP3A5 based dosing were more likely to be in the target C_0 compared with the control group [235]. This paper demonstrated that peri-transplant genotyping is both feasible and

effective in achieving a therapeutic C_0 level more rapidly in CYP3A5 expressers but failed to demonstrate any real clinical benefit from this as the rejection rates and eGFR were similar between the two groups. Furthermore, patients in this study were low immunological risk (most receiving their first transplant), but despite that were significantly immunosuppressed, 82% received induction with rabbit antithymocyte globulin (ATG) which in UK practice is reserved for the higher immunological risk transplants.

It is conceivable that higher immunological risk patients such as those who are highly sensitised, those with a less well-matched graft or HLA/ABO incompatible transplants may benefit more from targeted dosing of tacrolimus based on the CYP3A5 genotype but this remains to be evaluated.

In 2011 *Passey et al* described a dosing model that considered both genetic and clinical factors found to be important in predicting the tacrolimus clearance of individual patients [236]. In addition to CYP3A5 expression they found that transplantation at a steroid sparing centre, patient's age, time from transplant and concomitant use of a calcium channel blocker influenced the dose of tacrolimus required to achieve a therapeutic trough level. This equation is complex and would be difficult for every day clinical use (although an electronic calculator in a spreadsheet would be easy

enough to develop) and also requires the pre-emptive genotyping of all potential recipients for CYP3A5 to identify expressers.

It is clear that genetic polymorphism expression is not the only variable which explains the inter-patient variability of tacrolimus clearance, and studies have shown that the haematocrit level, steroid use, drugs such as fluconazole and patient's age, can influence the required dose in order to achieve a therapeutic trough level. Nevertheless, despite these other influences on tacrolimus metabolism, repeated studies have shown that CYP3A5 expression appears to have the largest impact of any one single variable. What has not been shown convincingly is a significant impact of CYP3A5 expression on major clinical outcomes.

7.7 Variability, Genetic Polymorphisms and Tac-QD

The final question in this thesis was to explore a more recent phenomenon that has been described and may significantly impact on graft survival; high **intra**-patient variability of tacrolimus levels. This is not a description of the different tacrolimus levels between different patients (which may or may not be related to genetic polymorphisms) but rather describes the variation of tacrolimus trough levels within an individual. We also set out to explore

whether or not there was a relationship between intra-patient variability and the 3 genetic polymorphisms of interest in this study.

A seminal paper by *Borra et al* in 2010 described intra-patient variability (IPV) by calculating the percentage by which any individual patient deviates from their own mean tacrolimus trough levels. By taking the median value patients were either in a high or low variability group. The authors reported an increased graft loss in patients in the high IPV group [49].

In this study we examined a subgroup of renal transplant patients who had been converted from a twice-daily tacrolimus standard release preparation (Prograf®, Tac-BD) to a once-daily modified release preparation (Advagraf®, Tac-QD) and evaluated the impact of the 3 SNPs on the dose requirements and tacrolimus trough levels. In addition, we calculated the IPV before and after conversion to once-daily tacrolimus and related this to clinical outcome.

This study found that IPV was reduced by conversion to a modified release once-daily tacrolimus (Tac-QD) however this effect was only seen if the conversion took place within the first 12 months after transplantation given that the first year after transplantation is the most dynamic period for tacrolimus levels and dose changes [237].

There are some studies that have suggested a more significant impact on IPV from converting to Tac-QD. *Wu et al, 2011* [59] found that in stable kidney transplant patients, conversion to once-daily tacrolimus still reduced the IPV. The impact of once-daily tacrolimus on IPV remains controversial as other studies found that a conversion to the modified release preparation did not have any impact on IPV [218]. The studies investigating this issue were small and therefore were unable to account for all the potential variables which could influence the variability of tacrolimus (many difficult to measure). Regression towards the mean (RTM) is a statistical phenomenon seen when repeated measures of the same subjects can suggest an effect of an intervention when, in fact, the difference is merely natural variation. RTM can be corrected for, in terms of IPV, however very few studies do this and their results are perhaps misinterpreted as a result. Until a carefully designed and adequately powered multi-centred study is undertaken, the smaller studies (this one included) will struggle to answer the question definitively.

An aspect of IPV that is far less controversial is the impact of a high IPV on long term graft survival. This study found that patients who had a high IPV both before and after conversion to once-daily Tac-QD had significantly higher graft loss than those patients who had low IPV before and after

conversion. High IPV has been consistently shown in several studies to negatively impact renal allograft survival [49, 238, 239] however these studies are all limited by the fact that they are retrospective and it is difficult to tease out the many factors and in particular non-adherence. Nevertheless, they provide some convincing evidence that high IPV (whatever the cause) has a deleterious impact on graft survival.

We then went on to explore whether there was a relationship between CYP3A5 expression and tacrolimus dose requirements between those renal transplant patients who were taking the standard twice-daily preparation of tacrolimus (Tac-BD) and those taking the modified release once-daily preparation (Tac-QD). Those who were CYP3A5 expressers had consistently higher dose requirements than those who did not express CYP3A5. We also found that there was no impact on the clinical outcomes.

The impact of the CYP3A4*22 SNP on once-daily tacrolimus pharmacodynamics was also similar to renal transplant patients taking Tac-QD with a trend clearly seen towards lower dose requirements for T-variant allele expressers, although again not statistically significant. There were similarly no differences seen in the clinical outcome of renal transplant patients, relative to CYP3A4*22 genotype.

It is not surprising that the influence of CYP3A5 and CYP3A4*22 is similar in the modified release once-daily tacrolimus as it is in the standard twice-daily preparation. Although the rate of absorption will be different, the overall amount of tacrolimus exposure will be similar and therefore the impact from cytochrome P450 enzymes in terms of dose requirements and levels are likely to be the same.

In terms of ABCB1 SNP influences, this is where modified release tacrolimus and the distribution of P-gp in the gut may have been more likely to interact. Tac-QD is absorbed more distally in the gut than the immediate release formulation Tac-BD. Given that T allele expression of ABCB1 C3435T decreases the expression of P-gp which is most abundant in the distal gut (colon) then it seemed plausible that ABCB1 may have a greater impact on the pharmacodynamics of once-daily Tac-QD compared with Tac-BD. This was not the case in this study as there was no difference in the dose requirements or trough levels between the CC, CT or TT genotype.
This finding suggests that the expression of P-gp relative to the ABCB1 C3435T polymorphism has very little impact on the pharmacodynamics of tacrolimus, particularly when both the immediate release and modified release preparations behave in the same way.

The final part of this study was to try and bring these 2 elements of IPV and genotype expression together and explore whether there was an association between CYP3A5, CYP3A4*22 or ABCB1 genotype and IPV. In this smaller cohort of patients, we did not find any significant association between the different genotypes of CYP3A5, CYP3A4*22 or ABCB1 and IPV. Although there was a higher variability for those who were CYP3A5 expressers, this did not reach statistical significance. There have been conflicting data regarding the influence of CYP3A5 on IPV with some studies suggesting there is an association with CYP3A5 genotype [60] while others have found no link between CYP3A5 genotype and IPV [61].

For the patients converted from twice-daily Tac-BD to once-daily Tac-QD in this study, a large number were converted some time out from transplantation and therefore at steady state in terms of their tacrolimus dosing and trough levels reducing the likelihood that variability was due to multiple dose adjustments as seen in the early post transplant period.

The reasons for increased IPV are certainly multifactorial and almost certainly more complex than CYP3A5 (or any other SNP) expression alone.

7.8 Limitations of This Thesis

This study was undertaken carefully and diligently to ensure the data and results presented were as accurate as possible. Nevertheless there are several limitations that could significantly impact on the results. Firstly this study is retrospective and so all the data collected was done as part of routine clinical care and not with a study in mind. As such there are missing data, variation in how the data was collected, and adjustment to doses that were made as part of clinical interpretation of results rather than a rigid study protocol. Without randomisation, a proper control group that is matched on demographics is not possible and thus can introduce bias into the results.

This study is underpowered to detect 2 major clinical outcomes of graft loss and patient survival. As a result, the impact of genetic polymorphisms on graft and patient survival cannot be fully evaluated and a much larger study with adequate follow up is required in order to answer that question definitively.

This study had only a small number of patients in the SPK cohort and therefore the impact of the SNPs was less pronounced. A larger cohort of SPK patients would be desirable to evaluate whether there were any

differences in SPK transplant patients compared with the standard renal transplant cohort.

Where patients were converted from Tac-BD to Tac-QD this was done at clinician discretion rather than as part of a protocol which introduces the potential for bias, particularly as it was not possible to ascertain why these patients were chosen to switch from Tac-BD to Tac-QD. This study did not correct for a regression to the mean when measuring IPV so the reduction in IPV seen by converting from Tac-BD to Tac-QD if converted within the first 12 months may simply be a regression to the mean.

7.9 Summary

This thesis describes the distribution of certain genetic polymorphisms in Scotland which are relevant to transplant patients and their immunosuppression medication and explores the impact on the clinical outcomes. We took one of the largest samples of a single population ever studied in this particular area and found the that the distribution of polymorphisms of CYP3A5, ABCB1 and CYP3A4*22 were similar to other Caucasian groups.

The studies in this thesis confirmed the impact of CYP3A5 expression on renal transplant patients and produced one of the first studies of this kind in a cohort of simultaneous pancreas-kidney transplant patients, finding similar results to renal transplant patients. In addition, one of the studies in this thesis revealed that in liver transplant patients, the donor genotype is of a greater influence than the recipient's own genotype. Furthermore, donor expression of CYP3A5 led to increased acute rejection compared with the non-expressers, although variations in these polymorphisms had no impact on graft or patient survival.

The final part of this thesis examined a subset of renal transplant patients who had been converted to once-daily tacrolimus (Advagraf®) and showed that the genetic polymorphisms of CYP3A5, CYP3A4*22 and ABCB1 influenced the once-daily preparation in a similar way to the standard twice-daily (Prograf®).

In addition, this subset of patients converted to once-daily tacrolimus had reduced IPV if converted within the first 12 months following transplantation however this effect was not seen if conversion took place beyond 12 months. Finally, patients with high IPV both pre and post conversion to once-daily tacrolimus had significantly greater graft loss

compared to those patients with low IPV pre and post conversion to once-daily tacrolimus.

Careful maintenance of a therapeutic but non-toxic tacrolimus level is crucial for long term graft survival and it is clear many factors can influence the pharmacodynamics and pharmacokinetics. Individually tailored dosing which incorporates CYP3A5 genotype is an approach which is gaining interest within the transplant community however its impact on long term graft function is yet to be fully evaluated. While CYP3A5 genotype would be a powerful tool in predicting the dose requirements of tacrolimus in transplant patients, it does not seem to influence clinical outcome in a particularly meaningful way and it may be that other indicators such as IPV are more relevant.

As the long term sequelae of immunosuppression begins to emerge, alongside the increasing evidence of the detrimental impact of high IPV, strategies to minimise CNI exposure and individually tailor immunosuppression to each patient are becoming desirable. Genotyping of recipients and donors may well become part of this strategy to better tailor doses to individual patients. Genotyping alone is unlikely to have a significant clinical impact however as part of a wider strategy, including measurement of IPV, it may provide a useful adjunct to delivering a

personalised immunosuppression regimen to each patient. To date CYP3A5 expression has been the most influential SNP discovered relative to tacrolimus pharmacodynamics and I think it is the only one that will have any significant clinical impact as part of a personalised dosing strategy. While CYP3A5 expression undoubtedly has an impact on the dose requirements of tacrolimus in transplant patients, the clinical impact is much weaker and it may be that the extra time and expense to genotype patients cannot be justified. IPV appears to have a much greater clinical impact and would not require any additional testing, but it cannot be determined pre-transplant and it is not yet clear why some people have high IPV compared with others, which makes the development of a useful intervention difficult to implement.

Further prospective studies in these areas are required to better inform clinicians of ways of improving tacrolimus dosing and monitoring in the peri-transplant period and in the longer term.

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APPENDIX

9 APPENDIX

9.1 Data Tables for Chapter 3

Table 9.1 CYP3A5 genotypes full cohort

SUBJECT TYPE			CYP3A5			
			GG (*3/*3)	GA (*3/*1)	AA (*1/*1)	UNDETERMINED
GENERATION SCOTLAND	WHITE - SCOTTISH	Count	3726	495	14	82
		Percentage	86.3%	11.5%	.3%	1.9%
	WHITE - OTHER BRITISH	Count	392	61	1	9
		Percentage	84.7%	13.2%	.2%	1.9%
	WHITE - IRISH	Count	40	4	1	2
		Percentage	85.1%	8.5%	2.1%	4.3%
	WHITE - OTHER	Count	31	4	0	2
		Percentage	83.8%	10.8%	0.0%	5.4%
	MIXED	Count	12	3	0	0
		Percentage	80.0%	20.0%	0.0%	0.0%
	ASIAN - INDIAN	Count	2	1	0	0
		Percentage	66.7%	33.3%	0.0%	0.0%
	ASIAN - CHINESE	Count	2	1	0	0
		Percentage	66.7%	33.3%	0.0%	0.0%
	ASIAN - OTHER	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	OTHER	Count	3	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	UNKNOWN	Count	9	1	0	0
		Percentage	90.0%	10.0%	0.0%	0.0%
	TOTAL	Count	4218	570	16	95
		Percentage	86.1%	11.6%	.3%	1.9%
KIDNEY TRANSPLANT	WHITE - SCOTTISH	Count	232	43	7	7
		Percentage	80.3%	14.9%	2.4%	2.4%
	WHITE - OTHER	Count	5	2	0	0
		Percentage	71.4%	28.6%	0.0%	0.0%
	ASIAN - INDIAN	Count	3	3	2	0
		Percentage	37.5%	37.5%	25.0%	0.0%
	ASIAN - CHINESE	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
		Count	241	48	9	7
		Percentage	79.0%	15.7%	3.0%	2.3%
SPK TRANSPLANT	WHITE - SCOTTISH	Count	36	8	1	2
		Percentage	76.6%	17.0%	2.1%	4.3%
	ASIAN - INDIAN	Count	0	1	0	0
		Percentage	0.0%	100.0%	0.0%	0.0%
	TOTAL	Count	36	9	1	2
		Percentage	75.0%	18.8%	2.1%	4.2%
LIVER TRANSPLANT	WHITE - SCOTTISH	Count	214	26	0	5
		Percentage	87.3%	10.6%	0.0%	2.0%
	ASIAN - INDIAN	Count	4	1	1	0
		Percentage	66.7%	16.7%	16.7%	0.0%
	BLACK	Count	0	0	1	0
		Percentage	0.0%	0.0%	100.0%	0.0%
	TOTAL	Count	218	27	2	5
		Percentage	86.5%	10.7%	.8%	2.0%
ORGAN DONOR	WHITE - SCOTTISH	Count	98	10	0	2
		Percentage	89.1%	9.1%	0.0%	1.8%
	WHITE - OTHER BRITISH	Count	108	13	0	6
		Percentage	85.0%	10.2%	0.0%	4.7%
	WHITE - IRISH	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	WHITE - OTHER	Count	117	23	0	5
		Percentage	80.7%	15.9%	0.0%	3.4%
	ASIAN - INDIAN	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - CHINESE	Count	0	1	0	0
		Percentage	0.0%	100.0%	0.0%	0.0%
	TOTAL	Count	325	47	0	13
		Percentage	84.4%	12.2%	0.0%	3.4%
OVERALL COHORT	WHITE - SCOTTISH	Count	4306	582	22	98
		Percentage	86.0%	11.6%	.4%	2.0%
	WHITE - OTHER BRITISH	Count	500	74	1	15
		Percentage	84.7%	12.5%	.2%	2.5%
	WHITE - IRISH	Count	41	4	1	2
		Percentage	85.4%	8.3%	2.1%	4.2%
	WHITE - OTHER	Count	153	29	0	7
		Percentage	81.0%	15.3%	0.0%	3.7%
	MIXED	Count	12	3	0	0
		Percentage	80.0%	20.0%	0.0%	0.0%
	ASIAN - INDIAN	Count	10	6	3	0
		Percentage	52.6%	31.6%	15.8%	0.0%
	BLACK	Count	0	0	1	0
		Percentage	0.0%	0.0%	100.0%	0.0%
	ASIAN - CHINESE	Count	3	2	0	0
		Percentage	60.0%	40.0%	0.0%	0.0%
	ASIAN - OTHER	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	OTHER	Count	3	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	UNKNOWN	Count	9	1	0	0
		Percentage	90.0%	10.0%	0.0%	0.0%
	TOTAL	Count	5038	701	28	122
		Percentage	85.5%	11.9%	.5%	2.1%

Table 9.2 ABCB1 genotypes full cohort

SUBJECT TYPE			ABCB1 CC	CT	TT	UNDETERMINED
GENERATION SCOTLAND	WHITE - SCOTTISH	Count	881	2044	1236	156
		Percentage	20.4%	47.3%	28.6%	3.6%
	WHITE - OTHER BRITISH	Count	78	232	137	16
		Percentage	16.8%	50.1%	29.6%	3.5%
	WHITE - IRISH	Count	4	19	24	0
		Percentage	8.5%	40.4%	51.1%	0.0%
	WHITE - OTHER	Count	8	18	9	2
		Percentage	21.6%	48.6%	24.3%	5.4%
	MIXED	Count	2	9	4	0
		Percentage	13.3%	60.0%	26.7%	0.0%
	ASIAN - INDIAN	Count	0	2	1	0
		Percentage	0.0%	66.7%	33.3%	0.0%
	ASIAN - CHINESE	Count	1	1	1	0
		Percentage	33.3%	33.3%	33.3%	0.0%
	ASIAN - OTHER	Count	0	0	1	0
		Percentage	0.0%	0.0%	100.0%	0.0%
	OTHER	Count	0	3	0	0
		Percentage	0.0%	100.0%	0.0%	0.0%
	UNKNOWN	Count	0	6	4	0
		Percentage	0.0%	60.0%	40.0%	0.0%
	TOTAL	Count	974	2334	1417	174
		Percentage	19.9%	47.6%	28.9%	3.6%
KIDNEY TRANSPLANT	WHITE - SCOTTISH	Count	56	137	94	2
		Percentage	19.4%	47.4%	32.5%	.7%
	WHITE - OTHER	Count	3	3	1	0
		Percentage	42.9%	42.9%	14.3%	0.0%
	ASIAN - INDIAN	Count	3	3	2	0
		Percentage	37.5%	37.5%	25.0%	0.0%
	ASIAN - CHINESE	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	63	143	97	2
		Percentage	20.7%	46.9%	31.8%	.7%
SPK TRANSPLANT	WHITE - SCOTTISH	Count	9	24	14	0
		Percentage	19.1%	51.1%	29.8%	0.0%
	ASIAN - INDIAN	Count	0	0	1	0
		Percentage	0.0%	0.0%	100.0%	0.0%
	TOTAL	Count	9	24	15	0
		Percentage	18.8%	50.0%	31.3%	0.0%
LIVER TRANSPLANT	WHITE - SCOTTISH	Count	57	105	74	9
		Percentage	23.3%	42.9%	30.2%	3.7%
	ASIAN - INDIAN	Count	1	3	2	0
		Percentage	16.7%	50.0%	33.3%	0.0%
	BLACK	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	59	108	76	9
		Percentage	23.4%	42.9%	30.2%	3.6%
ORGAN DONOR	WHITE - SCOTTISH	Count	18	62	25	5
		Percentage	16.4%	56.4%	22.7%	4.5%
	WHITE - OTHER BRITISH	Count	29	60	31	7
		Percentage	22.8%	47.2%	24.4%	5.5%
	WHITE - IRISH	Count	0	1	0	0
		Percentage	0.0%	100.0%	0.0%	0.0%
	WHITE - OTHER	Count	29	64	40	12
		Percentage	20.0%	44.1%	27.6%	8.3%
	ASIAN - INDIAN	Count	0	0	0	1
		Percentage	0.0%	0.0%	0.0%	100.0%
	ASIAN - CHINESE	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	77	187	96	25
		Percentage	20.0%	48.6%	24.9%	6.5%
OVERALL COHORT	WHITE - SCOTTISH	Count	1021	2372	1443	172
		Percentage	20.4%	47.4%	28.8%	3.4%
	WHITE - OTHER BRITISH	Count	107	292	168	23
		Percentage	18.1%	49.5%	28.5%	3.9%
	WHITE - IRISH	Count	4	20	24	0
		Percentage	8.3%	41.7%	50.0%	0.0%
	WHITE - OTHER	Count	40	85	50	14
		Percentage	21.2%	45.0%	26.5%	7.4%
	MIXED	Count	2	9	4	0
		Percentage	13.3%	60.0%	26.7%	0.0%
	ASIAN - INDIAN	Count	4	8	6	1
		Percentage	21.1%	42.1%	31.6%	5.3%
	BLACK	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - CHINESE	Count	3	1	1	0
		Percentage	60.0%	20.0%	20.0%	0.0%
	ASIAN - OTHER	Count	0	0	1	0
		Percentage	0.0%	0.0%	100.0%	0.0%
	OTHER	Count	0	3	0	0
		Percentage	0.0%	100.0%	0.0%	0.0%
	UNKNOWN	Count	0	6	4	0
		Percentage	0.0%	60.0%	40.0%	0.0%
	TOTAL	Count	1182	2796	1701	210
		Percentage	20.1%	47.5%	28.9%	3.6%

Table 9.3 CYP3A4*22 genotype full cohort

SUBJECT TYPE			CYP3A4*22			
			CC	CT	TT	UNDETERMINED
GENERATION SCOTLAND	WHITE - SCOTTISH	Count	3801	414	7	95
		Percentage	88.0%	9.6%	.2%	2.2%
	WHITE - OTHER BRITISH	Count	404	48	4	7
		Percentage	87.3%	10.4%	.9%	1.5%
	WHITE - IRISH	Count	44	3	0	0
		Percentage	93.6%	6.4%	0.0%	0.0%
	WHITE - OTHER	Count	32	4	0	1
		Percentage	86.5%	10.8%	0.0%	2.7%
	MIXED	Count	15	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - INDIAN	Count	3	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - CHINESE	Count	3	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - OTHER	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	OTHER	Count	2	1	0	0
		Percentage	66.7%	33.3%	0.0%	0.0%
	UNKNOWN	Count	8	2	0	0
		Percentage	80.0%	20.0%	0.0%	0.0%
	TOTAL	Count	4313	472	11	103
		Percentage	88.0%	9.6%	0.2%	2.1%
KIDNEY TRANSPLANT	WHITE - SCOTTISH	Count	260	25	2	2
		Percentage	90.0%	8.7%	0.7%	0.7%
	WHITE - OTHER	Count	7	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - INDIAN	Count	8	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - CHINESE	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	276	25	2	2
		Percentage	90.5%	8.2%	0.7%	0.7%
SPK TRANSPLANT	WHITE - SCOTTISH	Count	43	3	0	1
		Percentage	91.5%	6.4%	0.0%	2.1%
	ASIAN - INDIAN	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	44	3	0	1
		Percentage	91.7%	6.3%	0.0%	2.1%
LIVER TRANSPLANT	WHITE - SCOTTISH	Count	218	16	0	11
		Percentage	89.0%	6.5%	0.0%	4.5%
	ASIAN - INDIAN	Count	6	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	BLACK	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	225	16	0	11
		Percentage	89.3%	6.3%	0.0%	4.4%
ORGAN DONOR	WHITE - SCOTTISH	Count	88	14	0	8
		Percentage	80.0%	12.7%	0.0%	7.3%
	WHITE - OTHER BRITISH	Count	110	9	1	7
		Percentage	86.6%	7.1%	0.8%	5.5%
	WHITE - IRISH	Count	0	1	0	0
		Percentage	0.0%	100.0%	0.0%	0.0%
	WHITE - OTHER	Count	127	14	0	4
		Percentage	87.6%	9.7%	0.0%	2.8%
	ASIAN - INDIAN	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - CHINESE	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	TOTAL	Count	327	38	1	19
		Percentage	84.9%	9.9%	0.3%	4.9%
OVERALL COHORT	WHITE - SCOTTISH	Count	4410	472	9	117
		Percentage	88.1%	9.4%	0.2%	2.3%
	WHITE - OTHER BRITISH	Count	514	57	5	14
		Percentage	87.1%	9.7%	0.8%	2.4%
	WHITE - IRISH	Count	44	4	0	0
		Percentage	91.7%	8.3%	0.0%	0.0%
	WHITE - OTHER	Count	166	18	0	5
		Percentage	87.8%	9.5%	0.0%	2.6%
	MIXED	Count	15	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - INDIAN	Count	19	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	BLACK	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - CHINESE	Count	5	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	ASIAN - OTHER	Count	1	0	0	0
		Percentage	100.0%	0.0%	0.0%	0.0%
	OTHER	Count	2	1	0	0
		Percentage	66.7%	33.3%	0.0%	0.0%
	UNKNOWN	Count	8	2	0	0
		Percentage	80.0%	20.0%	0.0%	0.0%
	TOTAL	Count	5185	554	14	136
		Percentage	88.0%	9.4%	0.2%	2.3%

9.2 Data tables for Chapter 4

Table 9.4 CYP3A5 recipient dose requirement

	CYP3A5 GG (*3/*3) – non expresser (R)		**CYP3A5 GA/AA (*1/*3-*1/*1) – expresser (R)**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p
WK1-1	3.4714	105	3.8235	17	0.363
WK1-2	4.0194	103	4.7647	15	0.086
WK1-3	4.5000	93	5.8235	17	**0.015**
WK2-1	4.7794	102	6.5588	17	**0.003**
WK2-2	4.9813	80	6.8929	14	**0.009**
WK2-3	4.9375	64	6.4500	10	0.113
WK3-1	5.2525	101	7.2500	14	**0.002**
WK3-2	5.2823	62	7.0000	10	**0.043**
WK3-3	5.7436	37	7.0000	7	0.342
WK4-1	5.3564	101	7.7813	16	**0.001**
WK4-2	5.2174	69	7.4500	10	**0.020**
3 M	4.3535	99	6.0667	15	**0.010**
6 M	4.2684	95	5.2667	15	0.135
12 M	4.2391	92	4.1333	15	0.868

Table 9.5 CYP3A5 donor dose requirement

	Donor CYP3A5 GG (*3/*3)		**Donor CYP3A5 GA/AA (*3/*1 - *1/*1)**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p value
WK1-1	3.3938	160	3.9688	16	0.106
WK1-2	3.9399	157	4.7667	16	**0.045**
WK1-3	4.3542	144	6.1667	16	**<0.0001**
WK2-1	4.6818	154	7.0625	16	**<0.0001**
WK2-2	4.9837	123	7.6786	14	**<0.0001**
WK2-3	4.7526	97	8.3571	14	**<0.0001**
WK3-1	5.1074	149	9.0000	16	**<0.0001**
WK3-2	5.2000	100	7.6111	9	**0.003**
WK3-3	5.1491	57	9.1818	11	**<0.0001**
WK4-1	5.0900	150	9.5833	18	**<0.0001**
WK4-2	4.8960	101	9.9167	12	**<0.0001**
3 M	4.1301	146	7.8438	16	**<0.0001**
6 M	4.0324	142	7.0313	16	**<0.0001**
12 M	3.9961	128	6.2857	14	**<0.0001**

Table 9.6 CYP3A5 recipient trough levels

	Recipient CYP3A5 GG (*3/*3)		**Recipient CYP3A5 GA/AA (*3/*1 - *1/*1)**		
	Mean C_0 (µg/L)	n	Mean C_0 (µg/L)	n	p value
WK1-1	4.86440	118	3.3600	15	0.116
WK1-2	6.55000	116	5.0562	16	0.125
WK1-3	7.25470	106	5.7000	17	0.149
WK2-1	7.27810	114	6.4412	17	0.409
WK2-2	6.76410	92	6.8643	14	0.901
WK2-3	6.51970	76	6.0600	10	0.700
WK3-1	7.39910	108	7.2500	14	0.898
WK3-2	7.29710	69	6.7545	11	0.556
WK3-3	7.22227	44	7.1857	7	0.976
WK4-1	7.80100	105	8.2500	16	0.617
WK4-2	8.40820	73	6.5200	10	0.140
3 M	7.74810	104	8.7438	16	0.228
6 M	5.86430	98	6.2250	16	0.581
12 M	6.69460	92	6.8250	16	0.896

Table 9.7 CYP3A5 donor trough levels

	Donor CYP3A5 GG (*3/*3)		**Donor CYP3A5 GA/AA (*3/*1 - *1/*1)**		
	Mean C_0 (µg/L)	n	Mean C_0 (µg/L)	n	p value
WK1-1	4.9565	168	3.1526	19	**0.036**
WK1-2	6.5655	158	3.6889	18	**0.003**
WK1-3	7.1293	157	4.3000	18	**0.003**
WK2-1	7.2596	166	5.4444	18	**0.047**
WK2-2	6.9692	133	5.6625	14	0.084
WK2-3	6.6899	109	4.4750	16	**0.007**
WK3-1	7.3186	156	5.4353	17	0.053
WK3-2	7.4283	106	5.1769	13	**0.003**
WK3-3	7.8115	61	6.9727	11	0.419
WK4-1	7.8902	153	7.1167	18	0.333
WK4-2	8.3952	105	7.8167	12	0.652
3 M	7.6960	151	8.5056	18	0.287
6 M	6.0232	142	6.1056	18	0.896
12 M	6.7789	128	6.6312	16	0.866

Table 9.8 CYP3A5 recipient dose corrected levels

	Recipient CYP3A5 GG (*3/*3)		**Recipient CYP3A5 GA/AA (*3/*1 - *1/*1)**		
	Mean C_0/D (µg/L per mg)	n	Mean C_0/D (µg/L per mg)	n	p value
WK1-1	1.55300	105	1.0033	15	0.097
WK1-2	1.94920	103	1.3785	16	0.191
WK1-3	2.11010	93	1.6398	17	0.492
WK2-1	2.01740	102	1.0736	17	0.086
WK2-2	1.80340	79	1.0942	14	0.081
WK2-3	1.87560	63	1.0746	10	0.194
WK3-1	1.73760	100	0.9834	14	**0.027**
WK3-2	1.73730	62	0.9823	14	0.065
WK3-3	1.80580	37	1.0664	7	0.276
WK4-1	1.95340	100	1.0833	16	0.158
WK4-2	2.10140	69	0.9179	10	**0.036**
3 M	2.50190	104	1.4695	16	0.102
6 M	1.90200	98	1.3165	16	0.168
12 M	2.07300	92	1.6078	16	0.214

Table 9.9 CYP3A5 donor dose corrected levels

	Donor CYP3A5 GG (*3/*3)		**Donor CYP3A5 GA/AA (*3/*1 - *1/*1)**		
	Mean C_0/D (µg/L per mg)	n	Mean C_0/D (µg/L per mg)	n	p value
WK1-1	1.5480	157	0.9047	16	**0.031**
WK1-2	1.9035	157	0.8127	15	**0.006**
WK1-3	2.1717	144	0.7639	15	**0.036**
WK2-1	2.0300	154	0.8579	16	**0.035**
WK2-2	1.7794	120	0.8776	14	**0.016**
WK2-3	1.9463	97	0.6512	14	**0.011**
WK3-1	1.7781	148	0.6645	16	**0.002**
WK3-2	1.7889	99	0.6264	12	**0.003**
WK3-3	1.9879	55	0.8155	11	**0.028**
WK4-1	1.9883	148	0.8121	18	**0.022**
WK4-2	2.1026	101	0.8341	12	**0.009**
3 M	2.5260	151	1.0853	18	**0.009**
6 M	1.9834	142	0.9361	18	**0.011**
12 M	2.0843	128	1.1950	16	**0.009**

Table 9.10 CYP3A5 grouped genotype dose requirements

	RnE / DnE		**RE / DnE**		**RnE / DE**		**RE / DE**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	Mean Dose (mg)	n	Mean Dose (mg)	n	P value
WK1-1	3.3647	85	3.8462	13	4.0909	11	3.500	2	0.389
WK1-2	3.8869	84	4.9231	13	4.9000	10	4.500	2	0.074
WK1-3	4.3108	74	5.8846	13	6.3000	10	5.750	2	**0.004**
WK2-1	4.5854	82	6.3077	13	6.9545	11	7.750	2	**<0.0001**
WK2-2	4.6825	63	6.5909	11	7.5500	10	8.000	2	**0.001**
WK2-3	4.2917	48	5.9375	8	8.3000	10	8.500	2	**<0.0001**
WK3-1	4.8110	82	7.3182	11	8.7273	11	10.000	2	**<0.0001**
WK3-2	4.8125	48	6.8750	8	8.0625	8	8.000	1	**0.001**
WK3-3	4.4259	27	6.5000	6	9.5625	8	10.000	1	**<0.0001**
WK4-1	4.6750	80	7.6538	13	9.6667	12	9.000	2	**<0.0001**
WK4-2	4.3113	53	7.0625	8	10.000	9	9.000	2	**<0.0001**
3 MTHS	3.8846	78	6.0000	13	7.2917	12	6.000	1	**<0.0001**
6 MTHS	3.8243	74	4.8462	13	6.5000	12	8.000	1	**0.001**
12 MTHS	3.7254	71	4.0000	13	6.2083	12	2.000	1	**0.003**

Table 9.11 CYP3A5 grouped genotype trough levels

	RnE / DnE		**RE / DnE**		**RnE / DE**		**RE / DE**		
	Mean C_0 (µg/L)	n	Mean C_0 (µg/L)	n	Mean C_0 (µg/L)	n	Mean C_0 (µg/L)	n	p value
WK1-1	5.0695	95	3.7909	11	3.0923	13	2.0500	2	0.121
WK1-2	6.7298	94	5.6833	12	3.5250	12	3.3500	2	**0.017**
WK1-3	7.5440	84	6.1462	13	4.7333	12	4.4500	2	0.101
WK2-1	7.6261	92	6.4769	13	5.5583	12	6.2500	2	0.297
WK2-2	6.8903	72	7.3273	11	6.0909	11	5.0500	2	0.602
WK2-3	6.6103	58	6.8500	8	5.3000	11	2.9000	2	0.252
WK3-1	7.7000	87	7.1000	11	5.5273	11	8.4500	2	0.404
WK3-2	7.6365	52	6.9250	8	6.2889	9	5.5500	2	0.396
WK3-3	7.4833	30	7.1667	6	6.5875	8	7.3000	1	0.910
WK4-1	7.7393	84	8.6308	13	7.6250	12	6.5000	2	0.748
WK4-2	8.5018	57	6.8125	8	7.5333	9	5.3500	2	0.428
3 MTHS	7.7349	83	8.6538	13	8.1250	12	9.5500	2	0.650
6 MTHS	6.0104	77	6.1462	13	5.0750	12	6.7000	2	0.599
12 MTHS	7.0000	71	6.1538	13	4.9917	12	10.150	2	0.167

Table 9.12 CYP3A5 grouped genotype dose corrected levels

	RnE / DnE		**RE / DnE**		**RnE / DE**		**RE / DE**		
	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	p value
WK1-1	1.6428	85	1.1545	11	0.8629	11	0.6000	2	0.100
WK1-2	2.0510	84	1.5810	12	0.7200	10	0.7917	2	0.071
WK1-3	2.2713	74	1.8997	13	0.8003	10	0.8558	2	0.385
WK2-1	2.1353	82	1.1460	13	0.8911	11	0.7845	2	0.114
WK2-2	1.9194	62	1.2225	11	0.9607	10	0.5983	2	0.079
WK2-3	2.1174	48	1.2531	8	0.7716	10	0.3607	2	0.084
WK3-1	1.9266	81	1.0221	11	0.6934	11	0.7979	2	**0.002**
WK3-2	2.0406	47	1.0585	8	0.7291	9	0.6113	2	**0.006**
WK3-3	2.2980	25	1.1224	6	0.7299	8	0.7300	1	0.074
WK4-1	2.1575	79	1.1441	13	0.8574	12	0.7438	2	0.173
WK4-2	2.3876	53	0.9949	8	0.8041	9	0.6100	2	**0.010**
3 MTHS	2.7512	83	1.5479	13	1.1968	12	1.6167	1	0.096
6 MTHS	2.1077	77	1.4641	13	0.9689	12	0.5500	1	0.076
12 MTHS	2.3560	71	1.5955	13	1.0437	12	2.3000	1	**0.012**

Table 9.13 ABCB1 recipient dose requirements

	CC		CT		TT		
	Mean Dose (mg)	n	Mean Dose (mg)	n	Mean Dose (mg)	n	p value
WK1-1	3.6406	32	3.7455	55	3.1286	35	0.144
WK1-2	4.2500	32	4.2909	55	3.8485	33	0.449
WK1-3	5.0862	29	4.8137	51	4.3500	30	0.388
WK2-1	5.3485	33	5.1887	51	4.7424	33	0.539
WK2-2	5.8478	23	5.3488	43	5.0517	29	0.535
WK2-3	6.2222	18	5.3919	37	4.4000	20	0.145
WK3-1	6.1406	32	5.8000	50	5.1212	33	0.335
WK3-2	6.2188	16	5.9333	30	5.0200	25	0.246
WK3-3	7.6538	13	5.7368	19	4.9286	14	0.439
WK4-1	6.0313	32	6.0700	50	5.2714	35	0.390
WK4-2	6.1364	22	5.9028	36	4.5000	20	0.133
3 M	4.8548	31	4.6304	46	4.6528	36	0.919
6 M	4.8000	30	4.0814	43	4.9644	36	0.370
12 M	4.7833	30	3.7381	42	4.4853	34	0.127

Table 9.14 ABCB1 donor dose requirements

	CC		CT		TT		
	Mean Dose (mg)	n	Mean Dose (mg)	n	Mean Dose (mg)	n	p value
WK1-1	3.5441	34	3.41670	90	3.4079	38	0.883
WK1-2	4.2188	32	4.01120	89	3.9079	38	0.703
WK1-3	4.7931	29	4.64460	83	4.2361	36	0.474
WK2-1	4.9355	31	5.03890	90	4.7568	37	0.811
WK2-2	5.5536	28	5.17610	71	5.2586	29	0.790
WK2-3	5.3696	26	6.99000	60	6.8241	29	0.916
WK3-1	6.0833	30	5.33150	89	5.3824	34	0.422
WK3-2	6.2174	23	5.24550	55	5.2800	25	0.228
WK3-3	7.0000	17	5.71430	28	5.2222	18	0.183
WK4-1	6.6613	31	5.16480	88	5.8971	34	0.028
WK4-2	6.7500	24	4.90520	58	5.5217	23	0.023
3 M	4.5333	30	4.38510	87	4.9063	32	0.605
6 M	4.2679	28	4.12050	83	4.3357	32	0.219
12 M	4.3704	27	3.92670	75	4.7586	29	0.790

Table 9.15 ABCB1 recipient trough levels

	CC		CT		TT		
	Mean C_0 Level (µg/L)	n	Mean C_0 Level (µg/L)	n	Mean C_0 Level (µg/L)	n	p value
WK1-1	4.5229	35	4.7368	57	4.5927	41	0.957
WK1-2	6.6229	35	6.4737	57	6.2625	40	0.916
WK1-3	6.2719	32	7.2574	54	7.4784	37	0.436
WK2-1	7.0857	35	7.0732	56	7.5875	40	0.803
WK2-2	6.7731	26	6.7646	48	6.6152	33	0.968
WK2-3	8.1316	19	5.7512	41	6.5269	26	0.051
WK3-1	8.3500	32	6.9755	53	7.1189	37	0.284
WK3-2	6.7778	18	7.2629	35	7.3963	27	0.742
WK3-3	7.8769	13	6.5850	20	7.4167	18	0.439
WK4-1	8.3152	33	7.4863	51	7.7757	37	0.532
WK4-2	9.0682	22	7.5892	37	7.8696	23	0.316
3 M	8.4576	33	8.0940	50	7.2811	37	0.246
6 M	5.6613	31	6.4457	46	5.6162	37	0.206
12 M	6.2207	29	6.8841	44	6.8857	35	0.705

Table 9.16 ABCB1 donor trough levels

	CC		CT		TT		
	Mean C_0 Level (µg/L)	n	Mean C_0 Level (µg/L)	n	Mean C_0 Level (µg/L)	n	p value
WK1-1	5.0432	37	4.79370	95	4.5341	41	0.823
WK1-2	5.6229	35	6.05370	95	7.2244	41	0.164
WK1-3	8.1438	32	6.34040	89	6.9650	40	0.083
WK2-1	7.3412	34	7.51170	94	6.5390	41	0.375
WK2-2	6.4290	31	7.50270	74	6.3344	32	0.068
WK2-3	5.3077	26	6.99000	60	6.8241	29	0.060
WK3-1	6.2875	32	7.91220	90	6.6222	36	0.064
WK3-2	6.8231	26	7.61720	58	6.9370	27	0.348
WK3-3	7.0900	20	8.13930	28	8.3778	18	0.386
WK4-1	8.4303	33	7.78220	90	7.8758	33	0.618
WK4-2	7.5654	26	8.49670	60	8.8522	23	0.529
3 M	7.4806	31	7.91210	91	8.4294	34	0.455
6 M	5.8966	29	6.10580	86	6.2152	33	0.883
12 M	6.1036	28	6.66350	74	7.2484	31	0.418

Table 9.17 ABCB1 recipient dose corrected levels

	CC		CT		TT		
	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	p value
WK1-1	1.3305	32	1.3079	54	1.7759	34	0.155
WK1-2	1.9467	32	1.7863	54	1.9487	33	0.866
WK1-3	1.3952	29	2.3802	51	2.0452	30	0.262
WK2-1	1.7396	33	1.5407	52	2.3280	34	0.195
WK2-2	1.6887	23	1.4321	43	1.9063	28	0.359
WK2-3	2.0606	16	1.2379	37	2.2807	20	0.069
WK3-1	1.6658	31	1.3740	50	1.9070	33	0.131
WK3-2	1.3372	17	1.3714	32	1.9679	24	0.143
WK3-3	1.3452	12	1.3692	18	2.3289	14	0.189
WK4-1	1.6392	32	1.4658	50	2.4139	34	0.157
WK4-2	1.8739	22	1.5805	36	2.4201	20	0.182
3 M	2.8313	33	2.0167	49	2.3073	37	0.311
6 M	1.6771	31	1.9610	45	1.7235	37	0.694
12 M	1.7228	29	2.1704	43	1.9373	35	0.405

Table 9.18 ABCB1 donor dose corrected levels

	CC		CT		TT		
	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	Mean C_0/D (µg/L/mg)	n	p value
WK1-1	1.5406	34	1.5243	89	1.4041	37	0.848
WK1-2	1.4868	32	1.7419	89	2.2314	37	0.107
WK1-3	1.8164	29	1.6566	83	3.0905	36	**0.016**
WK2-1	2.2564	31	1.7931	90	2.0936	37	0.542
WK2-2	1.5266	27	1.7992	70	1.6829	28	0.672
WK2-3	1.3916	23	1.8084	55	2.2979	28	0.226
WK3-1	1.2248	30	1.8251	88	1.8583	34	0.107
WK3-2	1.1132	24	1.8641	56	1.8158	26	0.057
WK3-3	1.1016	17	2.0860	26	2.1292	18	0.111
WK4-1	1.3636	31	2.1898	87	1.6624	33	0.143
WK4-2	1.2928	24	2.2704	58	2.0259	23	**0.048**
3 M	2.4904	30	2.4548	91	2.3498	34	0.966
6 M	1.9114	28	2.0090	86	1.6702	33	0.627
12 M	1.6969	27	2.2475	74	1.6572	31	**0.047**

Table 9.19 CYP3A4*22 recipient dose requirements

	Recipient CYP3A4*22 CC		**Recipient CYP3A4*22 CT/TT**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p value
WK1-1	3.5219	114	3.6500	10	0.794
WK1-2	4.1563	112	3.9000	10	0.640
WK1-3	4.7379	103	4.6111	9	0.861
WK2-1	5.0586	111	5.2500	10	0.804
WK2-2	5.3667	90	5.0000	7	0.715
WK2-3	5.3239	71	4.5000	5	0.541
WK3-1	5.6402	107	5.7500	10	0.908
WK3-2	5.5435	69	6.8750	4	0.302
WK3-3	6.0114	44	5.8333	3	0.926
WK4-1	5.7982	109	5.7500	10	0.959
WK4-2	5.6081	74	5.0833	6	0.670
3 M	4.7429	105	3.8000	10	0.248
6 M	4.5396	101	3.5000	10	0.192
12 M	4.2959	98	3.7000	10	0.431

Table 9.20 CYP3A4*22 donor dose requirements

	Donor CYP3A4*22 CC		**Donor CYP3A4*22 CT/TT**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p value
WK1-1	3.4830	147	3.3750	16	0.768
WK1-2	4.0868	144	3.6250	16	0.258
WK1-3	4.6617	133	3.7857	14	0.112
WK2-1	5.0769	143	3.9667	15	0.064
WK2-2	5.4435	115	4.2727	11	0.136
WK2-3	5.4728	92	3.4444	9	**0.042**
WK3-1	5.7794	136	4.0588	17	**0.015**
WK3-2	5.5263	95	4.3571	7	0.220
WK3-3	6.0339	59	3.8750	4	0.160
WK4-1	5.8705	139	4.0000	17	**0.008**
WK4-2	5.7500	92	3.6538	13	**0.010**
3 M	4.7293	133	2.8529	17	**0.003**
6 M	4.5155	129	2.9000	15	**0.008**
12 M	4.3761	117	3.1429	14	**0.047**

Table 9.21 CYP3A4*22 recipient trough levels

	Recipient CYP3A4*22 CC		**Recipient CYP3A4*22 CT/TT**		
	Mean C_0 Level (µg/L)	n	Mean C_0 Level (µg/L)	n	p value
WK1-1	4.5774	124	6.1273	11	0.165
WK1-2	6.3862	123	7.2455	11	0.467
WK1-3	6.7339	115	10.7700	10	**0.003**
WK2-1	7.0566	122	9.4909	11	0.053
WK2-2	6.5560	100	9.3222	9	**0.005**
WK2-3	6.1938	81	10.0429	7	**0.005**
WK3-1	7.2027	113	8.8636	11	0.194
WK3-2	7.1325	77	8.7400	5	0.215
WK3-3	7.0688	48	8.9500	4	0.214
WK4-1	7.6402	112	9.6455	11	0.053
WK4-2	7.7104	77	12.5000	7	**0.001**
3 M	7.9144	111	7.7636	11	0.876
6 M	5.9981	106	5.5200	10	0.544
12 M	6.6596	99	6.8636	11	0.859

Table 9.22 CYP3A4*22 donor trough levels

	Donor CYP3A4*22 CC		**Donor CYP3A4*22 CT/TT**		
	Mean C_0 Level (µg/L)	n	Mean C_0 Level (µg/L)	n	p value
WK1-1	4.7667	156	5.62780	18	0.341
WK1-2	6.2344	154	7.04210	19	0.405
WK1-3	6.8103	145	6.84120	17	0.976
WK2-1	6.9941	153	8.33890	18	0.154
WK2-2	6.5839	124	8.19230	13	0.051
WK2-3	6.3167	102	7.50910	11	0.226
WK3-1	7.0482	141	9.00530	19	**0.038**
WK3-2	7.0786	103	9.00000	8	**0.047**
WK3-3	7.9129	62	8.10000	5	0.899
WK4-1	7.8411	141	7.82780	18	0.968
WK4-2	8.0926	95	7.86430	14	0.826
3 M	7.9633	139	7.81110	18	0.843
6 M	6.0917	133	6.01880	16	0.914
12 M	6.8864	118	6.18670	15	0.448

Table 9.23 CYP3A4*22 recipient dose corrected level

	Recipient CYP3A4*22 CC		**Recipient CYP3A4*22 CT/TT**		
	Mean C_0/D Level (µg/L/mg)	n	Mean C_0/D Level (µg/L/mg)	n	p value
WK1-1	1.4339	112	1.9408	10	0.198
WK1-2	1.8481	111	2.2690	10	0.431
WK1-3	2.0105	103	2.2656	9	0.776
WK2-1	1.8869	111	1.7969	10	0.896
WK2-2	1.6239	89	2.2931	7	0.222
WK2-3	1.7060	70	2.2578	5	0.509
WK3-1	1.5805	106	1.9762	10	0.315
WK3-2	1.5597	71	1.8515	4	0.648
WK3-3	1.6511	42	1.9038	3	0.797
WK4-1	1.7734	108	2.0422	10	0.721
WK4-2	1.7631	74	3.5054	6	**0.010**
3 M	2.1815	105	2.6903	10	0.248
6 M	1.7896	105	2.0563	10	0.609
12 M	1.9335	98	2.3482	11	0.346

Table 9.24 CYP3A4*22 donor dose corrected levels

	Donor CYP3A4*22 CC		**Donor CYP3A4*22 CT/TT**		
	Mean C_0/D Level (µg/L/mg)	n	Mean C_0/D Level (µg/L/mg)	n	p value
WK1-1	1.4954	145	1.6472	15	0.631
WK1-2	1.7670	143	2.2337	16	0.243
WK1-3	2.0206	133	2.2052	14	0.799
WK2-1	1.8644	143	2.6448	15	0.190
WK2-2	1.5967	112	2.3444	11	0.084
WK2-3	1.7530	91	2.4211	9	0.311
WK3-1	1.5896	135	2.4916	17	**0.013**
WK3-2	1.6128	97	2.5393	7	0.078
WK3-3	1.8116	57	2.4454	4	0.469
WK4-1	1.8101	137	2.3035	17	0.368
WK4-2	1.8065	92	2.4469	13	0.136
3 M	2.2086	138	4.0506	18	**0.039**
6 M	1.7667	132	2.8488	16	**0.015**
12 M	1.8848	117	2.7263	15	**0.014**

9.3 Data tables Chapter 5

9.3.1 Renal transplant patients

Table 9.25 CYP3A5 dose requirement

	CYP3A5 GG (*3/*3)		**CYP3A5 GA/AA (*1/*3-*1/*1)**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p
WK1-1	6.5900	121	6.23000	31	0.436
WK1-2	7.0840	125	8.03230	31	**0.034**
WK1-3	7.4360	125	9.79030	31	**<0.0001**
WK2-1	7.9152	112	11.8793	29	**<0.0001**
WK2-2	8.3619	105	13.2581	31	**<0.0001**
WK2-3	8.3606	104	13.0400	25	**<0.0001**
WK3-1	8.4592	98	13.7308	26	**<0.0001**
WK3-2	8.3138	94	14.0417	24	**<0.0001**
WK3-3	8.3011	88	14.1875	24	**<0.0001**
1M	8.1105	86	14.2826	23	**<0.0001**
2M	7.1944	90	13.3250	20	**<0.0001**
3 M	6.4607	89	12.0250	20	**<0.0001**
6 M	5.4659	88	10.7647	20	**<0.0001**
12 M	4.6131	84	9.52940	17	**<0.0001**

Table 9.26 CYP3A5 trough levels

	CYP3A5 GG (*3/*3)		**CYP3A5 GA/AA (*1/*3-*1/*1)**		
	Mean Tac Level (µg/L)	n	Mean Tac Level (µg/L)	n	p
WK1-1	8.6000	149	4.1833	36	**<0.0001**
WK1-2	8.3743	148	5.1028	36	**<0.0001**
WK1-3	8.1930	143	5.3250	36	**<0.0001**
WK2-1	7.8814	129	5.8576	33	**0.001**
WK2-2	8.5797	118	6.3750	32	**0.002**
WK2-3	9.3973	111	6.8600	25	**0.007**
WK3-1	9.2825	103	7.7808	26	0.061
WK3-2	9.4879	99	7.3333	24	**0.005**
WK3-3	10.6110	91	8.9875	24	0.152
1M	10.1500	90	8.4000	23	**0.035**
2M	9.9750	92	8.4200	20	0.082
3 M	9.3099	91	8.3800	20	0.187
6 M	8.5798	89	8.4471	17	0.872
12 M	7.6081	86	6.9235	17	0.556

Table 9.27 CYP3A5 dose corrected levels

	CYP3A5 GG (*3/*3)		**CYP3A5 GA/AA (*1/*3-*1/*1)**		
	Dose Corrected Tac Level (µg/L per mg)	n	Dose Corrected Tac Level (µg/L per mg)	n	p
WK1-1	1.3876	121	0.6784	31	**<0.0001**
WK1-2	1.3311	125	0.6898	31	**0.001**
WK1-3	1.2935	122	0.6004	31	**<0.0001**
WK2-1	1.1854	112	0.5681	29	**<0.0001**
WK2-2	1.1958	105	0.5329	31	**<0.0001**
WK2-3	1.3531	103	0.5708	25	**<0.0001**
WK3-1	1.3491	98	0.6025	26	**<0.0001**
WK3-2	1.4262	94	0.5723	24	**<0.0001**
WK3-3	1.6576	88	0.6504	24	**<0.0001**
1M	1.5632	86	0.6571	23	**<0.0001**
2M	1.8237	90	0.7156	20	**<0.0001**
3 M	2.3480	89	0.8023	20	0.124
6 M	2.0449	87	0.8820	17	**0.001**
12 M	2.2429	84	0.8640	17	**0.042**

Table 9.28 ABCB1 dose requirements

	CC		CT		TT		
	Dose (mg)	n	Dose (mg)	n	Dose (mg)	n	p value
WK1-1	6.5400	27	6.5600	72	6.4500	52	0.990
WK1-2	7.3148	27	7.1600	75	7.4340	53	0.852
WK1-3	8.0385	26	7.8581	74	7.9455	55	0.840
WK2-1	9.4130	23	8.1591	66	9.2353	51	0.230
WK2-2	10.6458	24	8.5231	65	10.2717	46	0.068
WK2-3	10.2800	25	8.5492	61	9.6905	42	0.236
WK3-1	10.2955	22	9.0259	58	9.7674	43	0.244
WK3-2	10.2500	22	9.2130	58	9.1585	41	0.072
WK3-3	10.3056	18	9.4151	53	9.1625	40	0.117
1 M	10.2500	18	9.2500	52	9.0132	38	0.206
2 M	8.1667	18	8.0755	53	8.4474	38	0.238
3 M	7.8889	18	7.3774	53	7.4342	38	0.902
6 M	5.8750	16	6.0096	52	6.9595	37	0.461
12 M	5.2333	15	5.4804	51	5.4714	35	0.966

Table 9.29 ABCB1 trough levels

	CC		CT		TT		
	Tac Level (µg/L)	n	Tac Level (µg/L)	n	Tac Level (µg/L)	n	p value
WK1-1	7.7000	34	7.5729	85	7.9954	65	0.958
WK1-2	7.9788	33	7.6706	85	7.5923	65	0.423
WK1-3	7.5781	32	7.6691	81	7.5662	65	0.998
WK2-1	7.9345	29	7.3918	73	7.3898	59	0.650
WK2-2	8.3857	28	7.9478	69	8.2596	52	0.596
WK2-3	10.1000	26	8.1569	65	9.5091	44	0.084
WK3-1	9.0174	23	8.9250	60	9.1600	45	0.473
WK3-2	10.1087	23	8.7304	56	8.9116	43	0.383
WK3-3	12.2474	19	9.6407	54	10.0073	41	0.095
1 M	10.9474	19	9.8491	53	9.1875	40	0.366
2 M	9.8389	18	9.2833	54	10.2000	39	0.691
3 M	9.4211	19	8.6352	54	9.7237	39	0.176
6 M	9.6294	17	8.1283	53	8.6861	36	0.211
12 M	6.4063	16	7.1942	52	8.4400	35	0.237

Table 9.30 ABCB1 dose corrected levels

	CC		CT		TT		
	Dose Corr Tac Level (µg/L per mg)	n	Dose Corr Tac Level (µg/L per mg)	n	Dose Corr Tac Level (µg/L per mg)	n	p value
WK1-1	1.23500	27	1.1979	72	1.3117	52	0.892
WK1-2	1.18690	27	1.2329	75	1.1486	53	0.616
WK1-3	1.02450	26	1.1020	72	1.2780	54	0.673
WK2-1	0.99070	23	1.1216	66	1.0112	51	0.861
WK2-2	0.91910	24	1.1371	65	0.9909	46	0.514
WK2-3	1.17260	25	1.1452	61	1.3201	41	0.637
WK3-1	1.06280	22	1.2499	58	1.2044	43	0.662
WK3-2	1.17010	22	1.2382	54	1.3332	41	0.821
WK3-3	1.35020	18	1.3565	53	1.6099	40	0.730
1 M	1.34230	18	1.3452	52	1.4453	38	0.789
2 M	1.63760	18	1.4836	53	1.8366	38	0.501
3 M	1.69990	18	1.6444	53	2.8227	38	0.363
6 M	2.20520	16	1.8600	52	1.6915	36	0.423
12 M	1.58980	15	1.7236	51	2.6098	35	0.228

Table 9.31 CYP3A4*22 dose requirements

	CYP3A4*22 CC Genotype		**CYP3A4*22 CT/TT Genotype**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p
WK1-1	6.4600	141	7.3500	10	0.403
WK1-2	7.2639	144	7.5909	11	0.536
WK1-3	7.9757	144	7.0455	12	0.534
WK2-1	8.7500	130	8.3500	10	0.894
WK2-2	9.5000	124	8.8182	11	0.498
WK2-3	9.2949	117	8.5455	11	0.401
WK3-1	9.6404	114	8.2222	9	0.437
WK3-2	9.5509	108	8.2222	9	0.494
WK3-3	9.6716	102	7.8333	9	0.333
1M	9.5850	100	7.5000	9	0.187
2M	8.5650	100	5.7500	10	0.077
3 M	7.7374	99	4.9500	10	**0.044**
6 M	6.5105	95	4.5500	10	0.127
12 M	5.5824	91	4.1500	10	0.190

Table 9.32 CYP3A4*22 trough levels

	CYP3A4*22 CC Genotype		**CYP3A4*22 CT/TT Genotype**		
	Tac Level (µg/L)	n	Tac Level (µg/L)	n	p
WK1-1	7.5023	171	10.8462	13	0.058
WK1-2	7.6418	170	9.18460	13	0.314
WK1-3	7.6248	165	7.69230	13	0.798
WK2-1	7.3329	149	9.31670	12	0.101
WK2-2	8.0255	137	9.08330	12	0.621
WK2-3	8.8423	123	9.5250	12	0.589
WK3-1	8.8542	118	10.4100	10	0.433
WK3-2	8.9375	112	10.8100	10	0.169
WK3-3	10.188	104	11.3600	10	0.711
1M	9.8456	103	9.2600	10	0.621
2M	9.6725	102	9.9500	10	0.818
3 M	9.1168	101	9.4000	10	0.765
6 M	8.6844	96	7.3500	10	0.195
12 M	6.9882	91	8.4700	10	0.065

Table 9.33 CYP3A4*22 dose corrected level

	CYP3A4*22 CC Genotype		**CYP3A4*22 CT/TT Genotype**		
	Dose Corrected Tac Level (µg/L/mg)	n	Dose Corrected Tac Level (µg/L/mg)	n	p
WK1-1	1.2207	141	1.5191	10	0.471
WK1-2	1.1985	144	1.2962	11	0.906
WK1-3	1.1430	141	1.3183	11	0.785
WK2-1	1.0449	130	1.2846	10	0.568
WK2-2	1.0258	124	1.3015	11	0.423
WK2-3	1.1709	116	1.5369	11	0.451
WK3-1	1.1566	114	1.6989	9	0.249
WK3-2	1.2137	108	1.8046	9	0.176
WK3-3	1.4063	102	1.9386	9	0.361
1M	1.3439	100	1.6836	9	0.315
2M	1.5674	100	2.1705	10	0.157
3 M	1.9767	99	2.9320	10	0.480
6 M	1.8133	94	2.2444	10	0.320
12 M	1.6262	91	3.4913	10	**<0.0001**

9.3.2 Simultaneous kidney/pancreas patients

Table 9.34 SPK CYP3A5 dose requirements

	CYP3A5 GG (*3/*3)		**CYP3A5 GA/AA (*1/*3-*1/*1)**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p
WK1-1	6.1600	16	5.0000	1	0.573
WK1-2	6.4375	16	5.0000	1	0.487
WK1-3	5.9688	16	3.2500	2	0.801
WK2-1	6.3389	18	7.0000	2	0.760
WK2-2	6.1605	19	9.0000	2	0.369
WK2-3	7.3000	17	10.3000	2	0.318
WK3-1	8.4211	19	13.3333	3	0.055
WK3-2	9.7500	16	15.0000	3	0.074
WK3-3	9.0941	17	18.1667	6	**<0.0001**
1M	10.2222	18	18.6667	6	**<0.0001**
2M	9.8750	20	15.6000	5	**0.013**
3 M	10.1389	18	13.6000	5	0.161
6 M	7.7381	21	11.5000	4	0.080
12 M	5.7778	18	10.2500	4	**0.025**

Table 9.35 SPK CYP3A5 trough levels

	CYP3A5 GG (*3/*3) –		**CYP3A5 GA/AA (*1/*3-*1/*1)**		
	Tac Level (µg/L)	n	Tac Level (µg/L)	n	p
WK1-1	11.0808	26	6.4000	6	**0.033**
WK1-2	14.2615	26	12.5333	6	0.594
WK1-3	13.3692	26	9.8000	6	0.135
WK2-1	10.4269	26	6.4167	6	0.064
WK2-2	9.4500	26	5.6333	6	**0.009**
WK2-3	8.2417	24	6.2833	6	0.192
WK3-1	8.5652	23	7.0500	6	0.348
WK3-2	8.0667	21	7.5000	6	0.666
WK3-3	8.4400	20	6.6833	6	0.119
1M	9.6750	20	13.7167	6	0.072
2M	10.2048	21	8.8000	5	0.433
3 M	10.4833	18	9.7600	5	0.743
6 M	9.0864	22	4.9250	4	**0.005**
12 M	8.5053	19	6.2000	4	0.245

Table 9.36 SPK CYP3A5 dose corrected levels

	CYP3A5 GG (*3/*3)		**CYP3A5 GA/AA (*1/*3-*1/*1)**		
	Dose Corrected Tac Level (µg/L per mg)	n	Dose Corrected Tac Level (µg/L per mg)	n	p
WK1-1	1.7844	16	0.7400	1	0.224
WK1-2	2.3849	16	6.0000	1	**0.041**
WK1-3	2.5768	16	1.4770	1	0.371
WK2-1	2.2676	18	1.6258	2	0.754
WK2-2	1.6361	18	0.7518	2	0.351
WK2-3	1.7045	17	0.7303	2	0.475
WK3-1	1.4348	19	0.5503	3	0.382
WK3-2	1.6051	16	0.4946	3	0.488
WK3-3	0.9920	16	0.4134	6	0.051
1M	1.0576	18	0.7813	6	0.288
2M	1.1478	20	0.6767	5	0.050
3 M	1.1927	18	0.9042	5	0.390
6 M	1.3949	21	0.5663	4	**0.030**
12 M	1.8764	18	0.7074	4	0.126

Table 9.37 SPK ABCB1 dose requirements

	CC		CT		TT		
	Mean Dose (mg)	n	Mean Dose (mg)	n	Mean Dose (mg)	n	p value
WK1-1	6.170	3	6.1100	9	6.000	5	0.993
WK1-2	6.5000	3	6.0000	9	6.900	5	0.724
WK1-3	5.1667	3	6.1000	10	5.100	5	0.275
WK2-1	7.2500	4	6.9091	11	4.620	5	0.262
WK2-2	7.5000	5	7.1455	11	5.500	5	0.629
WK2-3	8.9200	5	7.4636	11	6.000	3	0.601
WK3-1	10.2000	5	9.4167	12	7.200	5	0.504
WK3-2	12.5000	5	10.5000	10	8.375	4	0.448
WK3-3	12.4617	6	10.6667	12	12.220	5	0.815
1 M	13.3333	6	10.9231	13	14.800	5	0.350
2 M	9.8333	6	11.7500	12	10.786	7	0.735
3 M	12.3333	6	10.9091	11	9.417	6	0.601
6 M	7.8000	5	8.5417	12	8.375	8	0.994
12 M	6.2500	4	7.0000	12	6.000	6	0.859

Table 9.38 SPK ABCB1 trough levels

	CC		CT		TT		
	Tac Level (µg/L)	n	Tac Level (µg/L)	n	Tac Level (µg/L)	n	p value
WK1-1	8.9714	7	9.4250	16	12.5444	9	0.244
WK1-2	14.7571	7	12.2563	16	16.2889	9	0.374
WK1-3	11.2286	7	13.9500	16	11.6222	9	0.411
WK2-1	8.5143	7	11.2875	16	7.7111	9	0.156
WK2-2	9.4143	7	8.8375	16	8.0222	9	0.711
WK2-3	6.7714	7	7.3875	16	9.9857	7	0.127
WK3-1	7.3286	7	7.9267	15	9.8714	7	0.351
WK3-2	7.4714	7	8.1286	14	8.0500	6	0.879
WK3-3	8.3000	6	7.8857	14	8.1167	6	0.940
1 M	13.8667	6	9.2214	14	10.5833	6	0.144
2 M	10.3500	6	10.0000	13	9.4571	7	0.904
3 M	10.1833	6	10.2636	11	10.5833	6	0.986
6 M	8.7000	5	8.0000	13	9.0125	8	0.731
12 M	8.1500	4	8.2231	13	7.8167	6	0.975

Table 9.39 SPK ABCB1 dose corrected levels

	CC		CT		TT		
	Dose Corr Tac Level (µg/L per mg)	n	Dose Corr Tac Level (µg/L per mg)	n	Dose Corr Tac Level (µg/L per mg)	n	p value
WK1-1	1.3433	3	1.6303	9	2.1175	5	0.404
WK1-2	3.1181	3	2.2259	9	2.9542	5	0.676
WK1-3	1.4950	2	2.5074	10	2.9282	5	0.353
WK2-1	1.4454	4	1.9140	11	3.4465	5	0.480
WK2-2	1.2586	5	1.4000	10	2.1321	5	0.489
WK2-3	0.8081	5	1.7913	11	2.2305	3	0.493
WK3-1	0.7345	5	1.0110	12	2.6214	5	0.100
WK3-2	0.7242	5	1.0366	10	3.2946	4	0.235
WK3-3	0.7431	6	0.9549	12	0.6088	4	0.602
1 M	1.0263	6	1.0207	13	0.8597	5	0.848
2 M	1.1400	6	0.9854	12	1.0963	7	0.801
3 M	0.9684	6	1.1432	11	1.2675	6	0.740
6 M	1.2792	5	1.2978	12	1.1986	8	0.957
12 M	1.5242	4	1.7272	12	1.6304	6	0.969

Table 9.40 SPK CYP3A4*22 dose requirements

	CYP3A4*22 CC Genotype		**CYP3A4*22 CT/TT Genotype**		
	Mean Dose (mg)	n	Mean Dose (mg)	n	p
WK1-1	5.9700	15	7.00	2	0.489
WK1-2	6.2667	15	7.00	2	0.629
WK1-3	5.9333	15	6.50	2	0.713
WK2-1	6.5278	18	5.75	2	0.698
WK2-2	7.0842	19	4.50	2	0.331
WK2-3	8.0941	17	4.00	2	0.159
WK3-1	9.5000	20	5.00	2	0.149
WK3-2	11.1765	17	5.50	2	0.107
WK3-3	11.1800	20	6.00	2	**0.004**
1M	12.1667	21	6.25	2	**0.001**
2M	10.5870	23	6.00	1	**0.001**
3 M	10.9762	21	8.00	1	0.826
6 M	8.4318	22	5.50	2	0.401
12 M	6.5250	20	2.50	1	0.191

Table 9.41 SPK CYP3A4*22 trough levels

	CYP3A4*22 CC Genotype		**CYP3A4*22 CT/TT Genotype**		
	Tac Level (µg/L)	n	Tac Level (µg/L)	n	p
WK1-1	9.6029	28	15.2000	3	0.184
WK1-2	13.4536	28	21.4667	3	0.067
WK1-3	12.5536	28	16.4333	3	0.187
WK2-1	9.0500	28	16.5000	3	**0.025**
WK2-2	8.4679	28	11.9667	3	0.179
WK2-3	7.6385	26	10.0000	3	0.487
WK3-1	8.2200	25	9.5333	3	0.568
WK3-2	7.9957	23	8.1667	3	0.783
WK3-3	7.8182	22	10.5000	3	0.101
1M	10.8545	22	9.8333	3	0.776
2M	10.3478	23	5.2500	2	0.144
3 M	10.2905	21	7.1000	1	0.501
6 M	8.5609	23	7.6500	2	0.860
12 M	8.4381	21	4.9000	1	0.355

Table 9.42 SPK CYP3A4*22 dose corrected levels

	CYP3A4*22 CC Genotype		**CYP3A4*22 CT/TT Genotype**		
	Dose Corrected Tac Level (µg/L/mg)	n	Dose Corrected Tac Level (µg/L/mg)	n	p
WK1-1	1.7079	15	1.8357	2	0.842
WK1-2	2.6030	15	2.5571	2	0.974
WK1-3	2.5089	15	2.5355	2	0.977
WK2-1	2.0701	18	3.4032	2	0.513
WK2-2	1.4202	18	2.6950	2	0.172
WK2-3	1.5051	17	2.4250	2	0.501
WK3-1	1.2418	20	2.0375	2	0.512
WK3-2	1.4015	17	1.6700	2	0.899
WK3-3	0.7415	19	2.0357	2	**0.006**
1M	0.9421	21	1.8361	2	**0.025**
2M	1.0882	23	0.9333	1	0.362
3 M	1.1386	21	0.8875	1	0.932
6 M	1.2324	22	1.9146	2	0.293
12 M	1.7143	20	1.9600	1	0.636

9.4 Publications and Presentations from Thesis

9.4.1 Publications

1. *Early or Late Conversion From Tac-BD to Tac-QD in Renal Transplantation: When is the right time?*
   Falconer SJ, Peagam WR, Oniscu GC.
   Transplant Proc. 2015 Jul-Aug;47(6):1741-5
   PMID: 26293044

2. *Conversion from twice-daily to once-daily tacrolimus in simultaneous pancreas-kidney transplant patients.*
   Falconer SJ, Jansen C, Oniscu GC.
   Transplant Proc. 2014 Jun;46(5):1458-62
   PMID: 24935313

9.4.2 Presentations

Oral Presentations

1. Pharmacogenomic Influence of CYP3A5, CYP3A4*22 and ABCB1 Polymorphisms on Tacrolimus Dose Requirements and Trough Levels in Scottish Renal Transplant Patients.

   European Society for Organ Transplantation (ESOT) Congress, Brussels 2015.

2. Impact of donor and recipient CYP3A5, CYP3A4*22 and ABCB1 polymorphisms on tacrolimus pharmacokinetics and clinical outcome in liver transplant recipients.

   British Transplantation Society Congress, Glasgow 2014.

3. Donor and Recipient CYP3A5, CYP3A4*22 and ABCB1 Polymorphisms on Tacrolimus Pharmacokinetics and their Impact on Clinical Outcome in Liver Transplant Recipients.

   European Society for Organ Transplantation (ESOT) Congress, Vienna 2013.

Poster Presentations

1. Genetic Polymorphisms that Influence the Pharmacokinetics of Immunosuppression; Their distribution in a Healthy Scottish Population and Across Liver, Kidney and Pancreas Transplant Patients.

   European Society for Organ Transplantation Congress, Brussels 2015

2. Scottish Renal Transplant Patients Converted to Once-Daily Tacrolimus and the Influence of Genetic Polymorphisms of CYP3A5, CYP3A4*22 and ABCB1 on Dose, Trough Levels and Clinical Outcome.

   European Society for Organ Transplantation Congress, Brussels 2015

3. The Impact of CYP3A5, CYP3A4*22 and ABCB1 Polymorphisms on Renal Transplant Patients Converted to Once-Daily Tacrolimus.

   British Transplantation Society Congress, Glasgow 2014

4. The Distribution of CYP3A5, CYP3A4*22 and ABCB1 Polymorphisms in a Healthy Scottish Population and Liver, Kidney and Pancreas Transplant Patients.

   British Transplantation Society Congress, Glasgow 2014

5. The Impact of CYP3A5, CYP3A4*22 and ABCB1 Polymorphisms on Tacrolimus Dosing and Levels in Scottish Renal Transplant Patients.

   British Transplantation Society Congress, Glasgow 2014

6. High intra-individual variability of tacrolimus clearance in renal transplant recipients on Prograf or Advagraf leads to increased graft loss.

   British Transplantation Society Congress, Bournemouth 2013

7. The clinical impact of conversion from Prograf to Advagraf in simultaneous pancreas-kidney transplant patients.

   The Transplantation Society Congress, Berlin 2012

8. Early vs Late conversion from Prograf to Advagraf in renal transplant recipients

   The Transplantation Society Congress, Berlin 2012

9.5 Regional Ethics Committee Approval