

Chapter 13

General discussion

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On 31 december 2006, in total 1394 patients were waiting for a kidney (1054), heart (44), lung (141) or liver (155) donor organ although in 2006 only 360 kidney, 37 heart, 52 lung and 83 liver transplantations were performed in the Netherlands¹. Once the patients have received a well-functioning donor organ, the quality of life will improve significantly. However, all patients that receive a donor organ have to cope with a lifelong treatment by immunosuppressants like the calcineurin inhibitors: cyclosporin and/or tacrolimus, the mTOR inhibitors: sirolimus and/or everolimus, mycophenolic acid and azathiopurin, which all have highly variable pharmacokinetic characteristics and a small therapeutic index.

The clinical consequences and impact for a patient with a sub-therapeutic or toxic tacrolimus blood level can be very serious as was discussed in CHAPTER 2. Therefore, therapeutic monitoring of the immunosuppressant blood concentrations currently performed by trough (C_0) concentrations is inevitable. In the last five year many studies appeared regarding tacrolimus trough (C_0) levels of renal, liver and heart transplant recipients in association with the genotypes of the CYP3A4 A-392G, CYP3A5 A6986G and ABCB1 C1236T, G2677T/A, C3435T polymorphisms². Although all studies described a significant impact of the CYP3A5 A6986G polymorphism on the tacrolimus blood levels, conflicting results are reported about the contribution of the individual ABCB1 polymorphisms or ABCB1 haplotypes. Due to the large variation in the transplant patient populations that have been examined in the different studies, it is possible that a minor influence of the ABCB1 polymorphisms has not been noticed. The studies differ in the type of transplantation performed, number of patients included, time after transplantation, co-medication received by the patients and whether a trough level or a complete 12 hour AUC is determined. So it is likely that a polymorphism, that has a minor influence on the tacrolimus blood concentrations, due to this large variety in transplant patient population demonstrates contrasting results among these studies.

Tacrolimus is for the most part metabolised into 13-O-demethyltacrolimus by members of the CYP3A family and specifically CYP3A4 and CYP3A5. Additionally CHAPTER 7, 8 and 9 clearly point out that the members CYP3A4 and CYP3A5 of the cytochrome P450 3A family are involved the most in the tacrolimus metabolism process whereby CYP3A5 is able to metabolise tacrolimus with a 60% higher efficiency than CYP3A4. Recently, Kamden *et al.*³ demonstrated with *in vitro* experiments that the CYP3A4 and CYP3A5 members of the CYP3A iso-enzyme family are predominantly responsible for the metabolism of tacrolimus. However, Strassburg *et al.*⁴ demonstrated that tacrolimus is mainly glucuronidated by UGT2B7. Within the UGT2B7 gene several single nucleotide polymorphisms (SNPs) have been identified. One of the first UGT2B7 polymorphisms identified is the polymorphism H268Y (C802T). Since UGT2B7 is responsible for the metabolism of the two most important metabolites of morphine,

several studies⁵⁻⁸ examined whether there is an association between patients carrying a wild type or variant allele for the UGT2B7 C802T polymorphism. Conflicting evidence has been reported regarding the contribution of this UGT2B7 C802T polymorphism on the blood concentrations of morphine. Differences between the results of these studies may be caused by the drug administration of morphine (oral or intravenous), the numbers of patients included and if co-medication is used by the patients. Duguay *et al.*⁶ recently identified two novel polymorphisms located in the promoter area of the UGT2B7 gene. Although two novel UGT2B7 polymorphisms in the UGT2B7 promoter area are identified, only for the UGT2B7 -79G variant allele a difference in UGT2B7 expression is found. However, no differences in the glucuronidation ratios of morphine have been found, after genotyping 50 cancer patients receiving morphine on a long-term basis. In CHAPTER 10 three UGT2B7 polymorphisms are correlated to the tacrolimus exposure in a Chinese renal transplant population. Although quantitative evidence regarding the amount of tacrolimus glucuronidation is still lacking, it remains interesting to see that these UGT2B7 polymorphisms may have a significant contribution on the variability of the tacrolimus exposure.

Despite the fact that CYP3A4 and CYP3A5 are mainly responsible for the metabolism of tacrolimus only one polymorphism present in the CYP3A5 gene namely the CYP3A5 6986G or CYP3A5*3 variant allele has a high allelic frequency in different ethnic populations and has a significant contribution on the tacrolimus C_0 concentrations or other pharmacokinetic tacrolimus parameters. Even though there are several other functional CYP3A5 polymorphisms identified⁹, the allele frequency is very low in different ethnic populations and thus the contribution of these polymorphisms on the tacrolimus exposure is minor. Although CYP3A4 plays, like CYP3A5, an important role in the tacrolimus metabolism only the CYP3A4*1B variant allele has a relatively high allele frequency in Caucasians. However, due to a high linkage equilibrium between the CYP3A4*1B and the CYP3A5*1 it is difficult to examine whether the gene-dose contribution obtained is solely due to the CYP3A4*1B variant allele, or that the effect is caused by the high linkage with the CYP3A5*1 allele.

Since polymorphisms in the CYP3A iso-enzymes can only partly explain the variation in the tacrolimus blood concentrations, it is likely that other genes are also (in)directly involved in the tacrolimus metabolism. The pregnane X receptor (PXR) is a member of the nuclear receptor (NR) family of ligand-activated transcription factors that include the steroid, retinoid and thyroid hormone receptors, as well as many orphan receptors from which physiological ligands have yet to be identified^{10,11}. PXR is highly expressed in the liver and intestine, where it binds as a heterodimer with the 9-*cis* retinoic acid receptor (RXR; NR2B) to previously characterized xenobiotic response elements in CYP3A gene promoters and importantly, PXR is activated by the spectrum of chemicals that are known to induce CYP3A gene expression¹². Previously, Zhang *et al.*¹³ identified 38 polymorphisms in the PXR gene of which only eight polymorphisms present in either the

3'-untranslated (UTR) or intronic region seem to demonstrate an effect on the erythromycin breath test, CYP3A4 mRNA/villin or ABCB1 mRNA/villin after rifampicin induction. However, this study was performed with a low number of patients. Op den Buijsch *et al.* (Submitted; CHAPTER 11) demonstrated after genotyping 103 Chinese renal transplant recipients for four PXR polymorphisms, that these PXR polymorphisms have a significant influence on the tacrolimus exposure. Additionally, Op den Buijsch *et al.* (Submitted; CHAPTER 12) examined the influence of these four PXR polymorphisms on the pharmacokinetic tacrolimus parameters C_0 , AUC_{0-12} , C_{max} , and T_{max} in both an early using corticosteroids and a late using no corticosteroids renal transplant recipient group. Although the PXR genotypes of these early and late posttransplant recipient group did not significantly differ from each other, a significant contribution for the PXR polymorphism is only found in the early transplant recipient group which used corticosteroids. Previously, three different studies reported an increase of the tacrolimus C_0 concentration when the corticosteroid therapy was stopped¹⁴⁻¹⁶. Possibly, PXR polymorphisms are responsible for the mechanism behind the difference in tacrolimus C_0 concentration observed.

Conclusions based on the research presented in this thesis are:

The commercial DNA isolation kits (QIAamp blood mini kit, Roche High Pure PCR Template Preparation Kit and Puregene) have an extraction efficiency of approximately 30%.

Real-time PCR FRET assays developed and validated in our laboratory can be used perfectly for genotyping in a clinical setting.

Most limited sampling strategies for tacrolimus that already appeared in literature give a better indication of the tacrolimus exposure than the morning tacrolimus C_0 concentrations used nowadays.

The single nucleotide polymorphism (SNP) A6986G in the CYP3A5 gene has a major impact on the tacrolimus exposure calculated with a limited sampling strategy, and on the dnC_0 , $dnAUC_{0-12}$, and dnC_{max} . This CYP3A5 A6986G polymorphism can explain the variation observed in the AUC_{0-12} calculated with a two time point limited sampling strategy for approximately 35% in a Chinese renal transplant population.

Conflicting results were observed for the ABCB1 polymorphisms C1236T, G2677T/A and C3435T in the Chinese and the Caucasian renal transplant recipient group which is in line with literature.

Although it is known that tacrolimus is also glucuronidated mainly by UGT2B7, the UGT2B7 polymorphisms G-79A, T-66C and C82T have no influence on the tacrolimus exposure in a Chinese renal transplant patient group that is calculated with a two time point limited sampling strategy.

An interesting finding was observed for the PXR polymorphisms A7635G, C8055T, A11156C and T11193C. In the Chinese renal transplant recipients a significant difference was observed for the PXR polymorphisms A7635G and A11156C, T11193C while a trend was observed for the PXR polymorphism C8055T. Although after categorizing the renal transplant patients in different groups based on their CYP3A5 and ABCB1 genotype no significant difference was observed, a trend regarding a lower tacrolimus exposure for renal transplant patients carrying a variant allele for these PXR polymorphisms remained.

The fact that these PXR polymorphisms only have an effect on the tacrolimus exposure in early posttransplant patients that use high corticosteroid dosages indicates that induction of PXR by corticosteroids is a prerequisite for a contribution of PXR polymorphisms on the tacrolimus exposure. This finding also confirms the results of several studies that found lower tacrolimus concentration in early transplant patients using a high daily corticosteroid dose while a higher tacrolimus concentration is observed for the late posttransplant patient using a lower daily corticosteroid dose.

Directions for future research

Because all patients that receive a donor organ have to undergo lifelong treatment by immunosuppressants, an accurate follow up with close monitoring of the immunosuppressant blood concentrations of these patients is required. By receiving a donor organ the patients' health and their quality of life increases enormously, although these patients have to ingest at least one immunosuppressant twice a day, with often several other medications. Due to their highly variable pharmacokinetic characteristics, all immunosuppressants, like the calcineurin inhibitors tacrolimus and cyclosporine, mTOR inhibitors sirolimus and everolimus, mycophenolic acid and azathiopurin all require close monitoring of the blood concentrations on a regular basis. Recently, Hoogtanders *et al.*¹⁷ demonstrated a dried blood spot (DBS) method which is able to determine the tacrolimus concentration with capillary blood which can be obtained with a fingerprick by the patients themselves. Additionally, Wijnen *et al.* (*Submitted*) described a non-commercial DBS method for isolating DNA from capillary blood. Both DBS methods have a lot of advantages regarding the patient, logistics, storage, time and total costs. Recently, several studies reported that a limited sampling strategy consisting of two or three time sample points within four hours after the morning tacrolimus ingestion, have firstly a better correlation with the complete 12 hour area

under the time tacrolimus concentration (AUC_{0-12}) curve and secondly according to a comprehensive study of Op den Buijsch *et al.* the absolute prediction error (APE) of most limited sampling strategies are better than the APE of the trough concentrations C_0 and C_{12} . Therefore, we concluded that all limited sampling strategies that already appeared in literature could generate a reliable prediction of the complete AUC_{0-12} although in new settings careful evaluation of their reliability with an own transplant population is advisable. It is known that the immunosuppressant blood concentrations are most variable in the early period after transplantation. A better indication of the initial starting dose for each individual patient may contribute to a decrease in the variation of the tacrolimus blood concentrations and thus to a lower incidence of subtherapeutic or toxic blood concentrations, which ultimately lead to rejection or adverse drug reactions. Tacrolimus is mainly metabolised due to cytochrome P450 (CYP450) 3A iso-enzymes into 13-O-demethyltacrolimus, which has almost no immunosuppressive activity. The most relevant single nucleotide polymorphism (SNP) in the CYP3A iso-enzymes is the CYP3A5 A6986G polymorphism. Kamden *et al.*³ recently demonstrated that the CYP3A subfamily is the only CYP450 iso-enzyme that has a significant contribution *in vitro* on the tacrolimus metabolism. Additionally, they demonstrated that CYP3A5 is able to metabolise tacrolimus with a 60% higher efficiency rate compared to CYP3A4.

Regarding the contribution of the adenosine triphosphate binding cassette B1 gene (ABCB1) polymorphisms C1236T, G2677T/A and C3435T or ABCB1 haplotypes there is conflicting evidence on their role in the tacrolimus metabolism. Although a few studies found a significant association between the individual ABCB1 polymorphisms or ABCB1 haplotypes, no unambiguous evidence is found for their contribution. Several reasons can justify a discrepancy in the results of the studies that already appeared such as pharmacokinetic (C_0 ; C_2 , C_4 ; or AUC_{0-12}) or genetic (SNP *versus* haplotype) analyses that were performed. Moreover, the sample size of the study cohorts and the time after transplantation points at which a possible ABCB1 genotype effect was assessed, differed between studies. Previously, Strassburg *et al.*⁴ demonstrated that cyclosporine and tacrolimus are also glucuronidated especially by UGT2B7. More recently, Bhasker *et al.*¹⁸ identified the UGT2B7 C816T polymorphism and Dugauy *et al.*⁶ reported about two novel UGT2B7 polymorphisms which are located in the UGT2B7 promoter area. However, Op den Buijsch *et al.* (submitted) first described that three possible relevant polymorphisms in the UGT2B7 gene have no significant influence on the tacrolimus exposure in a Chinese renal transplant population. Because, polymorphisms present in the different CYP3A iso-enzymes, the UGT2B7 gene and the ABCB1 gene can only partly elucidate the variation in the tacrolimus blood concentration, other genes may possibly also have a significant contribution to the tacrolimus blood concentration. However, even when transplant patients achieve therapeutic drug concentrations of the immunosuppressive drugs, rejection and drug toxicity are not eliminated¹⁹. Recently, Op den Buijsch *et al.* (submitted) demonstrated that polymorphisms located in the PXR gene may also explain the variability in the tacrolimus exposure. Especially, since the PXR polymorphisms seem to have influence in patients only early after transplantation

when the daily corticosteroid dose is highest. These studies indicate that genes are not only directly, but also indirectly involved in the tacrolimus metabolism may contribute to the variability of the tacrolimus blood concentration. More recently, a study examining new onset diabetes after transplantation in tacrolimus treated renal transplant patients, showed that the CYP3A5*3, ABCB1 3435T and 2677T/A polymorphisms did not appear to be risk factors. This was, however, a small cohort of 70 subjects, only 10 of which developed new onset diabetes after transplantation and therefore may not have been sufficiently powerful statistically²⁰. Polymorphisms in target proteins have been previously identified: FK binding protein for tacrolimus and mTOR inhibitors²¹, cyclophilin for cyclosporine²² and inosine monophosphate dehydrogenase for mycophenolic acid²³. However, there are as yet no published studies on any potential pharmacogenetic effect. After identification of a number of polymorphisms in several genes that are involved in the tacrolimus metabolism a microarray with all these polymorphisms included, may identify those transplant patients that need an aberrant daily tacrolimus dose or even another type of immunosuppressant. In the end this multi-pharmacogenetic approach will result in a better immunosuppressive pharmacotherapy for each individual transplant patients and lesser rejections and adverse drug reactions are likely to occur.

But even when patients are genotyped for polymorphisms in all these genes involved in both the metabolism (pharmacokinetics) and the drug receptor interaction (pharmacodynamics) of tacrolimus, still a large area of unsolved mysteries will remain. Numerous exogenic and endogenic compounds have a minor to large inhibitory or inductive effect on the CYP3A iso-enzymes, the UGT2B7 enzyme and the drug transporter P-glycoprotein which all are involved in the tacrolimus metabolism. To obtain an idea of the total amount of CYP3A iso-enzymes that are active in the liver an erythromycine breath test is developed^{1,24-26}. By using this erythromycine breath test one is able to determine the actual amount of CYP3A present in the patients' liver. This CYP3A amount determined by the erythromycin breath test may provide a better indication of the CYP3A activity than solely genotyping for CYP3A polymorphisms so that the tacrolimus pharmacokinetics for each individual patient may be predicted more accurately.

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