THE STUDY OF THE EFFECTS OF IMMUNOSUPPRESSIVE DRUGS ON LIPID METABOLISM

by

Rita Tory

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ABSTRACT

Introduction:

Lipid abnormalities including increased total cholesterol, triglycerides, and low-density lipoprotein-cholesterol have been frequently reported in renal transplantation and could be involved in the high frequency of cardiovascular disease in this population. Immunosuppressive therapy appears to be a main factor that influences the post-transplant lipid profile. Cyclosporine A (CsA), rapamycin (RAPA), tacrolimus (TAC) and mycophenolate mofetil (MMF) are commonly used immunosuppressant in solid organ transplant patients. Several of these immunosuppressive agents including CsA, RAPA and TAC appear to have a significant effect on patient lipid level. Although RAPA does not seem to cause nephrotoxicity as commonly seen in patients treated with CsA or TAC, it seems to be associated with an incremental increase in triglyceride level. However, the immunosuppressive-induced hyperlipidemia has not been sufficiently described.

Purpose:

Our aim was to determine the effects of these drugs in vitro on key regulatory enzymes of lipid metabolism; Cholesteryl Ester Transfer Protein (CETP), hepatic lipase (HL) and lipoprotein lipase (LPL) activity within human plasma, as well as the in vivo effects of TAC on these enzymes in renal transplant patients. In addition, we also investigated the effects of RAPA and TAC on cholesterol efflux from human THP-1 macrophages.
Methods:

The effects of CsA, TAC, RAPA and MMF on CETP, HL and LPL activity were first determined in vitro in human normolipidemic plasma and post-heparin normal human plasma, respectively. We further investigated the in vivo effects of TAC on these enzymes activities in renal transplant patients for one month following transplantation. The cholesterol efflux study was conducted independently to assess the effects of RAPA and TAC on ApoA-I- and HDL-mediated cholesterol efflux from human THP-1 macrophages, as well as adenosine-triphosphate binding cassette (ABC)A1 and ABCG1 protein expressions in these cells.

Results:

Our in vitro CETP study showed that CsA and RAPA induced CETP activity in human normolipidemic plasma in a dose-dependant manner. Although, none of these drugs, CsA, TAC, RAPA and MMF affected in vitro HL activity, these drugs suppressed the LPL activity in the post-heparin plasma. Unlike TAC, RAPA was shown to decrease apoAI-mediated cholesterol efflux and ABCA1 protein expression in human THP-1 macrophages. In agreement with our in vitro result, our clinical study demonstrated that TAC significantly increased triglyceride levels and reduced the LPL activity in the renal transplant patients, regardless of the patients were on statin or not.

Conclusions:

These findings suggest that the increase in CETP activity, suppression in LPL activity and inhibition in the cholesterol efflux following either CsA, RAPA or TAC treatments observed in the present study may be associated with hypercholesterolemia and hypertriglyceridemia seen in patients administered these drugs.
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<tr>
<td>ABC</td>
<td>Adenosine triphosphate-binding cassette</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>Apolipoprotein A-I</td>
</tr>
<tr>
<td>Apo E</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl Ester</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol Ester Transfer Protein</td>
</tr>
<tr>
<td>CNI</td>
<td>Calcineurin Inhibitor</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FK-506 or TAC</td>
<td>tacrolimus</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK-506 binding protein</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein – cholesterol</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic Lipase</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate Density Lipoprotein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein - cholesterol</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMF</td>
<td>mycophenolate mofetil</td>
</tr>
<tr>
<td>MTOR</td>
<td>Mammalian target of Rapamycin</td>
</tr>
<tr>
<td>MTS</td>
<td>5-(3-carboxy-methoxyphenyl)-2-(4.5-dimethylthiazolyl)-3- (4-sulphonyl)</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>PTDM</td>
<td>Post-transplant diabetes mellitus</td>
</tr>
<tr>
<td>RAPA</td>
<td>rapamycin; sirolimus</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SR-BI</td>
<td>Scavenger Receptor B-I</td>
</tr>
<tr>
<td>TC</td>
<td>Total Cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>
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Special thanks are owed to my parents for their prayers, encouragement and support throughout all these years of education.
DEDICATION

This work is dedicated to my beloved parents, Eddy Tory and Ang Bie Hiong and to my sisters and brother, Lina Tory, Lindawaty Tory and Rudy Tanaka Tory, as well as my fiancé, Alfian Sutojo.
CHAPTER 1

INTRODUCTION
I. INTRODUCTION

1.1 Immunosuppressive drug therapy and dyslipidemia

1.1.1 Dyslipidemia as a cardiovascular risk factor in post-transplant patients

The mortality rate due to cardiovascular disease (CVD) in transplant recipients is greater than in the general population \(^1\). Because many renal transplant recipients die with functioning grafts, deaths resulting from CVD have become an increasingly important cause of graft loss, particularly after the first post-transplantation year \(^2\). The excessive risk for cardiovascular disease is related to a high prevalence and accumulation of atherogenic risk factors before and after transplantation. Hypertension, post-transplantation diabetes and hyperlipidemia are well-recognized risk factors for the development of cardiovascular events after renal transplantation. Hyperlipidemia following successful renal transplantation is a frequent and persistent complication that contributes to cardiovascular morbidity and mortality and may influence the development of allograft vasculopathy. Therefore, reduction of cardiovascular morbidity and mortality can improve not only the life expectancy and quality of life of the transplant recipients but also their graft function and survival \(^2\).

In the general population, hyperlipidemia is a well-established risk factor for the development of CVD. In transplant patients, hyperlipidemia is a major complication after organ transplant and may contribute to post-transplant accelerated coronary artery disease \(^3\). However, the pathophysiology of post-transplant hyperlipidemia is multifactorial; it can be due to proteinuria, diabetes mellitus, obesity, and suboptimal renal function as well as the immunosuppressive agents, particularly steroids,
calcineurin inhibitors (CNIs; such as cyclosporine A (CsA) and tacrolimus (TAC)) and rapamycin (RAPA). It is important to know that post-transplant hyperlipidemia occurs partly because of the underlying condition causing the need for transplantation and partly because of the side effects of immunosuppressant agents. Although, it is obvious that several of the immunosuppressive agents used today have disadvantageous influences on risk factors for CVD such as hyperlipidemia, hypertension and post-transplantation diabetes mellitus (PTDM), the relative importance of immunosuppressant-induced increases in these risk factors is unknown. Despite the fact that several epidemiological and clinical factors are strongly implicated in post-transplant hyperlipidemia, the pathogenesis remains multifactorial and not fully understood.

1.1.2 Immunosuppressant therapy

Immunosuppressive therapy appears to be a primary factor that influences the post-transplant lipid profile. Since the first successful renal transplantation, there has been an extensive search for immunosuppressive agents that can render the immune mechanism unresponsive to the specific alloantigen stimulus of the engrafted organ, while sparing non-specific host resistance. Out of several immunosuppressive therapies, several pharmaceutical agents have played a principal role in transplantation. The discovery of immunosuppressive drugs such as corticosteroids, cyclosporine A (CsA), azathioprine and tacrolimus (TAC) has been associated with major advancement in the treatment of solid organ transplantation. These immunosuppressants were designed to focus their action selectively on T and/or B cells by inhibiting cytokine synthesis (CsA,
TAC), cytokine action (RAPA), or cell differentiation (MMF) pathways, rather than to act on the immune system in a non-selective fashion. In recent years, many new immunosuppressive drugs have been discovered, developed and applied to clinical trials for their use in combination therapy, expecting them to exert complimentary and synergistic effects, while having minimum toxicities (Figure 1).

**Timeline: The development of immunosuppressive agents for transplantation**

![Timeline Diagram](image)

**Figure 1** The development of immunosuppressive agents for transplantation Adapted from Kahan.⁶
For the purpose of our project, we will focus on four different widely used immunosuppressants in the transplantation clinics; CsA, TAC, RAPA and MMF (Table 1).

Table 1 Summarized comparison of different properties of currently used immunosuppressive drugs

<table>
<thead>
<tr>
<th>Property</th>
<th>cyclosporine A</th>
<th>rapamycin</th>
<th>tacrolimus</th>
<th>mycophenolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Property</td>
<td>Calcineurin inhibitor</td>
<td>mTOR (molecular target of Rapamycin) inhibitor</td>
<td>Calcineurin inhibitor</td>
<td>Inhibitor of inosine monophosphat e dehydrogenase</td>
</tr>
<tr>
<td>Mechanism</td>
<td>Inhibits lymphokine and IL release, thus reduced func. of effector T cells</td>
<td>Blocks signal transduction of T cells and inhibits cell cycle from G1 to S</td>
<td>Inhibits T-lymphocyte signal transduction and IL-2 transcription</td>
<td>Inhibits synthesis of guanosine nucleotides in T and B lymphocytes</td>
</tr>
<tr>
<td>Dosage</td>
<td>10 – 14 mg/kg/day</td>
<td>1 – 7 mg/kg/day</td>
<td>0.05 – 0.3 mg/kg/day</td>
<td>1 g twice daily</td>
</tr>
<tr>
<td>Major side effects</td>
<td>Nephrotoxicity, dyslipidemia, hypertension</td>
<td>Dyslipidemia, hypertension, anemia</td>
<td>Nephrotoxicity, hyperglycemia, dyslipidemia hypertension</td>
<td>gastrointestinal intolerance; haematological aberrations</td>
</tr>
</tbody>
</table>

1.1.2.1 Cyclosporine A (CsA)

CsA (Neoral, Novartis) is a metabolite generated by the fungus, *Toiyocoladium inflatum Gams*, and is a cyclic peptide with a molecular weight of 1,202 composed of 11 amino acids (Figure 3). Since five of its amino acids are methylated, CsA is extremely hydrophobic \(^7\). CsA was first approved for its clinical use in 1983. CsA was first used clinically by Calne et al. \(^8\) for kidney transplantation and Powles et al. \(^9\) for
bone marrow transplantation. Since then, this drug has attracted much attention because it has great immunosuppressive effects not only in kidney transplant patients, but also in recipients of other solid organs such as the heart and the liver.

The main advantage of CsA is its action is very specific to lymphocytes and its effects are reversible. CsA is thought to bind to the cytosolic protein, cyclophilin (immunophilin) of immunocompetent lymphocytes, especially T lymphocytes (Figure 2). This complex of CsA and cyclophonylin inhibits calcineurin, which under normal circumstances is responsible for activating the transcription of IL-2, through the activation of calcineurin by calcium. It also inhibits lymphokine production and interleukin release and therefore leads to a reduced function of effector T cells.

CsA is administered either orally or intravenously. When administered orally, about 40% of the dose is absorbed through the small intestine, while a part of it is transferred into the bile duct. Therefore, CsA is excreted mainly into bile and partly into urine.

The major side effects of CsA are nephrotoxicity and hyperlipidemia. Impairment of renal function due to nephrotoxicity and hyperlipidemia has a serious impact on the prognosis of renal grafts.
Figure 2. Mechanism of inhibitory action of cyclosporine A (CsA), tacrolimus (TAC), rapamycin (RAPA), and mycophenolic acid (MPA; an active form of mycophenolate mofetil (MMF)) on T cells activation and proliferation. [APC: antigen presenting cell, APC, antigen-presenting cell; CTLA4, cytotoxic T lymphocyte antigen 4; GMP, guanosine monophosphate; IκB, inhibitory κB; IMP, inosine monophosphate; JAK3, Janus kinase 3; L, ligand; MTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PKB, protein kinase B; R, receptor; STAT5, signal transducer and activator of transcription 5; TCR, T-cell receptor; TLR4, Toll-like receptor 4; ZAP70, ζ-chain-associated protein 70. Adapted from Kahan 6.}
1.1.2.2 Tacrolimus (TAC)

TAC (FK506; Prograf) is a potent immunosuppressive agent with a molecular weight of 822, which was originally obtained from Streptomyces tsukubaensis, and has been shown in preclinical and clinical studies to prevent allograft rejection. Although the molecular structure is quite different from CsA, which is a cyclic peptide, the mode of its immunosuppressive action is similar to that of CsA (Figure 2 and Figure 3). TAC also affects helper T cells and inhibits the differentiation of cytotoxic T cells by inhibiting the production of IL-2 and expression of IL-2 receptors.

TAC binds to another member of the cytoplasmic protein family (immunophylin), FK binding protein (FKBP), as CsA binds to cyclophilin (Figure 2). After binding to the intracellular FK-506 binding protein, TAC blocks intermediate steps in the pathway that links early membrane-associated events with gene expression and inhibits T and B cell proliferation. Its selective effect on the immune system appears to be due to selectivity for a subset of calcium-associated signal transduction pathway that is important in the T cell receptor-mediated cascade leading to cytokine production.

TAC is about 50 times more potent than CsA in inhibiting alloreactive T cell proliferation, B cell activation and the production of other cytokines such as IL-3, IL-4, interferon-γ and G-CSF, although it does not inhibit the secondary proliferation of activated T cells in response to IL-2.
TAC shows considerable variation in its pharmacokinetic profile among individuals. Since it is almost completely metabolized in the liver, hepatic dysfunction causes its accumulation in the body and elevated blood levels of TAC. In blood, TAC is sequestered by erythrocytes and its plasma concentrations are influenced by hematocrit, plasma protein levels and temperature. 13

The major side effects of TAC are very similar to those of CsA, possibly because of the similarity of modes of action, and include nephrotoxicity, neurotoxicity, diabetogenicity, and gastrointestinal symptoms. 6 Hyperlipidemia, which is common in CsA therapy, is less pronounced in patients treated with TAC. However, TAC’s effect on patient lipid level is still a clinical problem and this side effect, in most cases, resolves with dosage reduction or withdrawal.

1.1.2.3 Rapamycin (RAPA)

RAPA (Rapamune, Wyeth), a compound with a molecular weight of 914 produced by the actinomycetes, Streptomyces hygroscopicus, was reported for the first time in 1975 as a compound with potent inhibitory activity against Candida albicans. 14 RAPA is a macrolide antibiotic like TAC and has a chemical structure similar to TAC but acts at a different site (Figure 3).

Although RAPA also binds the cytoplasmic immunophylin, FKBP, it does not inhibit calcineurin (Figure 2). Since the RAPA-FKBP complex does not bind calcineurin and does not block its activity of G0 to G1 progression, it results in preservation of signal transduction to the IL-2 gene. The failure to form a supercomplex
with RAPA is reported to be caused by the difference of FKBP for binding. RAPA inhibits the IL-2 receptors interaction without affecting the production of IL-2.$^{15}$

T cell growth induced by IL-2 and IL-4 is only slightly inhibited by TAC and CsA, but is effectively inhibited by RAPA. These findings suggest that unlike TAC and CsA, RAPA affects T cell activation by lymphokines at a late stage. While TAC and CsA affect T cell activation at an early stage and inhibit the lymphokine necessary for T cell activation, RAPA exerts its immunosuppressive effects by inhibiting reactions to these lymphokines. Based on these modes of action, RAPA is expected to inhibit alloreactive cells which are resistant to TAC or CsA. Likewise, RAPA has also been shown to have toxic side effects on the kidney, GI system and central nervous system and to be diabetogenic in clinical studies.$^{16}$

1.1.2.4 Mycophenolate mofetil (MMF)

Mycophenolate mofetil (Cellcept, Roche) is a prodrug of mycophenolic acid (MPA) that is a fermentation product of several Penicillium products (Figure 3). In the early 1990s, as ester derivative of MPA, MMF was created with the intent to increase oral bioavailability and gastrointestinal tolerability. In 1995 MMF was approved by the FDA for the prevention of renal allograft rejection and is currently used in immunosuppressive regimens for various organ transplants. Guanosine triphosphate (GTP) is essential for the synthesis of protein and nucleic acid and for various enzymatic reactions. Inosine 5'-monophosphate dehydrogenase (IMPDH) is a rate-limiting enzyme necessary for the biosynthesis of GTP. Therefore, an increase of IMPDH activity and GTP production is necessary for the proliferation of lymphocytes.
The esterified prodrug MMF is rapidly metabolized to mycophenolic acid (MPA) and has better bioavailability, which has led to its use in heart transplants and other solid organs. MMF, is an inhibitor of IMPDH, and selectively decreases intracellular GTP and inhibits purine biosynthesis, thereby inhibiting i) the differentiation and growth of T cells and ii) antibody production by B cells (Figure 2).

During the early post-transplant stage, mycophenolate mofetil shows marked inter-individual pharmacokinetic differences in absorption, which might produce inadequate immunosuppression, therefore, it can only be used along with more potent agents at this stage. However, during maintenance therapy, it can provide the primary treatment for patients who experience toxic reactions to other immunosuppressive drugs and are at low risk for allograft rejection 6.

It has been reported that MMF causes gastrointestinal and hematological side-effects, whereas these appear usually dose related, responding quickly to dose reduction. 17. However, another study has also shown that MMF usage, after renal transplantation, induced hepatotoxicity in patients. If hepatotoxicity related to MMF is not considered, especially in the early period of renal transplantation, resolution of hepatotoxicity can be required in the long term 18.
Figure 3 Chemical structures of several immunosuppressive drugs
1.1.3 Immunosuppressive drug-induced dyslipidemia

Calcineurin inhibitors, CsA and TAC, are the principal immunosuppressants prescribed for renal transplantation. In the majority of patients, these calcineurin inhibitors have been used in combination with other immunosuppressive drugs, such as azathioprine or mycophenolate mofetil (MMF)\textsuperscript{19}. However, calcineurin inhibitors are associated with variable effects on lipid profile. CsA is more dyslipidemic than TAC, and conversion from CsA to TAC based regimen in patients who developed dyslipidemia, has been associated with favorable lipid profile\textsuperscript{20}. However, TAC has been preferred to CsA in patients with a high risk for rejection, such as those with ABO-incompatibility, delayed graft function, sensitization, and the African American race and patients with additional risk factors, such as hypertension and hyperlipidemia\textsuperscript{21}.

In addition to a more favorable cardiovascular risk profile than CsA, TAC is also less nephrotoxic. It has little or no effect on blood pressure and serum lipids; however, its diabetogenic effect is more prominent in the period immediately following transplantation, although at maintenance dosages, the diabetogenic effect appears to be comparable to that of CsA\textsuperscript{22}. The diabetogenic effect of TAC can be managed by reducing the dose of TAC and early corticosteroid withdrawal.

However, another study has also shown that TAC causes a greater degree of glucose homeostasis alteration than CsA\textsuperscript{23}. It is not known whether the diabetogenic potential of the two CNIs is different depending on the pre-transplant insulin-resistance state of the recipient. Finally, although the adverse effects of TAC slightly differ from
those of CsA, the drug particularly shows an improved profile with respect to hypertension, dyslipidemia, and long-term renal function. TAC has been shown particularly effective as a rescue treatment in cases where recurrent rejection has occurred with CsA.

Although calcineurin inhibitors are still the major immunosuppressive regimes for transplant recipients, calcineurin inhibitors are often associated with severe side effects such as nephrotoxicity, as was mentioned previously. On the other hand, with little or no nephrotoxic activity of its own, rapamycin (RAPA) is another potent immunosuppressive drug that has been shown to decrease the incidence of rejection post renal transplantation.

Dyslipidemia is a well recognized side effect of rapamycin therapy, which may have an impact on patient survival and post-transplant cardiac morbidity and mortality. RAPA-associated dyslipidemia has been reported in 45% of liver transplant patients and in about 40% of renal transplant patients. Clinically, it has been known to induce a considerable increase in cholesterol and triglyceride plasma levels. Hypertriglyceridemia is the most frequent and marked alteration in RAPA-treated patients and it can occur two to four weeks after starting maintenance immunosuppressive therapy. In a study performed in six renal transplant patients treated with RAPA, there was evidence that RAPA intake led to the increased production of TG-rich lipoproteins, which contributed to the observed hypertriglyceridemia.
Interestingly, despite the effects of RAPA on dyslipidemia, it has been thought
that RAPA may have an anti-atherogenic effect as well. In an atherosclerotic-prone
mouse model (apo E knockout), animals treated with RAPA were protected from
atherosclerotic changes of the aorta despite increased cholesterol. This paradox could
be explained by the blockade of the local inflammatory cytokines (interleukin 6 (IL-6),
IL-10, IL-12 and interferon γ), pro-atherogenic factors (monocyte chemotactic
protein-1), cell proliferation mechanism and modulation of T-cell response exerted
by RAPA. Varghese and colleagues have shown that RAPA reduced lipid accumulation
in human glomerular mesangial cells by suppression of expression of LDL and VLDL
receptors and CD36-inhibited pro-inflammatory cytokine expression, and increased
efflux of cholesterol.

The proliferation inhibitor, mycophenolate mofetil (MMF) has little effect on
the cardiovascular system. Yet, indirectly, by inducing anemia, it has been associated
with increased risk for cardiovascular events and left ventricular hypertrophy. MMF is
not usually used as a monotherapy in transplant patients. Its use is mostly combined
with calcineurin inhibitors or steroids, so its effects on lipid metabolism are not well
understood.

In a study using a rabbit model of hyperlipidemia, MMF has been shown to
reduce atherosclerosis in these animals. Besides that, it has been reported that MMF has
beneficial effects in cardiac transplant patients; patients with MMF regimens
experienced diminished intimal thickening and cardiac allograft vasculopathy
compared to patients not taking MMF. It suppresses the glycosylation of adhesion
molecules, blocks leukocyte adhesion and tissue penetration, inhibits the proliferation of aortic smooth muscle, and decreases the primary antibody response. It may also suppress the formation of nitric oxide at the sites of tissue rejection, leading to diminished organ damage.

I.2 Effects of immunosuppressive drugs on lipid metabolism

For the present research project, we have selected four different drugs of interest to assess their potential effects on some key regulatory enzymes of lipid metabolism (Figure 4).
1.2.1 **Immunosuppressive drugs and cholesteryl ester transfer protein (CETP)** (TARGET 1).

Cholesteryl Ester Transfer Protein (CETP) is a 476-amino acid hydrophobic glycoprotein that is secreted mainly from the liver and circulates in the plasma, bound mainly to HDL (Figure 4)\(^35\). Plasma CETP facilitates the transfer of cholesteryl ester (CE) from high-density lipoprotein (HDL) to apolipoprotein (apo) B-containing lipoproteins (e.g., LDL) via the CETP-mediated cholesterol efflux pathway.

**Figure 4** Lipoprotein metabolism with some of the targets (highlighted) that may be altered by immunosuppressive drugs. [TARGET 1, CETP: cholesteryl ester transfer protein, TARGET 2. HL: hepatic lipase, TARGET 3, LPL: lipoprotein lipase, TARGET 4, Cholesterol efflux, ABCA1: adenosine triphosphate-binding cassette A1]. Modified from Kaysen \(^34\).
lipoproteins with reciprocal transfer of triglycerides (Figure 5)\textsuperscript{36}. CETP may also play a role in certain disease processes such as atherosclerosis by redistributing cholesterol from the antiatherogenic HDL particles to the proatherogenic LDL particles. Conversely, CETP is also implicated in the process of reverse cholesterol transport, which removes cholesterol from peripheral tissues and is viewed as antiatherogenic. The precise role of CETP in atherosclerosis is not clear.

CETP can be regulated by cholesterol levels, with an increase in activity and expression seen in response to cholesterol. In different studies using human CETP-transgenic mice, the increase in CETP activity is attributed to an increase in transcription of the CETP gene in these animals. Conversely, CETP activity has been reduced in response to corticosteroids and lipopolysaccharides.
It has also been shown that CETP has the ability to transfer hydrophobic drugs, such as CsA, between different lipoprotein subclasses and therefore has a potential application for drug delivery\textsuperscript{38}. Previous work has suggested that CsA-administered transplantation patients, who exhibit plasma dyslipidemias including hypocholesterolemia and hypertriglyceridemia, might have an elevated CETP level\textsuperscript{39}.

A previous study by Atger et al. suggested that therapeutic agents used in post transplantation treatment such as glucocorticoids and/or CsA may affect both CETP
and HL activities and, by arresting the HDL cycle in a CE-saturated state, to decrease the efficiency of reverse cholesterol transport at the site of the graft 40.

One study has demonstrated that CETP activity is not increased in chronic kidney disease, but instead may be decreased, suggesting that the mechanisms responsible for establishing HDL levels may differ between renal failure and insulin resistance, despite superficial similarities. The data also indicated that a reduced reverse cholesterol transport in patients was caused by increased levels of an inhibitor to CETP activity in their serum 41. Despite the known role of CETP in lipid metabolism, studies analyzing the effects of immunosuppressive drugs on CETP function are scarce.

1.2.2 Immunosuppressive drugs and hepatic and lipoprotein Lipase (TARGET 2 and 3).

LPL is a member of triacylglycerol lipase protein family, others of which include hepatic lipase and endothelial lipase, which like LPL is released into the plasma by heparin (Figure 4, Figure 6) 42. LPL is synthesized and secreted in adipocytes, muscle cells, cardiomyocytes and macrophages, but the functional fraction of LPL is at the vascular endothelial surface, where it is bound by heparin-like glycosaminoglycans 43.

The primary function of LPL is to hydrolyze core triglycerides in triglyceride-rich lipoproteins and convert them into remnant particles, IDL and LDL. Owing to the large size of chylomicrons and VLDLs, lipolysis takes place at the vascular endothelial
LPL hydrolyzes the 1(3)-ester linkages of triacylglycerol of chylomicrons and VLDLs whose surface contains apo CII (Figure 6). Apo CII is the cofactor required for the activity of LPL. The primary product of LPL-mediated lipolysis is 2-monoacylglycerol. Further lipolysis is mediated by plasma and platelet monoacylglycerol hydrolases. The end products of LPL-mediated triacylglycerol hydrolysis are unesterified fatty acids, monoacylglycerol and glycerol. Fatty acids
originating from LPL activity are cleared by adipose tissue and re-esterified under postprandial conditions and stored. Under fasting conditions, hormone-sensitive lipase promotes the release of unesterified fatty acids into the circulation from adipocyte triacylglycerol.

A chylomicron or VLDL contains about 10 to 20 molecules of apo CII/molecule of apo B. Titration of apo CII levels against the rate of lipolysis has shown that no more than two to three apo CII molecules per particle are needed for maximal TG hydrolysis rates. The end products of chylomicron and VLDL lipolysis are removed by the liver via LDL receptors and other receptors. The catalytic rate of TG hydrolysis by LPL is about 10/s and this rate is generally determined by the level of lipase activity at the capillary endothelial surface.

The most important factors regulating the activity of LPL at the vascular endothelial surface of adipose and muscle tissues are post-translational. In particular, the distribution of LPL between parenchymal cell and endothelial cell fractions is highly regulated in response to nutritional and other physiological factors. Genetic deficiency of LPL is associated with a massive increase in the circulating levels of chylomicrons, and an absence of LPL activity from post-heparin plasma. However, there is less increase in VLDL levels than would be predicted from the role of LPL in VLDL catabolism. An alternative, low-capacity pathway probably exists for the clearance of intact VLDL particles by the liver, for example by the hepatic lipase.

LPL and HL are equally important for normal remnant catabolism. Humans lacking LPL or its activator, apoC-II, develop massive hypertriglyceridemia due to the
accumulation of both chylomicrons and large VLDL. Familial HL deficiency results in typical type III hyperlipoproteinemia with impaired clearance of chylomicrons remnants. Lipases regulate lipoprotein catabolism by two mechanisms, as lipolytic enzymes and as ligands for lipoprotein receptors. Felts, Itakura, and Crane first suggested that by associating with remnant particles, LPL may provide the recognition signal for uptake by hepatic receptors. However, as reported by another group, HL was less potent than LPL in stimulating degradation of normal VLDL.

It was formerly thought that the conversion of IDL to LDL was mainly a function of HL. This enzyme is located in the liver and although it is related structurally to LPL, it has greater activity towards partial glycerides and phospholipids than does LPL. HL lacks LPL’s dependence on the presence of apo CII. Besides that, HL is also involved in reverse cholesterol transport (Figure 6). In this process, it mediates the conversion of triglyceride-rich HDL₂ to triglyceride-poor HDL₃, a step that releases cholesteryl esters, phospholipids, fatty acids and glycerol, which are taken up by the liver.

While a few cases of congenital HL deficiency have been reported in humans, the evidence that this lipase is involved in the processing of chylomicron remnants and/or IDL has remained ambiguous. Currently, it seems that the major role played by this lipase is related to optimizing the surface lipid of these particles to promote CE transfer from HDL in exchange for TG in IDL, or in the selective uptake of CE from HDL by the scavenger receptor-BI (SR-BI), or in the hydrolysis of TG in HDL.
Although congenital HL deficiency is associated with increased levels of plasma total TG, the pattern of lipoprotein abnormalities is more variable and less clear-cut than that of LPL deficiency. It seems probably that other plasma and endothelial lipases can take over many of HL’s functions 50.

The fact that hypertriglyceridemia is more frequently observed after certain immunosuppressive treatments may be partly caused by changes in the synthesis and elimination of triglycerides involving lipoprotein lipase or some apolipoproteins which serve as its cofactors (apoC-II or apoC-III) 51. The results of a study by Vaziri et al (2000) showed marked reductions of both skeletal muscle and adipose tissue lipoprotein lipase abundance in CsA-treated rats, thus suggesting lipoprotein lipase deficiency 52. Another study has also shown that CsA-treated rats had an increase in plasma triglycerides and non-HDL-cholesterol, and a dose and time-dependent decrease of LPL activity and HDL-cholesterol, mainly because of a decrease in the HDL2-cholesterol subfraction 53.

A pilot study in renal transplant patients receiving RAPA treatment found that hyperlipidemia induced by RAPA is the result of reduced catabolism of apoB100-containing lipoproteins 54. In guinea pigs, RAPA also interferes with TG metabolism by altering the insulin signaling pathway, inducing increased secretion of VLDL and promoting deposition of TG in the aorta. A low dose of RAPA was found to decrease cholesterol accumulation in tissue (liver and aorta) compared to a high dose of RAPA, suggesting that lower doses could be less detrimental to transplant patients 28. Massy et al (2000) found increased levels of several apolipoproteins (significant only for apo C-
II) in the rapamycin treated group of transplant patients in comparison with the CsA group, suggesting that an increase in hepatic production of triglyceride- and cholesterol-rich lipoproteins might be involved in the hyperlipidemia observed in the rapamycin group.

The pathogenesis of mTOR inhibitor dyslipidemia is unclear. Increased plasma levels of apo B$_{100}$; a major component of VLDL and LDL, and a ligand for the LDL receptor, have been demonstrated in rapamycin-treated kidney transplant patients, resulted in elevated VLDL and LDL levels. These alterations appear to be due to the result of decreased catabolism rather than increased synthesis of apo B$_{100}$, which could be partially mediated by down-regulation of LDL receptors.

In addition, the inhibition of LPL activity by increased level of apoC-III and decreased level of apoC-II may result in an increase in VLDL and IDL levels, but data supporting this pathway are controversial. Morrisett and coworkers have also described increased free fatty acid concentration with decreased incorporation into VLDL, despite increased hepatic synthesis of triglycerides secreted as VLDL. Further research efforts are needed to better describe the pathways implicated in these phenomena.

However, as to our knowledge, there are no studies, which have investigated the effects of TAC and MMF on hepatic and lipoprotein lipase activity.
1.2.3 Immunosuppressive drugs and cholesterol efflux (TARGET 4).

Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which HDL particles play a crucial role to carry cholesterol derived from peripheral tissues to the liver. Cholesterol efflux is the first and most-likely rate-limiting step of reverse cholesterol transport. Several pathways of cholesterol efflux have been identified. As acceptors such as apoA-I and HDL approach macrophages in subintimal space, intracellular cholesterol can be released outside the cells for excretion, and a process termed cholesterol efflux of macrophages. In this pathway, ATP-binding membrane cassette transport protein A1 (ABCA1) and ABCG1 play a major role in translocating cholesterol into the extracellular space. In addition to ABCA1 and ABCG1, there are other factors that are known to be involved in the pathway, such as scavenger receptor B1 (SR-B1) and the passive diffusion pathway. Cholesterol efflux constitutes an efficient pathway by which excess cholesterol can be removed out of the body. Although extensive studies have recently been performed, it is a complicated process and its regulatory mechanisms are not completely understood.

In addition, the ATP-binding cassette transporter ABCA1, which mediates cholesterol and phospholipid efflux to lipid-poor HDL apolipoproteins, plays a key role in the elimination of cholesterol from macrophages in the arterial wall (Figure 4). Macrophage ABCA1 has specifically been shown to have an anti-atherogenic role in mouse models of atherosclerosis, as observed in bone marrow transplantation studies in which ABCA1-deficient marrow donors led to larger aortic lesions than wild-type donors. Le Goff et al. (2004) have provided evidence that CsA inhibits ABCA1-
mediated lipid efflux to apo A-I, reduces ABCA1 turnover and increases total and cell-surface ABCA1 in the RAW264.7 cells and THP1 cells \(^{59}\). It has been reported that ABCA1 gene expression is up-regulated in human THP1 macrophages when they are treated with TAC, while the protein expression of ABCA1 remains relatively unchanged \(^{61}\). On the other hand, other experiments have demonstrated that RAPA increased cholesterol efflux from vascular smooth muscle cells (VSMC) and overrode the suppression of cholesterol efflux induced by IL-1 beta. RAPA also increased ABCA1 and ABCG1 gene expression, even in the presence of IL-1 beta \(^{62}\).

A recent paper by Ma et al. (2007) showed that in HepG2 cells, rapamycin increased cholesterol efflux mediated by ABCA1 gene expression by increasing peroxisome proliferator-activated receptor-alpha and liver X receptor-alpha gene and protein expression. Increased cholesterol efflux from HepG2 cells may increase high-density lipoprotein cholesterol levels and also contribute to apo B lipoprotein formation by enhancing transfer of high-density lipoprotein cholesterol to apo B lipoproteins. Moreover, this study also demonstrated that the increase of LDL cholesterol by sirolimus is partly due to the reduction of LDL receptors on hepatocytes \(^{62}\). However, no experimental results have been shown to assess the effect of MMF on the cholesterol efflux in human THP-1 macrophages.
CHAPTER 2

SUMMARY OF RESEARCH PROJECT
2 SUMMARY

2.1 Purpose

The overall purpose of my research is to address mechanisms as to why specific immunosuppressive drugs cause dyslipidemia in post-transplant patients. In order to answer our research question, we have investigated the effects of four different immunosuppressant drugs; cyclosporine A (CsA), rapamycin (RAPA), tacrolimus (FK-506) and mycophenolate mofetil (MMF), on some key components of lipid metabolism, primarily on Cholesteryl Ester Transfer Protein (CETP) activity, hepatic lipase and lipoprotein lipase activity and cholesterol efflux.

2.2 Hypotheses

Based on the introduction above, it is evident that potential alterations in the activities of CETP, HL, LPL and cholesterol efflux by immunosuppressive drugs may lead to the dyslipidemia seen in post-transplant patients. Therefore we hypothesize that;

2.2.1 Hypothesis I

CsA, tacrolimus, rapamycin and mycophenolate decrease CETP-mediated transfer of cholesteryl esters (CE) between lipoproteins.

2.2.2 Hypothesis II

CsA, tacrolimus, rapamycin and mycophenolate decrease the hepatic and lipoprotein lipase activity.
2.2.3 Hypothesis III

Tacrolimus and rapamycin inhibit cholesterol efflux to ApoA-I and HDL from human THP-1 macrophages by decreasing the protein expression of ABCA1 and ABCG1 respectively.

2.2.4 Hypothesis IV

Tacrolimus increases cholesterol and triglyceride levels and suppresses CETP, hepatic lipase and lipoprotein lipase activities in renal transplant patients within one month post-TAC administration.

2.3 Specific Objectives

In order to test our hypotheses, there are four different objectives:

2.3.1 Objective I:

To determine if CsA, RAPA, TAC and MMF alter CETP activity in vitro in human normolipidemic plasma.

2.3.2 Objective II:

To investigate if CsA, RAPA, TAC and MMF affect the hepatic and lipoprotein lipase activity in vitro in post-heparinized human plasma.
2.3.3 Objective III:
To determine if RAPA and TAC affect cholesterol efflux to ApoA-I and HDL from the oxidized human THP-1 macrophage cells and if it occurs due to the change in the protein expressions of ABCA1 and ABCG1 respectively.

2.3.4 Objective IV:
To investigate if TAC affects renal transplant patients lipid profile and their plasma CETP mass and activity, lipoprotein lipase activity and hepatic lipase activity in a period of one month post-transplant.

2.4 Rationale

2.4.1 Objective I:
In our previous work, we have demonstrated that CETP not only can influence the lipoprotein distribution of CsA, but also facilitate the transfer of hydrophobic drugs, such as CsA, between different lipoprotein subclasses. Further investigation showed that CETP-mediated transfer of CsA between lipoproteins may be a result of the potentially direct binding of CsA along the molecule of CETP itself.

Hence, we started to investigate the in vitro effects of the immunosuppressive drugs on CETP activity in human normolipidemic plasma. This was done by evaluating radiolabelled $^3$H-CE transfer between lipoproteins, using a monoclonal antibody against the neutral binding site of CETP, and two different sources of CETP; lipoprotein-
deficient plasmas or a recombinant CETP. To date, the interaction or the effect of immunosuppressive drugs on CETP activity has not been investigated. Objective 1 helps us to understand whether CETP plays a role in immunosuppressant-induced dyslipidemia.

2.4.2 Objective II:

In addition, CETP was directly or indirectly related to LPL activity. LPL hydrolyzes TG-rich lipoproteins, thereby providing surface material to be integrated into HDL. Subsequently, lecithin:cholesterol acyltransferase esterifies unesterified cholesterol on the HDL surface, resulting in the formation of a hydrophobic core as CE are formed. HDL CEs may alternatively be diverted to TG-rich lipoproteins in exchange for their major core lipid, i.e. TG, in a process catalyzed by CETP. HDL TGs may subsequently be hydrolyzed by HL, resulting in a reduction of the HDL core size and the conversion of larger HDL2 to smaller HDL3 particles. Thus, processes that augment HDL mass (driven by LPL and lecithin:cholesterol acyltransferase) are balanced by processes that reduce HDL mass by the combined actions and interactions of post absorptive and postprandial TG-rich lipoproteins, CETP, and HL.

Therefore, based on the results of the experiments completed in objective 1 and objective 2, we would be able to understand if any effect of these immunosuppressive drugs on CETP activity is related to the effects that we observe in LPL and HL activities. Since LPL and HL are involved in plasma triglyceride clearance, the suppression of LPL and HL activities by any of these immunosuppressive drugs would
explain the hypertriglyceridemia observed in post-transplant patients administered those particular drugs.

### 2.4.3 Objective III:

CETP, HL, LPL, lecithin:cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP) are some of the major constituents of the reverse cholesterol transport (RCT). A critical part of RCT is cholesterol efflux, in which accumulated cholesterol is removed from macrophages in the subintima of the vessel wall by ATP-binding membrane cassette transporter A1 (ABCA1), ABCG1 or by other mechanisms, including passive diffusion, scavenger receptor B1 (SR-B1), caveolins and sterol 27-hydroxylase, and collected by HDL and apoA-I. Impairment of RCT and cholesterol efflux have been associated with an increase in CVD incidence.

The purpose of this objective was to determine if any of the immunosuppressive drugs that we studied would affect cholesterol efflux. This was accomplished by the determination of the amount of radiolabeled cholesterol transferred to the exogenous apoA-I and HDL from the oxidized human THP-1 macrophages when the cells were coincubated with any of the immunosuppressive drugs. If our hypothesis is true, this finding can be an explanation as to why we see low plasma HDL cholesterol levels in some post-transplant patients treated with these drugs.

### 2.4.4 Objective IV:

Finally, in order to determine the clinical relevance of the results that we observed in vitro, in objective 4, we conducted a clinical study, in which we
investigated the effects of one of our immunosuppressive drugs; tacrolimus (TAC) on renal transplant patient lipid profile as well as other key regulatory proteins of lipid metabolism, including CETP, HL and LPL.

This objective was done by recruiting pre-scheduled living donor renal transplant patients at St. Paul’s hospital renal program, in collaboration with Dr. David Landsberg. Patients whole blood and serum were collected at six different time points; pre-transplant, post-transplantation surgery, post-TAC administration 1 week, 2 weeks, 3 weeks and 4 weeks.

2.5 Significance of research

Finally, understanding the effects of CsA, RAPA, TAC and MMF on CETP activity, LPL and HL activity and cholesterol efflux will provide us with some mechanistic knowledge related to how these immunosuppressive drugs cause dyslipidemia and increase CVD-risk factors in post-transplant patients administered these drugs.

Several considerations limit the options for drug treatment of dyslipidemia in patients after transplantation, including drug-drug interactions and the potential for overlapping toxicities between lipid-lowering agents and immunosuppressive therapy. Although several studies show that statins can be used safely and effectively to treat hypercholesterolemia in post-transplant patients, further understanding of immunosuppressive-induced hyperlipidemia is still needed.
Furthermore, understanding the mechanism by which CsA, RAPA, TAC and MMF cause dyslipidemia in transplant patients will allow improved administration of immunosuppressive drugs and dosing regimen. In addition, this may be a strong argument for the selective use and individual tailoring of immunosuppressive agents based upon the risk factor profile of the patient, without jeopardizing the function of the graft.
CHAPTER 3

MATERIALS AND METHODS
3 MATERIALS AND METHODS

3.1 Materials

Radiolabeled CE [1α,2α(n)-3H-cholesteryl oleate, specific activity 68.8 mCi/mg], dissolved in toluene solution, was purchased from Amersham Biosciences (Piscataway, New Jersey, USA). Radiolabeled glycerol tri [9,10 (n)-3H] oleate (specific activity 19.5 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Tris-HCl, sodium chloride, sodium bicarbonate, sodium bromide, sodium azide, sodium phosphate, sodium hydroxide, ethylenediaminetetraacetate (EDTA), potassium phosphate monobasic, potassium chloride, bovine serum albumin and CsA were purchased from Sigma Chemical Company (St. Louis, MO). T150 buffer was made from 50mM Tris-HCl, 150mM NaCl, 0.02% NaN₃, 0.01% disodium EDTA at pH 7.4. RAPA, FK-506 and MMF were purchased from Tecoland Corporation (Edison, NJ).

Normolipidemic human plasmas (total plasma cholesterol concentrations in the range of 135-150 mg/dL) were obtained from Bioreclamation (Hicksville, NY). Post-heparin normal human plasma was a gift from St. Paul’s Atherosclerosis Specialty Lab (Vancouver, BC, Canada) and used as received. TP2, a monoclonal antibody that specifically binds to the CE-binding site of CETP, was obtained from the Ottawa Heart Institute (Ottawa, ON, Canada). Recombinant CETP (rCETP) was purchased from Roar Biomedical Inc. (New York, USA). Plasma and lipoprotein total cholesterol and triglycerides were determined using enzymatic assays obtained from Sigma Diagnostics (St. Louis, MO).
All tissue culture plates were purchased from Corning Incorporated (NY, USA). CytoTox96® Non-Radioactive Cytotoxicity Assay was obtained from Promega Corporation (Madison, WI, USA). MTS CellTiter 96® Aqueous One Solution Cell Proliferation Assay was purchased from Promega Corporation (Madison, WI, USA). BCA™ Protein Assay kit was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Amplex Red Cholesterol Assay kit was bought from Invitrogen (Eugene, OR, USA).

ApoA-I was obtained from Sigma-Aldrich (Oakville, ON, Canada) and HDL was purchased from Fitzgerald (Concord, MA, USA) Nitrocellulose membrane, 0.45 mM, was purchased from Bio-Rad (Hercules, CA, USA). High range markers, anti-ABCG1 and anti-Actin (I-19) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-ABCA1 primary antibody was obtained from Novus (Novus Biologicals, Littleton, CO, USA). Anti-goat HRPO conjugate was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-rabbit HRPO conjugate was obtained from BioRad (BioRad, Mississauga, ON, Canada). T01901317 (575310; Calbiochem) was used as synthetic activators of LXRα. Triton X-100 was obtained from Sigma-Aldrich (St. Louis, MO, USA).

The equipment for electrophoresis, such as the gel cassette, electrode apparatus, glass plates, comb, etc. were bought from Bio-Rad (San Fransisco, CA, USA). Tween-20, protease inhibitor cocktail, phenylmethylsulphonylfluoride (PMSF), Na-deoxycholate, isopropanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). NP-40 was purchased from Roche Applied Science (Laval, QC, Canada). Acrylamide, Laemmli sample buffer, Tris buffered saline, 2% Bis solution N, N, N’, N’-Tetra-
methyl-ethylenediamine (TEMED), nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA).

CETP Fluorescence assay kit was obtained from Roar Biomedical Inc. (New York, NY, USA). CETP Elisa kit and LPL Elisa kit were purchased from ALPCO Diagnostics (Salem, NH, USA). Total cholesterol, triglycerides, LDL-C, and HDL-C enzymatic kits were obtained from Wako Pure Chemicals Industries (Richmond, VA, USA).

3.2 CETP in vitro study

3.2.1 CETP radioactive assay

CETP was isolated from human lipoprotein-deficient normolipidemic plasma as previously described 66. Citrated human plasma was made lipoprotein-deficient by the dextran sulfate-MnCl₂ precipitation procedure of Burstein et al. 67.

The human normolipidemic plasma was separated into very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and lipoprotein deficient plasma (LPDP) fractions by density gradients and ultracentrifugation as has been previously described 68. Human plasma (3 mL) was placed in the centrifuge tubes and its density was adjusted to 1.25 g/mL by adding sodium bromide. Discontinuous density gradients were constructed by under layering the following NaBr solutions: 1.006 g/mL, 1.063 g/mL and 1.21 g/mL respectively. Plasma sample was then spun at 40,000 rpm for 18 hours at 15°C in a SW41 Ti rotor to separate the fractions (L8-30 M; Beckman, Toronto, Ontario, Canada). The HDL
fractions were further dialyzed against 0.9% saline solutions at 4°C overnight. The molecular weight cut-off of the dialysis tubing used was 500 daltons. After dialysis, these fractions were filtered through a 0.2 μm filter.

Human LDL fractions were labeled by the lipid dispersion technique as previously described. Human plasma was incubated with [³H]-CE (17.5 μCi) overnight at 37°C. Then the radiolabeled LDL fractions were isolated from the plasma by ultracentrifugation as previously described and further dialyzed against 0.9% saline solutions at 4°C overnight. The molecular weight cut-off of the dialysis tubing used was 1000 daltons. After dialysis, these fractions were filtered through a 0.2 μm filter.

The drug concentrations used in all our experiments were chosen below and above the blood concentrations in the body (Table 1). However, for CsA, higher drug concentrations are used, because CsA is highly lipophilic, is known to distribute in lipoproteins, and could potentially accumulate in atherosclerotic foam cells. CE transfer activity between lipoproteins by CETP was measured using the method that has been previously described. Briefly, 10 μg (total cholesterol) of radiolabeled LDL and unlabeled HDL were incubated with delipidated plasma as the source of CETP and with or without the drug in T150 buffer for 90 mins at 37°C. TP2, a monoclonal antibody specific against the CE binding site on CETP, was used as a positive inhibition control in this experiment. Enzymatic assay kit from Sigma Diagnostics (St. Louis, MO) was used to determine total and lipoprotein cholesterol concentrations. In order to measure the CE transfer from [³H] LDL to HDL, the donor particle was precipitated with the addition of PO₄³⁻ and Mn²⁺ prior to the scintillation counting for
the radioactivity. The CETP activity which is represented as the amount of CE transferred is calculated as the following:

\[
\text{% of CE transferred} = \frac{[^{3}H]\text{sample} -[^{3}H]\text{blank}}{[^{3}H]\text{- total}} \times 100\%
\]

where \([^{3}H]\text{- sample}\) is the radioactivity in the sample, \([^{3}H]\text{- blank}\) is the radioactivity in the absence of CETP and \([^{3}H]\text{- total}\) is the total radioactivity without precipitating out donor particles. The data were expressed as a percentage of control.

3.2.2 CETP fluorescence assay

In the CETP Fluorescence assay, CETP activity was measured with a commercial kit as directed (Roar Biomedical Inc., New York, NY). The CETP activity kit includes donor (without apoA-I) and acceptor particles. Incubation of donor and acceptor with a CETP source results in the CETP mediated transfer of fluorescent neutral lipid from donor to acceptor. This method measures CETP activity without interference from endogenous plasma lipoproteins. The specific activity of the donor does not vary with the HDL concentration of the plasma sample. The addition of excess acceptor normalizes any variability associated with endogenous LDL or VLDL. The CETP mediated transfer is determined by the increase in fluorescence intensity as the fluorescent neutral lipid is removed from donor to acceptor. The amount of fluorescent substrate transferred was expressed as pmoles of fluorescent substrate transferred and as percentage of control.
3.3 Hepatic lipase and lipoprotein lipase assay

In order to assess LPL and HL activity, we used a triolein emulsion containing radiolabeled \[^3\text{H}\] triolein as described previously. Post-heparin normal human plasma was used for the assay. Plasma sample was diluted ten times using NaCl (0.15M)-Tris HCl (0.2 M, pH 8.2) for LPL assay, or NaCl (1M)-Tris HCl (0.2M, pH 8.8) for HL assay respectively. In order to prepare for the substrates, a mixture of 100 µL triolein (75mg/ml), 100 µL phosphatidylcholine (10mg/ml) and 10 µL of \[^3\text{H}\] triolein (50µ Ci) was dried under a stream of nitrogen for both LPL and HL substrate. 2.1 mL of either LPL or HL buffer and 0.4 mL of 1% BSA dissolved in either LPL or HL buffer were added into each mixture. The mixture was placed in an ice bath and was sonicated for 8 minutes at a 50% pulse and lowest power setting. After sonication, 0.5 mL of 4% BSA dissolved in each buffer was added into each tube, except for the LPL substrate, in which we added 1 µg/mL of Apo C-II.

For a single assay for total lipase and HL, 80 µL of buffer, 20 µL of diluted samples, and 100 µL of substrate were mixed together and incubated for 30 minutes at 37°C. The blank or background was the mixture without any addition of samples. The reaction was terminated by addition of 3.25 ml of chloroform:methanol:heptane (1.25:1.41:1) mixture. For the extraction of the lipids, 1.05 ml of 0.1M boric acid and 0.1M potassium carbonate were added, and the tube was shaken on vortex for 15 seconds and then centrifuged at 1500 g for 10 minutes. 1 mL aliquot of the upper phase was added to 4 mL of scintillation cocktail and the radioactivity was counted in Liquid Scintillation Counter.
Total lipase activity was measured in the presence of apoC-II at a physiological salt concentration. LPL activity was represented as total lipase activity subtracted by salt resistant lipase activity (HL). To assay for HL activity, LPL was inhibited by raising the NaCl concentration of the incubation mixture to 1 mol/L and omitting the source of apoC-II.

3.4 Cholesterol efflux Study

3.4.1 Cell culture and treatment

THP-1 monocytes (American Type Culture Collection) (Rockville, MD, USA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% antibiotics-antimycotics (Invitrogen), 1 mM sodium pyruvate, and 1.5% sodium bicarbonate at 37°C, 95% air and 5% CO₂, and used within 20 passages for experiments. Phorbol 12-myristate 13-acetate (PMA; P8139; Sigma-Aldrich) was added at a final concentration of 100 nM for 72 h to differentiate THP-1 monocytes into macrophages.

3.4.2 Cell cytotoxicity study

To access the cytotoxicity profile of each drug concentrations on THP-1 macrophages, 10,000 THP-1 monocytes were seeded per well in a 96-wells plate and differentiated with PMA as previously described. Cells were washed several times
before each drug treatment, and incubated in a humidified chamber at 37°C, 5% CO₂ for 24 h. The cytotoxicity profile of both RAPA and TAC was assessed at 1, 5, 10 and 20 ng/ml. For a positive control group, cells were treated with 0.1% Triton X-100. Cytotoxicity in these cells was determined using commercially available enzymatic assay CytoTox96® Non-Radioactive Cytotoxicity Assay kit, which is often referred as LDH assay. LDH assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released into the medium upon cell lysis. Then, LDH presented in the culture medium participates in series of coupled reactions, leading to the formation of formazan-dye, which is measured by absorbance at 492nm. Drug’s toxicities in each group were compared to the Triton X-100 treated group. The amount of formazan is directly proportional to the amount of LDH in the culture, which is in turn directly proportional to the number of dead or damaged cells.

3.4.3 Cell viability study

In order to confirm the non-toxic range of our drug treatments, the CellTiter 96® Aqueous One Solution Assay (also called MTS Assay) was used to determine cell viability in response to the drug treatment. The CellTiter 96® Aqueous Assay uses the novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulfate (PMS). MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium. The measurement of the absorbance of the formazan was carried out using 96 well microplates at 492nm.
The assay measures dehydrogenase enzyme activity found in metabolically active cells. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viability of the cells. The cell viability in each group was compared to the control group without any drug treatment.

3.4.3 Cholesterol efflux assay

Human THP-1 macrophages were cultured and differentiated in RPMI1640 medium (serum free) as previously described. Cells were then treated with 50μg/mL oxidized LDL (Fitzgerald, Concord, MA, USA) and labeled with 1 μCi/ml [3H]-cholesterol, respectively, for overnight. Following the equilibration, cells were incubated in RPMI1640 medium containing 0.2% BSA with the various concentrations of RAPA or TAC (24 h) and in the presence of acceptor [apoA-I (10 μg/ml) or HDL (25 μg/ml)]. The radioactivity was determined by liquid scintillation counting. The background for the efflux was in the presence of medium alone. Specific HDL- or ApoA-I-induced [3H]-cholesterol efflux was calculated by subtracting the radioactivity in supernatants without apoA-I and HDL from the counts in supernatants containing apoA1. The data were normalized by determining total [3H]-cholesterol radioactivity (including in supernatant and cell) and were expressed as a percentage of control.

Furthermore, the amount of cholesterol presence in both of the medium and cell lysates was determined using Amplex Red Cholesterol Assay kit. This fluorometric kit was based on an enzyme-coupled reaction that detected both free cholesterol and
cholesteryl esters. Each sample was corrected from background fluorescence by subtracting the values derived from the no-cholesterol control group. Data was expressed as a percentage of control.

3.4.4 ABCA1 and ABCG1 protein expression study

One million THP-1 monocytes were seeded per well in a 6-well plate. Cells were differentiated as previously described (Section 3.4.1). After 72 hours, cells were washed with Phosphate-buffered saline (PBS; Invitrogen; NY, USA) before 24 hours treatment in the presence or absence of drugs, RAPA and TAC. The concentrations used were 1, 5, 10 and 20 ng/ml. Macrophages were treated with LXRα activators (1 μM T0901317; Calbiochem) as a positive control.

Cells were washed three times with PBS and incubated with RIPA lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.5% Na-deoxycholate, 1% NP-40) containing protease inhibitor (1:100 dilution) and 1 mM phenylmethanesulphonylfluoride (PMSF) for 30 mins. Whole cell protein content in each sample was determined using BCA™ assay and 100 μg of protein from each sample were separated by electrophoresis with a 7% SDS-polyacrylamide gel (SDS-PAGE) gel at 25mA for 1 h and then 35 mA for another 3h. The gel was then transferred to a nitrocellulose membrane using a wet-transfer system at 100V for 1 h. After 2 h of blocking with 5% non-fat milk (in 1x TBS-T), membrane was incubated overnight at 4°C with primary antibody (1:300 dilution) of rabbit polyclonal anti-
ABCA1 (Novus; NB400-105), rabbit polyclonal anti-ABCG1 (Santa Cruz; H-65) in 1:300 dilution with 5% non-fat milk, and goat anti-human actin (I-19) in 1:4000 dilution with 3% BSA as the internal control. Afterwards, membrane was washed four times with TBS-T before 90 minutes incubation in a 1:3000 dilution of anti rabbit HRPO conjugate and 1:4000 dilution of anti goat HRPO conjugate for ABCA1, ABCG1 and actin respectively. After several washings, membrane was visualized using ECL Western blotting detection reagent from Amersham Biosciences (Piscataway, NJ, USA), exposed to an X-Omat film from Kodak™ and quantified using UVP-Labworks Software.
3.5 Tacrolimus-renal transplant patients study

3.5.1 Patient recruitment

This study was done in collaboration with Dr. David Landsberg from St. Paul's Hospital Renal Program, and Ethics Approvals was obtained from UBC and Providence Health Care (Appendix A). The twenty eight patients involved in this study received living donor renal transplant between March 2008 and October 2008 in St. Paul's hospital.

Only the patients, who had been scheduled for living donor renal transplant surgery, were approached for consent. Subjects were approached only when they came into the clinic for pre-admission. After being informed of the purpose, design, and potential risks of the study, subjects gave written informed consent to participate (Appendix B). Among these 28 renal transplant recipients, we were able to recruit 18 patients, with one patient passed away during the follow-up. Duration of follow-up was 1 month after transplantation. All patients recruited receive tacrolimus regimen post-transplant. Tacrolimus has been a standard therapy for renal transplantation across the province of British Columbia, except for patients who have diabetes mellitus, in which they will be given alternative immunosuppressant, such as CsA.

Initial immunosuppressant therapy included methylprednisolone: 100-120 mg pre- and post-operatively, anti-IL-2 receptor, basiliximab, 20 mg, and anti-infectives, cefazolin 1g (pre- and post-operatively) and cotrimoxazole 400/80 mg post-operatively. Maintenance immunosuppressive regimen included methylprednisolone with a gradual
taper and mycophenolate mofetil (MMF). All patients received tacrolimus (TAC) at a 0.0375 mg/kg daily dose.

The study population was divided into two groups, depending on whether or not the patient received any lipid-lowering drug treatment. Tacrolimus dose was adapted according to the trough blood levels: 10 to 15 ng/ml during the first 4 weeks. Before transplantation, the following variables were recorded: age and gender of the recipient, body height, body weight, medications, biochemical parameters including serum cholesterol, HDL-C, LDL-C and triglycerides level.

### 3.5.2 Patient inclusion and exclusion criteria

**Inclusion criteria**

Patients, who will receive renal transplantation and either cyclosporine A or tacrolimus treatment after the transplantation.

**Exclusion criteria**

1. Age less than 19 years old.
2. Pregnancy or lactation.
3. Patients that previously received either cyclosporine A or tacrolimus.
4. Patients who are receiving other immunosuppressant drugs.
5. Liver function abnormalities above the following limits: bilirubin 3mg/dL. AST or ALT 5 times greater than upper limits of normal.
6. Baseline serum creatinine concentration is more than 120 μmoles/L.
7. Patients receiving any potential agents that cause kidney toxicity within the preceding 3 days (i.e. Antibiotics Gentamycin, tobramycin, Antifungals Amphotericin, Ketoconazole, Chemotherapy Mephalan (Alkeran) Eroposide (VePesid) and aminoglycosides).

8. Patients with a life expectancy of less than 1 month.

3.5.3 Patient lipid analysis

After a patient was recruited into the study, the whole blood sample and pre-heparin plasma sample from each patient would be taken on pre-transplant, post-transplant surgery, post-tacrolimus administration 1 week, post-TAC 2 week, post-TAC 3 week, and post-TAC 4 week. Collected samples were stored in -80°C freezer until further analysis. However, patients were not given any intravenous or sub-cutaneous heparin during those six time points, except for the blood samples that were collected couple of hours after the transplantation surgery, at which heparin was given to patients.

Lipid parameters including total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides level were measured from each sample using enzymatic assay from Wako Diagnostics kit (Wako, Richmond, VA, USA). CETP activity in the sample was determined using CETP Fluorescence kit as previously described. Hepatic lipase and lipoprotein lipase activity were measured using radioactive triolein assay, as mentioned before.
3.5.4 Measurement of CETP concentration

CETP mass or concentration was determined using ALPCO CETP Elisa kit (ALPCO, Salem, NH, USA). The CETP Elisa kit is a quantitative assay for CETP in human serum or plasma. These wells were coated with anti-CETP MoAb. CETP in the sample is captured by the antibody in the first incubation. After the first incubation and washing to remove all of the unbound material, HRP-labeled anti-CETP MoAb was added. After the second incubation and subsequent washing, substrate solution was added. Next, stop reagent was added. The intensity of the color that developed was read by a microplate reader. The absorbance was proportional to the concentration of CETP in the sample.

3.5.4 Measurement of LPL concentration

Patient serum LPL mass levels were measured by the sensitive sandwich enzyme-linked immunosorbent assay (ELISA). LPL ELISA, as originally described by Kobayashi et al., is commercially available as a test-kit from ALPCO Diagnostics (ALPCO, Salem, NH, USA). The kit contains polyclonal and monoclonal antibodies against bovine milk LPL.

These wells were coated with anti-LPL MoAb. LPL in the sample is captured by the antibody in the first incubation. After the first incubation and washing to remove all of the unbound material, anti-LPL PoAb was added. After the second incubation and subsequent washing, enzyme-labeled PoAb was added. After the third incubation and
subsequent washes, the antibody/LPL/enzyme complex was incubated with a substrate solution and terminated with stop reagent. The intensity of the color that developed was read by a microplate reader. The absorbance was proportional to the concentration of LPL in the sample.

3.6 Statistical analysis

Differences in the mean values of in vitro CETP, HL and LPL activities in the controls and drug treated plasmas were compared using the One Way ANOVA test. The one way repeated measures ANOVA was used to analyze the in vivo clinical data. Statistically significant differences between treatment groups versus control group were further assessed by the Tukey post hoc tests. A difference in the mean values was considered to be significant if p < 0.05. All statistical analysis was done using SigmaStat version 3.5 from Systat Inc. Data were presented as mean ± SD.
CHAPTER 4

RESULTS
RESULTS

4.1 The effect of immunosuppressive drugs on in vitro CETP activity

As shown in Figure 7, the in vitro human normolipidemic plasma CETP activity was significantly increased in CsA or RAPA-treated (90 minutes) samples compared to the control condition. However, at maximum blood concentrations, TAC (FK-506) and MMF did not result in a significant difference in plasma CETP activity relative to untreated plasma. When the samples were pre-treated with TP2, a monoclonal antibody against CE binding site of CETP, for 90 minutes prior to the incubation with the drugs, all samples showed a significant decrease in CETP activity (Figure 7). Figure 8 and Figure 9 show that CsA and RAPA significantly induce in vitro CETP activity in a concentration dependent manner, respectively.
Figure 7. Cyclosporine A (CsA) and rapamycin (RAPA) significantly induced CETP activity in human normolipidemic plasma, at average and maximum blood concentrations, while tacrolimus (FK-506-) and mycophenolate mofetil (MMF-) treated plasmas were not significantly different, compared to untreated plasma. The TP2, monoclonal antibody against the CE binding site, significantly inhibited CETP-mediated transfer of cholesteryl esters (CE) in the untreated plasma and the drugs treated plasma, respectively. (n = 3, *p < 0.001). Values expressed as mean ± SD.
Figure 8. Cyclosporine A (CsA) significantly increased CETP activity within human normolipidemic plasma, after the incubation for 90 minutes, in a concentration dependant manner, relative to the control (n=6, *p < 0.001 versus control). The inhibition by TP2, a monoclonal antibody against the CE binding site, showed that the transfer of CE was due to a facilitated transfer by CETP, not to a spontaneous transfer among lipoprotein classes. Values expressed as mean ± SD.
Figure 9. Rapamycin (RAPA) significantly induced CETP activity in human normolipidemic plasma, in a concentration dependent manner, compared to untreated plasma (n=3, *p < 0.001 versus control). The co-incubation with TP2, a monoclonal antibody against the CE binding site, significantly inhibited the CE transfer (data not shown). Values expressed as mean ± SD.

Similarly, we assessed the effects of CsA, RAPA, TAC (FK-506) and MMF on the in vitro activity of rCETP using the CETP fluorescence assay. As can be observed in Table 2, the effects of these drugs on the rCETP activity corresponded to the results shown for human normolipidemic plasma. Similarly, co-incubation of rCETP with TP2 resulted in a significant reduction of the CE transfer activity by rCETP.
Table 2. Corresponding to the previous result shown for human normolipidemic plasma, CsA and RAPA also increased CE transfer activity of recombinant CETP (rCETP). No significant difference was observed in TAC- and MMF-treated samples, compared to the control. TP2, a monoclonal antibody against the CE binding site, was also shown to inhibit the CE transfer of rCETP. (n = 3, *p < 0.001). Values expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Activity (%) of Control</th>
<th>% of Control ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>CE transfer by rCETP</td>
<td>100±1</td>
</tr>
</tbody>
</table>

4.2 The effect of immunosuppressive drugs on *in vitro* hepatic lipase and lipoprotein lipase activity.

Using post-heparinized normal human plasma, Figure 10 shows the effects of CsA, RAPA, TAC (FK-506) and MMF on the *in vitro* activity of LPL. LPL activity was decreased significantly when co-incubated with CsA, RAPA, TAC and MMF at each chosen concentration (Figure 10). However, no differences in *in vitro* HL activity were observed for the same drugs incubations compared to untreated plasma (Table 3).
Figure 10. At average and maximum blood concentrations, cyclosporine A (CsA), rapamycin (RAPA), tacrolimus (FK-506) and mycophenolate mofetil (MMF) significantly suppressed lipoprotein lipase activity in post-heparin normal human plasma, compared to untreated plasma (n = 4, \( p < 0.001 \) versus control). Values expressed as mean ± SD.

Table 3. At chosen concentrations, CsA, RAPA, TAC and MMF did not significantly change the hepatic lipase activity in post-heparin normal human plasma, relative to control (n = 4, \( p < 0.001 \) versus control). Values expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Activity ( % of Control)</th>
<th>% of Control ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>Hepatic Lipase Activity</strong></td>
<td>100±12</td>
</tr>
</tbody>
</table>
Furthermore, as shown in Figure 11, CsA significantly reduced the *in vitro* LPL activity in post-heparinized normal human plasma at concentrations of 1, 5, 10 and 20 μg/mL, as compared to control. Interestingly, the other calcineurin inhibitor, TAC, also showed a significant suppression of LPL activity *in vitro*, but in a dose dependent manner (Figure 13). On the other hand, RAPA only showed significant reduction of LPL activity at the higher concentration, 20 ng/mL, as compared to the control (Figure 12). Likewise, MMF also suppressed LPL activity at the higher concentrations, 10 and 20 μg/mL (Figure 14).
Figure 11. Cyclosporine A (CsA) significantly reduced the in vitro lipoprotein lipase activity in post-heparinized normal human plasma, compared to untreated plasma. N = 3, *p < 0.05 versus control. Values represent as mean ± S.D.
Figure 12. Rapamycin (RAPA) at 20ng/mL significantly reduced the *in vitro* lipoprotein lipase activity in post-heparinized normal human plasma, compared to untreated plasma. N = 3, *p < 0.05 versus control. Values represent as mean ± S.D.
Figure 13. Tacrolimus (TAC) significantly reduced the in vitro lipoprotein lipase activity in post-heparinized normal human plasma in a dose dependent manner, compared to untreated plasma. N = 3, *p < 0.05 versus control. Values represent as mean ± S.D.
Figure 14. Mycophenolate mofetil (MMF) at 10 and 20 μg/mL significantly reduced the \textit{in vitro} lipoprotein lipase activity in post-heparinized normal human plasma, compared to untreated plasma. N = 3, *p < 0.05 versus control. Values represent as mean ± S.D.
4.3  The effects of RAPA and TAC on cholesterol efflux in human THP-1 macrophages.

4.3.1 The effects of TAC and RAPA on THP-1 cell cytotoxicity.

The cytotoxicity profile of THP-1 macrophages in response to each concentrations of the drug treatment was obtained using an enzymatic assay, called lactate dehydrogenase (LDH) assay. This assay is a colorimetric alternative enzymatic assay to $^{51}$Cr release cytotoxicity assay. It quantitatively measures lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis, thus it is considered as an indirect measure of membrane integrity. Triton X-100 was used as a positive control, which lysed the cell completely. As shown in Figure 11, there was no toxicity associated with both tacrolimus and rapamycin treatment at the chosen concentrations for this study.
Figure 15. The 24-hours toxicity profile of THP-1 macrophages cells treated with different concentrations of tacrolimus (A) or rapamycin (B) using LDH assay. The positive control group is the group treated with 0.1% of Triton X-100. (n = 3, *p<0.001 vs. control). Six replicates for each treatment group per experiment. The control group is the one treated with medium alone. Values were expressed as mean ± SD.
4.3.2 The effects of TAC and RAPA on THP-1 cell viability.

The viability profile of THP-1 macrophages in response to each concentrations of the drug treatment was obtained using an enzymatic assay, MTS assay. This assay is a colorimetric alternative enzymatic assay that measures dehydrogenase enzyme activity found in metabolically active cells, which is a good indication of cell viability. Triton X-100 was used as a negative control, which lysed the cell completely. As shown in Figure 12, there was no toxicity associated with both tacrolimus and rapamycin treatment at the chosen concentrations for this study. The cytotoxic and viability profiles generated by LDH and MTS for each of these drug treatments were in agreement with each other.
Figure 16. The 24-hours viability profile of THP-1 macrophages cells treated with different concentrations of tacrolimus (A) or rapamycin (B) using MTS assay. The negative control group is the group treated with 0.1% of Triton X-100. (n = 4, *p<0.001 vs. control). Six replicates for each treatment group per experiment. The control group is the one treated with medium alone. Values were expressed as mean ± SD.
4.3.3 The effects of TAC and RAPA on cholesterol efflux from human THP-1 cells.

Using THP-1 macrophage cell models, Figure 13B and Figure 14A show the effects of rapamycin and tacrolimus on cholesterol efflux to the acceptor ApoA-I (blue bar) and HDL (red bar) from these cells, respectively. At physiological concentrations, 10 and 20 ng/ml, RAPA seems to inhibit cholesterol efflux to ApoA-I, but not to HDL. On the other hand, tacrolimus does not affect the cholesterol efflux to ApoA-I and HDL, at the same concentrations.

To ensure the accuracy of the data obtained from the radioactive cholesterol efflux study, we performed a cholesterol mass study in order to determine the amount of cholesterol in both the medium and cell lysate in response to the cellular cholesterol efflux. Figure 13A demonstrates that there was less cholesterol content in the medium, compared to the cell lysate, when cells were treated with 10 and 20 ng/ml of RAPA, in the presence of acceptor Apo A-I. The decrease of amount of cholesterol being effluxed to exogenous ApoA-I in these cells was consistent with the results that we observed in Figure 13B. However, with tacrolimus treatment at 20 ng/ml, there was a significant reduction of cholesterol being effluxed to acceptor ApoA-I (Figure 14B), but we did not see the similar effect in the radioactive cholesterol efflux study (Figure 14A).
Figure 17. Effects of different concentrations of rapamycin on the cholesterol efflux to apoA-I- (blue bar) and HDL- (red bar) from human THP-1 macrophages. THP-1 monocytes (4 x 10⁵ cells) were differentiated into macrophages by 72 h of stimulation with phorbol 12-myristate 13-acetate (PMA), and then treated with oxidized LDL and subsequently with RAPA at various concentrations for 24 h. Cholesterol efflux ratio was calculated as; A. ratio of amount of radioactivity in medium to total, B. as ratio of amount of cholesterol mass in medium to total, using Amplex Red Cholesterol kit (Invitrogen). Values represent mean ± SD. N = 5, *p<0.05 vs. control.
Figure 18. Effects of different concentrations of tacrolimus on the cholesterol efflux to apoA1 (blue bar) and HDL (red bar) from human THP-1 macrophages. THP-1 monocytes (4 x 10⁵ cells) were differentiated into macrophages by 72 h of stimulation with phorbol 12-myristate 13-acetate (PMA), and then treated with oxidized LDL and subsequently with RAPA at various concentrations for 24 h. Cholesterol efflux ratio was calculated as: A. ratio of amount of radioactivity in medium to total, B. as ratio of amount of cholesterol in medium to total, using Amplex Red Cholesterol kit (Invitrogen). Values represent mean ± SD. N = 5, *p<0.05 vs. control.
4.3.4 The effects of rapamycin and tacrolimus on ABCA1/ABCG1 protein expression in THP-1 macrophages

Since previous experiments showed a decrease in cholesterol efflux when cells were treated with rapamycin, we also investigated the effects of both rapamycin and tacrolimus on the ABCA1 and ABCG1 protein expression in these cells. Cholesterol efflux to ApoA-I in THP-1 cells is usually mediated by ABCA1, while efflux to HDL is mediated by ABCG1. In this experiment, ABCA1 and ABCG1 protein expression was normalized using actin as the house-keeping protein. LXR agonist (T0901317) was used to induce the expression of ABCA1 and ABCG1 protein expression.

As shown in Figure 15A and B, at the concentration of 10 and 20 ng/ml, there was a significant reduction in ABCA1 and ABCG1 (at 20 ng/ml only) protein expression in THP-1 macrophages treated with tacrolimus. Moreover, Figure 16A and B demonstrates that rapamycin, at the concentration of 20 ng/ml, reduced the ABCA1 and ABCG1 protein expression in these cells.
Figure 19. ABCA1 and ABCG1 expression in THP-1 macrophages after LXRα activation (10 μM T0901317) and tacrolimus treatment, as demonstrated using Western Blot. The insert is a representative image of 4 independent blots. Each bar represents the mean ± SD (n = 4; *p<0.05 vs. control) of protein expression normalized by actin expression.
Figure 20. ABCA1 and ABCG1 expression in THP-1 macrophages after LXRα activation (10 μM T0901317) and rapamycin treatment, as demonstrated using Western Blot. The insert is a representative image of 4 independent blots. Each bar represents the mean ± SD (n = 4; * p<0.05 vs. control) of protein expression normalized by actin expression.
4.4 The effect of tacrolimus on renal transplant patient lipid metabolism

Our previous *in vitro* work has shown us that tacrolimus did not affect the CETP activity and hepatic lipase in human normolipidemic plasma, but suppressed its lipoprotein lipase activity. The clinical study that we carried out will help us to investigate whether the *in vitro* effects that we have observed are clinically relevant. Table 4 and Table 5 show the demographic of living donor renal transplant patients that we have recruited since February 2008 until October 2008. These patients are divided into two groups based on whether or not they receive any lipid lowering drug treatment.

**Table 4. Baseline data for the living donor renal transplant patients at the time of pre-admission or pre-transplant surgery.** The following patients did not receive any lipid lowering drug treatments.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>DMI (kg/m²)</th>
<th>TG (mmol/l)</th>
<th>TC (mmol/l)</th>
<th>HDL (mmol/l)</th>
<th>LDL (mmol/l)</th>
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</thead>
<tbody>
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<td>56</td>
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<tr>
<td>M.D.</td>
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<td>48</td>
<td>94.4</td>
<td>29.9</td>
<td>0.97</td>
<td>4.36</td>
<td>1.65</td>
<td>2.26</td>
</tr>
<tr>
<td>K.W.</td>
<td>M</td>
<td>56</td>
<td>100</td>
<td>36.5</td>
<td>0.8</td>
<td>5.4</td>
<td>2.1</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Table 5. Baseline data for the living donor renal transplant patients at the time of pre-admission or pre-transplant surgery. The following patients receive lipid lowering drug treatments.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>TG (mmol/l)</th>
<th>TC (mmol/l)</th>
<th>HDL (mmol/l)</th>
<th>LDL (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.W.</td>
<td>F</td>
<td>59</td>
<td>51.4</td>
<td>20.1</td>
<td>0.8</td>
<td>6.5</td>
<td>2.56</td>
<td>3.58</td>
</tr>
<tr>
<td>B.S.</td>
<td>F</td>
<td>68</td>
<td>90</td>
<td>32.3</td>
<td>1.59</td>
<td>3.39</td>
<td>0.92</td>
<td>1.74</td>
</tr>
<tr>
<td>W.B.</td>
<td>M</td>
<td>60</td>
<td>70.2</td>
<td>23.5</td>
<td>0.9</td>
<td>3.0</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>J.R.</td>
<td>M</td>
<td>65</td>
<td>82</td>
<td>25.3</td>
<td>1.0</td>
<td>4.78</td>
<td>1.86</td>
<td>2.47</td>
</tr>
<tr>
<td>N.B.</td>
<td>M</td>
<td>28</td>
<td>91.1</td>
<td>27.2</td>
<td>1.3</td>
<td>4.21</td>
<td>0.99</td>
<td>2.6</td>
</tr>
<tr>
<td>M.K.</td>
<td>M</td>
<td>51</td>
<td>70</td>
<td>23.4</td>
<td>1.82</td>
<td>5.81</td>
<td>1.48</td>
<td>3.49</td>
</tr>
<tr>
<td>K.I.</td>
<td>M</td>
<td>55</td>
<td>73</td>
<td>26.0</td>
<td>1.33</td>
<td>2.64</td>
<td>0.91</td>
<td>1.12</td>
</tr>
<tr>
<td>A.R.</td>
<td>M</td>
<td>40</td>
<td>83.5</td>
<td>26.4</td>
<td>1.46</td>
<td>4.42</td>
<td>1.07</td>
<td>2.68</td>
</tr>
<tr>
<td>M.R.</td>
<td>M</td>
<td>35</td>
<td>44.5</td>
<td>17.4</td>
<td>1.8</td>
<td>4.2</td>
<td>1.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

In both groups, TAC trough levels were in the range between 5 to 20 ng/ml.

Table 6 shows that in the group of patients who were not receiving any lipid lowering drug treatment, TAC trough level in these patients were quite dispersed. On the other hand, as shown in Table 7, the patients who were on lipid lowering drug treatment had similar TAC concentration in their blood.
Table 6. Tacrolimus (TAC) trough levels in renal transplant patients, who were not receiving any lipid lowering drug treatment. TAC was given after the transplantation surgery and each patient was followed up for 4 weeks after TAC administration.

<table>
<thead>
<tr>
<th>Patients without statin</th>
<th>Patient Tacrolimus Trough Levels (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-Tac 1 day</td>
</tr>
<tr>
<td>J.H.</td>
<td>5</td>
</tr>
<tr>
<td>M.D.</td>
<td>5</td>
</tr>
<tr>
<td>S.V.</td>
<td>12</td>
</tr>
<tr>
<td>C.S.</td>
<td>16</td>
</tr>
<tr>
<td>R.H.</td>
<td>15</td>
</tr>
<tr>
<td>W.M.</td>
<td>5</td>
</tr>
<tr>
<td>T.M.</td>
<td>7</td>
</tr>
<tr>
<td>D.P.</td>
<td>4</td>
</tr>
<tr>
<td>K.W.</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 7. Tacrolimus (TAC) trough levels in renal transplant patients, who were receiving lipid lowering drug treatment. TAC was given after the transplantation surgery and each patient was followed up for 4 weeks after TAC administration.

<table>
<thead>
<tr>
<th>Patients with statin</th>
<th>Patient Tacrolimus Trough Levels (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-Tac 1 day</td>
</tr>
<tr>
<td>L.W.</td>
<td>5</td>
</tr>
<tr>
<td>B.S.</td>
<td>9</td>
</tr>
<tr>
<td>W.B</td>
<td>6</td>
</tr>
<tr>
<td>J.R</td>
<td>6</td>
</tr>
<tr>
<td>N.B.</td>
<td>6</td>
</tr>
<tr>
<td>M.K.</td>
<td>-</td>
</tr>
<tr>
<td>K.I.</td>
<td>8</td>
</tr>
<tr>
<td>A.R.</td>
<td>9</td>
</tr>
<tr>
<td>M.R.</td>
<td>5</td>
</tr>
</tbody>
</table>
As expected, in the group of patients who received statin or any lipid lowering drugs, there was no significant difference in their total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, compared to the pre-transplant (Table 12). No significant difference was observed in terms of their pre-heparin HL activity, CETP mass and CETP activity. Since post-heparinized blood samples are not usually collected from renal transplant patients at St. Paul’s hospital, we are not able to get these blood samples for this study. However, there was a significant decrease in their pre-heparin lipoprotein lipase activity after TAC administration, as compared to the pre-transplant baseline level (Table 12 and Table 10). There were seven out of nine patients who were receiving lipid lowering drug treatment showed a significant decrease in their pre-heparin LPL activity, after post-TAC administration 4 weeks as compared to the pre-transplant.

Several studies have shown a good correlation between pre-heparin LPL mass and triglyceride levels in patients (as we will discuss later), therefore measuring pre-heparin LPL mass in these samples will be important in order to support the results that we already observed with pre-heparin LPL activity. Likewise, we could see a slight increase in these patients triglyceride level, although the increase was not significant (Table 12 and Table 8). Table 8 and Table 10 show each individual patient triglyceride level and pre-heparin lipoprotein lipase activity at pre-transplant and post-TAC administrations, respectively. In consistency with the decrease in LPL activity and an increase in triglyceride levels, there was also a decrease in pre-heparin LPL mass in this group of patients at post-tacrolimus administration (Table 12).
Interestingly, similar results were also seen in the group of patients who did not receive statin or lipid lowering drug treatment (Table 13). There was no difference in these patients lipid profile, CETP mass, CETP activity and their pre-heparin hepatic lipase activity. There was also a significant reduction in the pre-heparin lipoprotein lipase activity post-TAC administration compared to the pre-transplant (Table 13 and Table 11). There were five out of nine patients in this group had a significant reduction in their pre-heparin LPL activity four weeks after TAC administration, as compared to the pre-transplant. In addition, there was also a trend of decrease in preheparin LPL mass post-tacrolimus administration in these patients, although the degree of the decrease was less than that of the patients on statin (Table 13 and Table 12). On the contrary, TG level in these patients seemed to be no change in comparison to the group with statin or lipid lowering drug treatment (Table 13 and Table 9). Table 9 and Table 11 show each individual patient triglyceride level and pre-heparin lipoprotein lipase activity at pre-transplant and post-TAC administrations, respectively.
Table 8. Effect of Tacrolimus on plasma triglyceride (TG) concentrations in renal transplant patients, receiving lipid lowering drug treatments. Each patient was followed up for 4 weeks after Tacrolimus administration. Each samples were compared to the baseline level or pre-transplant samples and there was no significant difference between the groups. [Pre-Tx: pre-transplant, Post-Tac 1 wk, 2 wk, 3 wk, 4 wk: post-Tacrolimus administration 1 week, 2 weeks, 3 weeks, 4 weeks].

<table>
<thead>
<tr>
<th>Patients with statin</th>
<th>Patient Triglyceride levels at different time points (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Tx</td>
</tr>
<tr>
<td>L.W.</td>
<td>0.90</td>
</tr>
<tr>
<td>B.S.</td>
<td>3.05</td>
</tr>
<tr>
<td>W.B</td>
<td>1.27</td>
</tr>
<tr>
<td>J.R</td>
<td>0.44</td>
</tr>
<tr>
<td>N.B.</td>
<td>0.70</td>
</tr>
<tr>
<td>M.K.</td>
<td>2.59</td>
</tr>
<tr>
<td>K.I.</td>
<td>0.19</td>
</tr>
<tr>
<td>A.R.</td>
<td>1.36</td>
</tr>
<tr>
<td>M.R.</td>
<td>4.20</td>
</tr>
</tbody>
</table>
Table 9. Effect of Tacrolimus on plasma triglyceride (TG) concentrations in renal transplant patients, not receiving any lipid lowering drug treatments. Each patient was followed up for 4 weeks after Tacrolimus administration. Each samples were compared to the baseline level or pre-transplant samples and there was no significant difference between the groups. [Pre-Tx: pre-transplant, Post-Tac 1 wk, 2 wk, 3 wk, 4 wk: post-Tacrolimus administration 1 week, 2 weeks, 3 weeks, 4 weeks].

<table>
<thead>
<tr>
<th>Patients without statin</th>
<th>Patient Triglyceride levels at different time points (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Tx</td>
</tr>
<tr>
<td>J.H.</td>
<td>1.35</td>
</tr>
<tr>
<td>M.D.</td>
<td>0.13</td>
</tr>
<tr>
<td>S.V.</td>
<td>0.71</td>
</tr>
<tr>
<td>C.S.</td>
<td>1.17</td>
</tr>
<tr>
<td>R.H.</td>
<td>0.74</td>
</tr>
<tr>
<td>W.M.</td>
<td>0.53</td>
</tr>
<tr>
<td>T.M.</td>
<td>1.46</td>
</tr>
<tr>
<td>D.P.</td>
<td>0.55</td>
</tr>
<tr>
<td>K.W.</td>
<td>1.43</td>
</tr>
</tbody>
</table>
Table 10. Effect of Tacrolimus (TAC) on pre-heparin serum lipoprotein lipase activity in renal transplant patients, receiving lipid lowering drug treatment. Each patient was followed up for 4 weeks after Tacrolimus administration. Each samples were compared to the baseline level or pre-transplant samples. [Pre-Tx: pre-transplant, Post-Tac 1 wk, 2 wk, 3 wk, 4 wk: post-Tacrolimus administration 1 week, 2 weeks, 3 weeks, 4 weeks].

<table>
<thead>
<tr>
<th>Patients with statin</th>
<th>Patient lipoprotein lipase activity at different time points (nmol/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Tx</td>
</tr>
<tr>
<td></td>
<td>1 wk</td>
</tr>
<tr>
<td>L.W.</td>
<td>38.10</td>
</tr>
<tr>
<td>B.S.</td>
<td>14.32</td>
</tr>
<tr>
<td>W.B</td>
<td>47.60</td>
</tr>
<tr>
<td>J.R</td>
<td>26.46</td>
</tr>
<tr>
<td>N.B.</td>
<td>25.70</td>
</tr>
<tr>
<td>M.K.</td>
<td>18.04</td>
</tr>
<tr>
<td>A.R.</td>
<td>10.30</td>
</tr>
<tr>
<td>M.R.</td>
<td>19.60</td>
</tr>
</tbody>
</table>
Table 11. Effect of Tacrolimus (TAC) on pre-heparin serum lipoprotein lipase activity in renal transplant patients, not receiving lipid lowering drug treatment. Each patient was followed up for 4 weeks after Tacrolimus administration. Each samples were compared to the baseline level or pre-transplant samples. [Pre-Tx: pre-transplant, Post-Tac 1 wk, 2 wk, 3 wk, 4 wk: post-Tacrolimus administration 1 week, 2 weeks, 3 weeks, 4 weeks].

<table>
<thead>
<tr>
<th>Patients without statin</th>
<th>Patient lipoprotein lipase activity at different time points (nmol/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Tx</td>
</tr>
<tr>
<td>J.H.</td>
<td>52.80</td>
</tr>
<tr>
<td>M.D.</td>
<td>74.00</td>
</tr>
<tr>
<td>S.V.</td>
<td>12.39</td>
</tr>
<tr>
<td>C.S.</td>
<td>52.00</td>
</tr>
<tr>
<td>R.H.</td>
<td>19.13</td>
</tr>
<tr>
<td>W.M.</td>
<td>24.70</td>
</tr>
<tr>
<td>T.M.</td>
<td>15.21</td>
</tr>
<tr>
<td>D.P.</td>
<td>21.50</td>
</tr>
<tr>
<td>K.W.</td>
<td>15.38</td>
</tr>
</tbody>
</table>
Table 12. Lipid profile of renal transplant patients administered tacrolimus with lipid lowering drug treatment. There was no difference in patients lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides), as well as, their CETP mass, CETP activity and hepatic lipase activity, as compared to pre-transplant group. However, there were significant differences in pre-heparin LPL activity and mass between the post-TAC groups and the pre-transplant group, n = 9, *p<0.050. Values were expressed as mean ± SD. [Pre-Tx: pre-transplant, Post-Tac 1 wk, 2 wk, 3 wk, 4 wk: post-tacrolimus administration 1 week, 2 weeks, 3 weeks, 4 weeks].

<table>
<thead>
<tr>
<th>Total Cholesterol (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>LPL act. (nmol/min/ml)</th>
<th>LPL mass (ng/ml)</th>
<th>HL act. (nmol/min/ml)</th>
<th>CETP mass (ug/ml)</th>
<th>CETP act. (pmoles of CE trans./3 hr)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.87±0.47</td>
<td>1.19±0.35</td>
<td>2.80±0.94</td>
<td>1.63±1.35</td>
<td>29.66±18.18</td>
<td>106.35±36.49</td>
<td>72.77±57.56</td>
<td>3.50±2.31</td>
<td>28.52±17.82</td>
<td>Pre-Tx</td>
</tr>
<tr>
<td>1.32±0.57</td>
<td>0.86±0.22</td>
<td>2.57±0.94</td>
<td>2.72±1.63</td>
<td>11.61±7.8*</td>
<td>50.30±9.20*</td>
<td>76.02±55.98</td>
<td>2.91±1.71</td>
<td>31.43±19.31</td>
<td>Post-Tac 1 wk</td>
</tr>
<tr>
<td>1.22±0.48</td>
<td>0.94±0.32</td>
<td>2.84±1.12</td>
<td>2.69±1.56</td>
<td>10.55±6.60*</td>
<td>57.86±1.95*</td>
<td>80.36±57.95</td>
<td>3.28±1.97</td>
<td>29.27±20.73</td>
<td>Post-Tac 2 wk</td>
</tr>
<tr>
<td>1.29±0.72</td>
<td>1.07±0.35</td>
<td>2.67±1.05</td>
<td>2.45±1.55</td>
<td>10.14±6.71*</td>
<td>56.83±9.61*</td>
<td>70.00±59.87</td>
<td>2.90±1.52</td>
<td>35.74±23.58</td>
<td>Post-Tac 3 wk</td>
</tr>
<tr>
<td>1.23±0.69</td>
<td>1.08±0.32</td>
<td>2.73±1.00</td>
<td>2.15±0.76</td>
<td>11.32±9.23*</td>
<td>59.42±2.78*</td>
<td>71.82±53.33</td>
<td>3.19±1.99</td>
<td>36.99±18.79</td>
<td>Post-Tac 4 wk</td>
</tr>
</tbody>
</table>
Table 13. Lipid profile of renal transplant patients administered tacrolimus, without any lipid lowering drug treatment.
There was also no difference in this patients group lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides), as well as, their CETP mass, CETP activity and hepatic lipase activity, as compared to the pre-transplant group. However, there were significant differences in pre-heparin LPL activity and mass between the post-TAC groups and the pre-transplant group, n = 9, *p<0.050. Values were expressed as mean ± SD. [Pre-Tx: pre-transplant, Post-Tac 1 wk, 2 wk, 3 wk, 4 wk: post-tacrolimus administration 1 week, 2 weeks, 3 weeks, 4 weeks].

<table>
<thead>
<tr>
<th>Total Cholesterol (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>LPL act. (nmol/min/ml)</th>
<th>LPL mass (ng/ml)</th>
<th>HL act. (nmol/min/ml)</th>
<th>CETP mass (ug/ml)</th>
<th>CETP act. (pmoles of CE trans./3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.53±0.73</td>
<td>1.02±0.51</td>
<td>2.14±0.53</td>
<td>0.90±0.47</td>
<td>31.90±21.99</td>
<td>108.80±40.89</td>
<td>38.01±21.84</td>
<td>3.76±2.01</td>
<td>45.74±34.99</td>
</tr>
<tr>
<td>2.46±1.01</td>
<td>0.76±0.25</td>
<td>2.13±0.44</td>
<td>1.21±0.55</td>
<td>12.94±2.22*</td>
<td>47.61±26.03*</td>
<td>48.96±25.4</td>
<td>2.25±1.07</td>
<td>37.34±19.62</td>
</tr>
<tr>
<td>2.54±1.22</td>
<td>0.73±0.43</td>
<td>2.30±0.58</td>
<td>1.31±0.51</td>
<td>12.43±6.35*</td>
<td>59.97±45.87</td>
<td>54.27±42.28</td>
<td>2.22±1.25</td>
<td>40.18±20.35</td>
</tr>
<tr>
<td>2.69±1.47</td>
<td>0.84±0.39</td>
<td>2.39±0.89</td>
<td>1.38±0.84</td>
<td>12.91±6.82*</td>
<td>65.11±39.06</td>
<td>35.83±27.45</td>
<td>2.76±0.81</td>
<td>44.40±25.93</td>
</tr>
<tr>
<td>1.94±0.73</td>
<td>0.80±0.34</td>
<td>1.96±1.10</td>
<td>1.08±0.59</td>
<td>12.20±5.46*</td>
<td>64.07±21.45</td>
<td>33.45±25.14</td>
<td>2.76±0.82</td>
<td>42.06±24.25</td>
</tr>
</tbody>
</table>
CHAPTER 5

DISCUSSION
5 DISCUSSION

5.1 CsA and RAPA induced in vitro CETP activity.

The pathogenesis of post-transplant hyperlipidemia is poorly understood, but the most important factors leading to hyperlipidemia are inappropriate diet in combination with reduced physical activity and, most relevant, post-operative immunosuppressive therapy. Previous studies have suggested that immunosuppressive-administered transplantation patients, who exhibit plasma dyslipidemia including hypercholesterolemia and hypertriglyceridemia, might have some changes in their lipid metabolism. However, available data on the mechanisms of immunosuppressive-induced dyslipidemia are limited. The present study was undertaken to investigate the effects of CsA, RAPA, TAC and MMF on the in vitro activity of key lipid regulatory enzymes including CETP, HL, LPL and cholesterol efflux. The type of calcineurin inhibitor plays an important role in the hyperlipidemia; lipid profiles in renal transplant recipients treated with TAC were usually better than those in patients treated with CsA. We are also investigating the in vivo effect of Tacrolimus on these key enzymes in renal transplant patients.

We have previously demonstrated that the transfer of CsA between HDL and LDL is partially facilitated through CETP, CE and TG transfer activities. Since CETP facilitates the exchange of CE from CE-rich lipoproteins for TG from TG-rich lipoproteins, these data suggested that the presence of CsA may affect the transfer of neutral lipids between lipoproteins. However, the interaction or the effect of other immunosuppressive drugs on CETP has not been addressed previously.
In our study, we have demonstrated that CsA and RAPA induced CE transfer activity by CETP in a concentration dependant manner. By contrast, we did not find any significant differences in amounts of CE transferred between untreated and TAC (FK-506)- or MMF-treated plasma, suggesting that TAC and MMF do not affect the CE transfer activity of CETP. This was further confirmed by the observation of similar results using rCETP.

In order to determine if CsA and RAPA increased CE transfer by CETP specifically, the CE binding site on CETP was blocked by using the TP2 monoclonal antibody. We observed that when CETP was co-incubated with TP2 and CsA or RAPA, there was a significant reduction in the CETP activity. In addition, based on the overall structure of CETP, we know that there is no direct interaction between CsA or RAPA and the CE binding site within the CETP molecule. We hypothesize that CsA and RAPA may bind to site(s) separate from the CE binding site on CETP, causing a conformational change that enhances its CE transfer activity.

5.2 CsA, TAC, RAPA and MMF suppressed LPL activity and did not affect HL activity.

On the other hand, lipoprotein lipase deficiency has been associated with hypertriglyceridemia and impaired chylomicron and VLDL clearance, as well as triglyceride enrichment of various lipoproteins. The results of our study had shown significant reductions in in vitro LPL activity of CsA-, RAPA-, TAC- and MMF-treated plasma. This observation may correspond to a reduction in the hydrolysis of
triglycerides contained within triglyceride-rich lipoproteins and the observed hypertriglyceridemia in patients receiving these drugs.

Interestingly, although we observed a significant decrease in the lipoprotein lipase activity in CsA-, RAPA-, TAC- and MMF-treated plasma, the hepatic lipase activity appeared unaffected, indicating that the effects of these drugs on lipid metabolism might not be mediated by this enzyme. By contrast, a previous study reported an increase in VLDL and LDL level in CsA-treated patients that was associated with a decreased HL activity.

5.3 RAPA inhibited cholesterol efflux to ApoA-I and not HDL, in part by decreasing the protein expression of ABCA1.

Furthermore, Le Goff et al. (2004) demonstrated that CsA treatment decreased ABCA1 turnover and yielded a two-fold increase in cell-surface ABCA1. Despite of the increase in cell-surface ABCA1, cyclosporin A decreased apolipoprotein A-I uptake, resecretion, and degradation in RAW cells and THP-1 macrophages. This finding explains in part the low HDL and apoA-I levels observed in some patients with transplants. On the other hand, based on our cholesterol efflux study, tacrolimus did not affect the cholesterol efflux to ApoA-I and HDL in the THP-1 macrophages. While the efflux of labeled cholesterol to lipid-free ApoA-I reflects the movement of cholesterol mass, the efflux mediated by HDL is accompanied by the exchange of cholesterol between cells and HDL, therefore not necessarily reflecting the movement of cholesterol mass.
Surprisingly, although we did not find a significant effect on the cholesterol efflux, there was a significant reduction in the ABCA1 and ABCG1 protein expression in the cells treated with tacrolimus. Our finding is also in agreement with that of Jin S et al. (2004), which shows that tacrolimus regulates MSRs, nuclear hormone receptors, and ABCA1 in THP-1 macrophages. Although, it has been suggested that ABCA1 and ABCG1 work in sequence and synergy in cholesterol efflux, leading to the formation of cholesterol-rich HDL, there is another mechanism, such as SR-B1, which competes with ABCA1 and ABCG1 for cholesterol destined for efflux. Similarly to what we have discussed previously, lesser effect of tacrolimus on cholesterol efflux, compared to CsA, agrees with the less profound effect of tacrolimus on patients’ cholesterol levels. However, the mechanism by which tacrolimus inhibits ABCA1 and ABCG1 still needs further investigation.

Furthermore, our cholesterol efflux study has shown that at the blood concentrations, 10 and 20ng/ml, rapamycin inhibited cholesterol efflux to ApoA-I in the human THP-1 macrophages, but not to the HDL. Since ABCA1 and ABCG1 are major players in mediating cellular efflux of phospholipids and cholesterol from peripheral cells to apoA-I and HDL, we examined their protein expression in the rapamycin-treated cells. As we have demonstrated, rapamycin also decreased the ABCA1 protein expression in these cells, which provides an explanation for the decreased efflux to ApoA-I. As indicated by LDH assays, the concentrations selected for this study were not cytotoxic to the cells.

Furthermore, in another study using HepG2 cells, rapamycin has been shown to increase cholesterol efflux mediated by ABCA1 gene expression by increasing
peroxisome proliferator-activated receptor-alpha and liver X receptor-alpha gene and protein expression. Increased cholesterol efflux from HepG2 cells may increase high-density lipoprotein cholesterol level and also contribute to apolipoprotein B lipoprotein formation by enhancing transfer of high-density lipoprotein cholesterol to apolipoprotein B lipoproteins. Besides that, rapamycin also inhibited LDL receptor (LDLr) in the liver, resulting in a delay of LDL-cholesterol clearance from circulation causing an increase of plasma cholesterol concentration.

Taken together, these findings suggest that rapamycin may result in an accumulation of LDL cholesterol in plasma by inhibiting the ABCA1-mediated cholesterol efflux from the foam cells or reducing hepatic LDLr expression. This may provide an extra explanation to hyperlipidemia in the plasma caused by rapamycin.

However, since these studies were performed in vitro, there are multiple factors that would also lead to drug-induced dyslipidemia, specifically the influence on fatty acid homeostasis in the body. Another possible factor is the activation of peroxisome proliferator-activated receptors (PPARs), which may lead to enhanced fatty acid uptake and oxidative fatty acid metabolism. In vitro RAPA treatment on macrophages showed concentration and/or time dependent effects of PPARγ. In addition, Jin et al. had found the opposing effect of TAC and CsA on CD36 and PPARγ in macrophages. Further, the regulation of CD36 and PPARγ observed with these drugs may suggest a direct effect of the immunosuppressant on PPARγ expression through a yet unknown mechanism. PPARγ activation can lead to upregulation of CD36, which results in accumulation of cholesterol by macrophages, yet PPARγ activation also stimulates ABCA1, which then prevents atherosclerosis through enhanced efflux.
5.4 **TAC decreased LPL activity in renal transplant patients, regardless of any lipid lowering drug treatment.**

In agreement with our *in vitro* results, tacrolimus has also been shown to significantly decrease LPL activity in both treatment groups, with and without statin or lipid lowering drug treatment. Surprisingly, no significant difference in total cholesterol, HDL-C, LDL-C, CETP mass, CETP activity and pre-heparin HL activity has been noted in both groups. Although there was a slight increase in TG level in group of patients receiving statin, it was not significant. The TG level in group of patients without statin remained unchanged. The pattern and the degree of decreased pre-heparin LPL activity were similar throughout the study period in both treatment groups, a finding which further supports TAC’s major role in the development of dyslipidemia in post-transplant patients.

These current results suggest that tacrolimus might alter the insulin signaling pathway by decreasing the LPL activity, resulting in limited clearance of plasma triglycerides, and increased hypertriglyceridemia in the transplant patients. The accumulation of triglycerides observed might be due to a high production rate and a low fractional catabolic rate. However, due to the limited number of patients for this study, the effect of tacrolimus on a patient’s lipid profile and LPL activity still needs further investigation.

The mechanism of the effect of tacrolimus on lipid metabolism is not well understood yet. However, we proposed that it exerts an inhibitory effect on heparin-induced LPL activity. We believe that tacrolimus might share the same mechanism as
rapamycin in suppressing the LPL activity. Tacrolimus administration might increase apoC-III levels, as observed with rapamycin administration. ApoC-III then down regulates LPL activity and interferes with hepatic uptake of VLDL remnants particles.

The two calcineurin inhibitors, CsA and TAC, seem to have different effects on lipid metabolism. The effect of TAC on lipid metabolism is similar to that of CsA, although the rise in total cholesterol levels may be less pronounced. The current study also supports improvement in total and LDL cholesterol levels upon conversion from CsA to TAC. Besides that, it has also been reported that patients on TAC showed an increased risk of new-onset diabetes after transplantation (NODAT) as compared to CsA, only in the presence of high TG levels. CsA, significantly increases incidence and prevalence of high TC and LDL-C, while TAC causes a greater degree of glucose homeostasis alteration.

A recent study has shown that patients with post-transplant diabetes mellitus (PTDM) had higher serum triglyceride levels in the period before the onset of diabetes. The rate of acute rejection episodes in this group was also higher compared to the non-diabetic transplant controls. TAC-based therapy also led to higher peripheral insulin resistance and hyperinsulinemia in comparison to CsA-based regimen in these patients. Even after adjustments on recipient age, BMI and initial fasting glucose, recent multivariate analysis indicated that triglycerides remained significantly associated with new-onset diabetes mellitus. Abnormalities of triglycerides storage have been shown to lead to impaired pancreatic β-cell function, and hypertriglyceridemia is a known risk factor for type 2 diabetes mellitus in nontransplanted populations.
In addition, some studies had shown that pre-heparin LPL mass level correlated negatively with TG, positively with HDL-C, and not at all with TC or LDL-C. In addition, preheparin LPL mass levels were found lower in patients with hypertriglyceridemia, indicating that preheparin LPL mass might reflect the amount of LPL working in the body. Tornvall et al. also reported that postheparin LPL mass was not correlated with VLDL triglycerides in CHD patients, but preheparin LPL mass was. It has been consistently been reported that post-heparin LPL has a closer correlation with HDL-C than with TG.

Furthermore, LPL also acts as a ligand of chylomicrons or VLDL remnants for the VLDL receptors, and enhances the uptake of remnants into the cells. Therefore, in low preheparin LPL mass group, chylomicrons or VLDL remnants might have a tendency to remain in the plasma longer. Preheparin LPL mass was apparently higher in women than in men, but when serum lipid levels were adjusted, preheparin LPL mass was identical.

It is also known that LPL production is controlled by insulin. If this would be true, preheparin LPL mass is expected to decrease in diabetes mellitus, in which insulin action is decreased. And insulin therapy would enhance the preheparin LPL mass, which then reflects an increase in LPL activity in the body. Shirai and colleagues demonstrated that the preheparin LPL mass is decreased in hypertriglyceridemic or diabetic subjects and is increased by the administration of bezafibrate or insulin, which are known to stimulate LPL synthesis. These data also suggest that although serum...
LPL is catalytically inactive, its mass reflects the level of systemic LPL biosynthesis. Serum LPL is usually about one fifth of the post-heparin LPL concentration.

It is important to note that the LPL activity that we measured from the patients samples was pre-heparin LPL activity, which was supposed to be much lower than the LPL activity that would be seen in post-heparinized serum. However, in order to further support our results, we were able to measure the preheparin LPL mass in both groups of our transplant patients. As shown in Table 12 and Table 13, the increase of triglyceride levels in both groups was accompanied by the significant decrease of preheparin LPL mass in these patients. As expected, the group of patients who were not on any lipid lowering drug treatment had less amount of decrease of serum LPL mass, compared to the group with statin. This finding further suggests that tacrolimus might not only affect the LPL activity, but also the LPL biosynthesis, rendering it as a diabetogenic immunosuppressive drug.

Recently, it has been reported that a series of statins, such as pravastatin, atorvastatin and pitavastatin may produce significant increase in serum LPL mass after treatment \(^91,92\). In addition, it was also suggested that the main mechanisms by which atorvastatin produced considerable TG reductions may be due to their inhibition of the production and secretion of VLDL from the liver.

In this study, we can completely rule out effects of MMF on lipid metabolism, since concomitant use of MMF for every patient was the same. In addition, it has also been reported that there are no alterations in glucose metabolism or lipid profile due to the use of MMF \(^93\).
5.5 Conclusion

Lipid abnormalities are frequent in chronic renal failure and after renal transplantation. The pathophysiology of post-transplant dyslipidemia is multifactorial, and immunosuppressive drugs play an important role. Our study has demonstrated that CsA and RAPA can induce in vitro CETP activity and, along with TAC and MMF, suppress LPL activity. In vitro, RAPA can also inhibit ABCA-1 mediated cholesterol efflux to ApoA-I. Increased CE transfer by CETP could result in the enrichment of the apolipoprotein (apo) B-containing lipoproteins (VLDL, LDL) with cholesteryl esters, which is also a proatherogenic step in reverse cholesterol transport. On the other hand, reduction in LPL activity may lead to elevated triglyceride levels, low total cholesterol and a pronounced decrease in HDL-cholesterol plasma levels. Meanwhile, inhibition of cholesterol efflux results in the low HDL and ApoA-I levels in the plasma.

Since many of the post-transplant patients who are administered these drugs experience dyslipidemia, including hypertriglyceridemia and hypercholesterolemia, these results may provide a possible explanation as to why it occurs. Our findings are really exciting since we were able to show the suppression of LPL activity by TAC, both in vitro and in vivo in renal transplant patients administered TAC.

However, when secondary hyperlipidemia due to the immunosuppressant therapy is treated with statins, there is a potential drug interaction problem. This is because statins are metabolized via the metabolic enzyme, cytochrome P450 (CYP), as are the immunosuppressants CsA and TAC, and concurrent administration may result in delayed metabolism and increased blood concentrations of the statins. Hence,
understanding the mechanism by which CsA, RAPA, TAC and MMF cause dyslipidemia in transplant patients will not only allow improved administration of immunosuppressive drugs therapy and dosing regimen, but also find the potential target to prevent or treat dyslipidemia in these patients.

5.6 Limitations

The current clinical study is limited by small sample size, lack of an untreated control group, and exclusively tacrolimus therapy. This study was a general analysis of lipid and lipoprotein profile in renal transplant patients, receiving tacrolimus therapy. Considering that change in lipoprotein lipase activity was seen in these patients in such a short time, more extensive analysis of the effect of tacrolimus on lipoprotein lipase and its diabetogenic effects in this patient group may be warranted in future studies. Since we are not able to obtain post-heparinized blood samples from any of our patients, it will be important to measure the pre-heparin LPL mass in correlation to the triglyceride levels in these patients. This experiment will be done and further results will be shown on the defense.

5.7 Future research

A longer clinical study will be necessary in order to evaluate the clinical implications of our in vitro and in vivo findings. Our study investigating the effects of tacrolimus on the CETP and LPL activity in a population of renal transplant patients is still ongoing.
Since LPL mass has been reported to be different between women and men, the effect of TAC on LPL activity and mass in different genders can be explored in the future.

Another key enzyme, LCAT, is an enzyme necessary for extracellular cholesterol metabolism. LCAT may facilitate the uptake of cholesterol from peripheral tissues into HDL particles by maintaining a concentration gradient for the efflux of free cholesterol, and may play a major role in RCT.\textsuperscript{95, 96} The effect of immunosuppressive drugs on this enzyme is warranted in the near future.
References


75. Kwong M, Wasan KM. Cyclosporine binds to the neutral lipid and potentially other binding sites of lipid transfer protein I. *Pharm Res.* 2003;20:1009-1014.


Appendices

Appendix A  PHC Institutional Certificate of Final Approval

Providence Health Care
Institutional Certificate of Final Approval

<table>
<thead>
<tr>
<th>Principal Investigator:</th>
<th>Department:</th>
<th>Reference Number:</th>
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<tbody>
<tr>
<td>K. M. Wasan</td>
<td>Pharmaceutical Sciences</td>
<td>PHC H07-01728</td>
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</table>

Sponsoring Agencies:
Canadian Institutes of Health Research (CIHR)

Project Title:
Title: The Role of Serum Lipoproteins in Optimizing Amphotericin B Therapy
Subset: The Effect of Cyclosporine A (CSA) and Tacrolimus on Cholesterol Ester Transfer Protein (CETP)-mediated Transfer of Cholesterol Ester (CE) in Renal Transplantation Patients.

Date Ethical Approval:
September 21, 2007

The UBC-PHC Research Ethics Board granted ethical approval for the above-referenced research project on the date stated above. I am now pleased to inform you that all necessary hospital department/facilities approvals and institutional agreements/contracts are now in place and that you have permission to begin your research.

Dr. Yvonne Lefebvre
Vice President Research and Academic Affairs, Providence Health Care
President, Providence Health Care Research Institute

Date: December 12, 2007

St. Paul's Hospital
Holy Family Hospital
Mount St. Joseph's Hospital
St. Vincent's Hospital-Brock Fahrni Pavilion
St. Vincent's Hospital-Langara

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Appendix B Patient Informed Consent Form

THE UNIVERSITY OF BRITISH COLUMBIA

Faculty of Pharmaceutical Sciences
2146 East Mall
Vancouver, B.C. Canada V6T 1Z3

Kishor M. Wasan, Ph.D.
Professor & Chair
Distinguished University Scholar
Division of Pharmaceutics & Biopharmaceutics
Phone (604) 822-4889
Fax (604) 822-3035
E-mail: kwasan@interchange.ubc.ca

Informed Consent Form

Project Title: The Role of Serum Lipoproteins in Optimizing Amphotericin B Therapy.

Subset: The Effect of Cyclosporine A (CSA) and Tacrolimus on Cholesteryl Esters Transfer Protein (CETP)-mediated Transfer of Cholesteryl Esters (CE) in Renal Transplantation Patients

Principal Investigator: Kishor M. Wasan, R.Ph, Ph.D; Faculty of Pharmaceutical Sciences, University of British Columbia, (604) 822-4889

Co-Investigator(s): John S. Hill, Ph.D; Department of Pathology and Lab Medicine University of British Columbia, (604) 806-8616
David N. Landsberg, Ph.D; Faculty of Medicine University of British Columbia, (604) 806-8970

Sponsor: Canadian Institutes of Health Research (CIHR)*

*CIHR is Canada's premier health research funding agency that receives its funding from the Federal Government. The investigators have no conflict of interest as they are related to this study.

Emergency Telephone Number: Kishor M. Wasan, R.Ph, Ph.D (604-272-9250) or David N. Landsberg, Ph. D (604-806-8970)

Reason for Your Participation in this Study:

You understand that you are being invited to take part in this research study because you will be receiving either Cyclosporine A (CSA) or Tacrolimus to prevent acute rejection in the early post-transplantation period. Cyclosporine A (CSA) or Tacrolimus is given until

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the transplanted organ is functioning well or you no longer can tolerate the drug (due to
decrease in your kidney function).

Your participation is entirely voluntary. You have the right to refuse to participate in this
study. If you decide to participate, your decision is not binding and you may choose to
withdraw from the study at any time without any negative consequences to the medical
care, education, or other services you may receive from this clinic or this hospital.

The study is being sponsored by the Canadian Institutes of Health Research (CIHR).

Background:

Recent evidence has indirectly implicated some of immunosuppressive agents, which
have been used to prevent the body from rejecting a transplanted organ, in the
development of heart disease and atherosclerosis after kidney transplantation. However, it
is currently uncertain what mechanisms contribute to the observed disease profile.
Cholesteryl Ester Transfer Protein (CETP) has been considered as a key component in
regulating cholesterol exchange as it transfers cholesteryl esters and triglycerides between
lipoproteins. It is currently unknown what effect these immunosuppressant drugs have on
Cholesteryl Ester Transfer Protein (CETP) activity, known to participate in the
development of cardiovascular diseases.

Purpose:

The purpose of this study is to investigate whether Cyclosporine A (CSA) or Tacrolimus
will have any effects on your lipid profile by inducing the cholesteryl esters transfer
between different lipoproteins. This will be done by determining your plasma cholesteryl
esters transfer protein (CETP) activity before and after you are receiving the drug
treatment.

Subject Inclusions:

Inclusion criteria from participation in this study include:
Patients who will receive renal transplantation and either Cyclosporine A or Tacrolimus
treatment prior to and after the transplantation.

Subject Exclusions:

Exclusion criteria from participation in this study include:
1. Age less than 19 years old.
2. Pregnancy or lactation.
3. Patients that previously received either Cyclosporine A or Tacrolimus.
4. Patients who are receiving other immunosuppressant drugs.
5. Liver function abnormalities above the following limits: bilirubin 3mg/dL, AST
   or ALT 5 times greater than upper limits of normal.
6. Baseline serum creatinine concentration is more than 120 μmoles/L.
7. Patients receiving any potential agents that cause kidney toxicity within the

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preceding 3 days (i.e. Antibiotics Gentamycin, tobramycin, Antifungals Amphotericin, Ketoconazole, Chemotherapy Mephalan (Alkeran) Eroposide (VePesid) and aminoglycosides).

8. Patients with a life expectancy of less than 1 month.

Overview of the Study Procedures:

For this study, we are asking your permission to use, for the purposes of this research, left over blood collected from you in the pre-transplant clinic and post-transplant clinic (before and after you receive Cyclosporine A or Tacrolimus treatment). These left over blood samples will be collected from St. Paul’s Laboratory and then further analyzed at the faculty of Pharmaceutical Sciences at the University of British Columbia. The investigators will know that you will be receiving or have received either Cyclosporine A or Tacrolimus treatment. The investigators will determine the low-density and high-density lipoprotein cholesterol, triglycerides, cholesteryl esters transfer protein (CETP) activity, hepatic lipase and lipoprotein lipase activity. No additional time or blood is required of you in this study beyond that normally needed for your standard medical care.

If You Decide to Join This Study: Specific Procedures

1.) Left over blood samples (3 to 4 mL of whole blood and 1 to 1.5 mL of post heparin plasma) will be collected from St. Paul’s Laboratory at least 6 hours after being withdrawn from the transplant clinic or ward by the nurse.

2.) The whole blood samples will be centrifuged at 4°C, 3000 rpm for 15 minutes in order to collect the plasma.

3.) The plasma collected will be used to determine the following:
   a.) Lipid profile: low-density and high-density lipoprotein cholesterol, as well as triglycerides using enzymatic assay.
   b.) Cholesteryl Esters Transfer Protein (CETP) activity using a CETP Fluorescence activity assay (from Roar Biomedical Inc.).
   c.) Hepatic and Lipoprotein Lipase activity will be measured in post-heparin plasma using trioleate-lyosphosphatidylcholine emulsion.

Pregnancy:

Participants and their partners must avoid pregnancy. Failure to do so may result in potential harm to your fetus. You should discuss the issues surrounding this necessity with your study doctors, and find an acceptable solution that will address this matter to ensure that you avoid pregnancy.

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Risks:

If you experience uncomfortable (but not serious) side effects after taking the oral medication such as chills, fever, nausea, etc., these must be noted and you may be given appropriate medications. **Risks involved with the oral medication of Cyclosporine A or Tacrolimus are part of standard care and that there are no actual risks from this study.**

Benefits:

**You will not receive any direct clinical benefit from participating in this study.** The physicians could determine the maximum tolerated dose of Cyclosporine A or Tacrolimus where kidney function is not affected. The findings would be useful, not only to you, who are presently receiving Cyclosporine A or Tacrolimus treatment, but also to serve as a tool to reduce the side effects of Cyclosporine A and Tacrolimus in future renal transplant patients.

Rights and Compensation:

By signing this form, you do not give up any of your legal rights and you do not release the study doctor or other participating institutions from their legal and professional duties. There will be no costs to you for participation in this study. You will not be charged for any research procedures. If you become ill or physically injured as a result of participation in this study, medical treatment will be provided at no additional cost to you. The costs of your medical treatment will be paid by your provincial medical plan.

Compensation: you will not receive any compensation for participating in this study.

Withdrawal From the Study:

If you are not complying with the requirements of the study, or for any other reason, the study doctor may withdraw you from the study and will arrange for your care to continue. On receiving new information about the treatment, your research doctor might consider it to be in your best interests to withdraw you from the study without your consent if they judge that it would be better for your health.

New Findings:

You will be advised of any new information that becomes available that may affect your willingness to remain in this study.

Confidentiality:

Your confidentiality will be respected. Information that discloses your identity will not be released without your consent unless required by law or regulation. However, research records and medical records identifying you may be inspected in the presence of the
investigators (Drs. Wasan, Hill or Landsberg) or their designate, by representatives of the sponsor (CIHR), Health Protection Branch (HPB, Canada), and the UBC-PHC Research Ethics Board for the purposes of monitoring the research. No records that identify you by name or initials will be allowed to leave the investigator’s office.

Contact:

You understand if you have any questions or desire further information with respect to this study, or if you experience any adverse effects, you should contact either Dr. Wasan at (604) 822-6772, or Dr. Hill (604) 806-8616 or Dr. Landsberg (604) 806-8970.

If you have any concerns about your treatment of rights as a research subject and/or your experiences while participating in this study, you may call the Research Subject Information Line at the University of British Columbia Office of Research Services at 604-822-8598 or the Chair of the UBC-PHC Research Ethics Board at 604-682-2344 ext. 62325.
PRIMARY CARE PHYSICIAN/SPECIALIST NOTIFICATION

Please indicate, by checking the applicable box, whether you want us to notify your primary care physician(s) or specialist(s) of your participation in this study.

☐ Yes, I want the study investigator to advise my primary care physician(s) or specialist(s) of my participation in this study. My primary care physician(s) and/or specialist(s) name(s) is/are:

The name of the medical clinic I attend is:

Subject Initials: __________________

☐ No, I do not want the study investigator to advise my primary care physician(s) or specialist(s) of my participation in this study.
Subject Initials: __________________

☐ I do not have a primary care physician or specialist.
Subject Initials: __________________

☐ The study investigator is my primary care physician/specialist.
Subject Initials: __________________

I understand that if I choose not to advise my primary care physician(s) or specialist(s) of my participation in this study, there may be potential medical consequences which may affect my comprehensive medical care or treatment. I understand that the study investigator may not be responsible for these consequences.

You may wish to discuss the consequences of your decision with the study staff.
SUBJECT CONSENT TO PARTICIPATE

Title of Study: The Role of Serum Lipoproteins in Optimizing Amphotericin B Therapy.
Subset: The Effect of Cyclosporine A (CSA) and Tacrolimus on Cholesteryl Esters Transfer Protein (CETP)-mediated Transfer of Cholesteryl Esters (CE) in Renal Transplantation Patients

You understand that participation in this study is entirely voluntary and that you may refuse to participate or you may withdraw from the study at any time without providing any reasons without any consequences to your continuing medical care.

You consent to participate in this study. However, as a subject in this study, you understand that the consent form is not a contract and you do not waive any of your legal rights against the sponsor, investigators, or anyone else by signing this consent form.

As a subject in this study,

- I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential.
- I understand that my participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in any way the quality of care that I receive.
- I understand that I am not giving up any of my legal rights as a result of signing this consent form.
- I have read this form and I freely consent to participate in this study. I understand that I will be given a copy of this signed and dated consent form.

Printed name of subject ______________________ Signature ______________________ Date ______________________

Printed name of subject’s legally acceptable representative ______________________ Signature ______________________ Date ______________________

Printed name of Witness ______________________ Signature ______________________ Date ______________________

Printed name of Person Conducting Consent Process ______________________ Signature ______________________ Date ______________________

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