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„Effects of renal transplantation and
cross gender hormone therapy on
macrophage cholesterol efflux“

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List of abbreviations

ABC -	ATP binding cassette transporter
ac-LDL-	acetylated LDL
Apo-	apolipoprotein
AR-	androgen receptor
ATP-	adenosinetriphosphate
BA-	Bile acid
CAC-	cholesterol acceptor capacity
cAMP-	cyclic adenosinemonophosphate
CC-	CKD controls
CD36-	cluster of differentiation 36
CE-	cholesterol ester
CEC-	cholesterol export capacity
CEH-	cholesteryl ester hydrolase
CES1-	carboxylesterase 1
CETP-	cholesterol ester transfer protein
CKD-	chronic kidney disease
CP-	CKD patients
CVD-	cardiovascular disease
EC-	endothelial cell
EDTA-	ethylene diamine tetra-acetic acid
eGFR-	estimated glomerular filtration rate
ER α,β -	estrogen receptor α,β
FBS-	fetal bovine serum
FC-	free cholesterol
FtoM-	female to male

HC-	hemodialysis controls
HDL-C –	high density lipoprotein cholesterol
HERS-	estrogen/progestin Replacement Study
HL-	hepatic lipase
HMDM-	human monocyte derived macrophages
HP-	hemodialysis patients
HRT-	hormone replacement treatment
HSL-	hormone sensitive lipase
LCAT-	lecithin:cholesterol acyltransferase
LDL-C –	low density lipoprotein cholesterol
LDL-R-	LDL- receptor
LOX-1-	lectin-type oxidized LDL receptor 1
Lp(a)-	lipoprotein (a)
LXR α,β -	liver X receptor α,β
M-CSF-	macrophage colony-stimulating factor
MHT-	menopause hormone treatment
MtoF-	male to female
mTOR-	mechanistic target of rapamycin
Nceh1-	neutral cholesterol ester hydrolase 1
ox-LDL-	oxidized LDL
PAF-	platelet activating factor acetyl hydrolase
PBS-	phosphate buffered saline
PL-	phospholipid
PLTP-	phospholipid transfer protein
PR-A,B-	progesterone receptor A,B
RCT-	reverse cholesterol transport
SAA-	serum amyloid A
SR-B1-	scavenger receptor class B type 1
SR-A-	scavenger receptor class A
TC-	transplant controls/ total cholesterol
TG-	triglycerides
TICE-	transintestinal cholesterol excretion
UC-	unesterified cholesterol
VCAM-1-	vascular adhesion protein 1
VLDL-	very low density lipoprotein
VSMC-	vascular smooth muscle cells
WHI-	Women's Health Initiative
WHO-	World Health Organisation

1. Introduction

Cardiovascular diseases (CVD) are among the leading causes of death worldwide (Ross, 1999). The World Health Organization (WHO) predicts about 23.6 million CV deaths per year caused by 2030, increasing the number of cardiovascular deaths by nearly two thirds compared to 2008 (www.who.int, 2013a). Men and postmenopausal women are more prone to CVDs than pre-menopausal women (Reslan and Khalil, 2012). CVD's also are the main cause of mortality in renal transplant recipients partly due to adverse effects of immunosuppressive drugs (Jardine et al., 2011; Watorek, et al., 2011). Atherosclerosis represents one of the main underlying pathogenic processes of many CVDs such as cerebrovascular disease (e.g. stroke), ischaemic heart disease or coronary artery disease (e.g. heart attack), and diseases of the aorta and arteries, including peripheral vascular disease (Glass and Witzum, 2001; Navab et al., 1996; Ross, 1999).

1.1 Cardiovascular disease in renal transplant recipients

Renal transplantation is the routine treatment of choice for end stage chronic kidney disease patients. Mortality caused by CVDs in renal transplant recipients amounts up to 55 % and is still considerably higher than in the general population, though lower compared to hemodialysis patients (Jardine et al., 2011; Ostovan et al., 2006; Watorek et al., 2011). Most CVD risk factors in renal transplant recipients are similar to those in the general population, but also include nontraditional factors like allograft dysfunction, insulin resistance, proteinuria, elevated inflammatory markers and plasma homocysteine, and viral infections (Watorek et al., 2011). Renal transplantation is accompanied by immunosuppressive treatment to prevent acute allograft rejection and to ensure optimal early graft function (Ekberg et al., 2007). Indeed the incidence of acute allograft rejection nowadays lies close to only 10 % and more than 90 % of patients have a functioning transplant after 1 year with an average estimated glomerular

filtration rate (eGFR) of $\sim 60 \text{ mL/ min/ } 1.73 \text{ m}^2$ (Baigent et al., 2000; Ekberg et al., 2007; Foley et al., 1998). Although immunosuppressants improved the outcome of renal transplantation, they also have adverse effects on the development of atherosclerosis by potentiating CVD risk factors (Arias and Fernández-Fresnedo, 2001; Chung-Wen Chang and Hricik, 2010; Jardine et al., 2011; Ostovan et al., 2006; Watorek et al., 2011).

1.1.1 Immunosuppressive drugs- advantages and disadvantages

Although there are a small number of reports of recipients who only require little or no immunosuppressive medications at all, most patients need a life-long immunosuppression to avoid allograft rejection (Arias and Fernández-Fresnedo, 2001; Chung-Wen Chang and Hricik, 2010).

There are several different combinations of immunosuppressive drugs, which are in use currently, each having its own advantages and disadvantages. These multidrug approaches minimize the toxicity of the single agent and inhibit the recipient's immune system independently or in a synergistic manner (Chung-Wen Chang and Hricik, 2010; Jardine et al., 2011).

Antibodies, such as lymphocyte depleting antibodies or non-depleting antibodies, which bind to interleukin 2 receptors on activated T-cells, are commonly used for induction therapy. Immunosuppressants used for maintenance include corticosteroids, acting against lymphocytes, and calcineurin inhibitors, like cyclosporine or tacrolimus, which currently represent the cornerstone of solid-organ transplant immunosuppression and affect calcineurin, an intracellular phosphatase.

The third branch of the multidrug immunosuppressant regimen is represented by antiproliferating drugs, which essentially block the proliferation of lymphocytes; lymphocytes recognize foreign donor antigens. There are three anti-proliferating drugs available: azathioprine, mycophenolic acid derivatives, and the mechanistic target of rapamycin (mTOR)-inhibitors (Chung-Wen Chang and Hricik, 2010; Jardine et al., 2011).

As already mentioned, immunosuppressants can potentiate CVD risk factors. Hyperlipidemia, which is almost invariably associated with kidney transplantation, is

characterized by elevated total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), triglycerides (TG), and reduced high-density lipoprotein-cholesterol (HDL-C), and can be enhanced by mTor inhibitors, cyclosporine, tacrolimus, and corticosteroids (Jardine et al., 2011; Watorek et al., 2011). Corticosteroids and calcineurin inhibitors can also potentiate hypertension. New-onset diabetes, which can be induced by corticosteroids, cyclosporine, and tacrolimus, and weight gain are also common adverse effects of immunosuppressants in renal transplant recipients (Jardine et al., 2011; Watorek et al., 2011).

1.2 Cardiovascular disease: differences between men and women

There is a large body of evidence of sex dependent differences in CVDs; men and postmenopausal women show higher incidence of CVD than premenopausal women. Several studies confirm sex differences in development, progression and risk of atherosclerosis, the main underlying cause of CVDs, in men and pre- and postmenopausal women of the same age. This effect was long thought to be mainly caused by the beneficial effect of higher estrogen levels in premenopausal women, but nowadays is assumed to be more complex, involving differences in sex steroids as well as sex specific tissue and cell mediated responses to a broad range of stimuli (Badeau et al., 2009; Badeau et al., 2013; Mendelsohn and Karas, 2005; Reslan and Khalil, Rossi et al., 2002; 2012; Vitale et al., 2009; Vitale et al., 2010). Not only does the lipid profile differ between men and women, but it also changes during menopause in women. After onset of menopause plasma LDL-C levels, pro-atherogenic small dense LDL, and triglycerides tend to increase, whereas some studies report a decrease of HDL-C (Hall et al., 2002; Matthews et al., 1989; Reslan and Khalil, 2012).

1.2.1 Sex hormones and atherosclerosis risk

For a long time estrogen and its receptors have gained more attention concerning their role in cardiovascular physiology and disease than other sex steroid hormones like testosterone and progesterone and their receptors as well as the enzyme aromatase, which is responsible for conversion of testosterone to estrogen in certain tissues (Mendelsohn and Karas, 2005) (Figure 1).

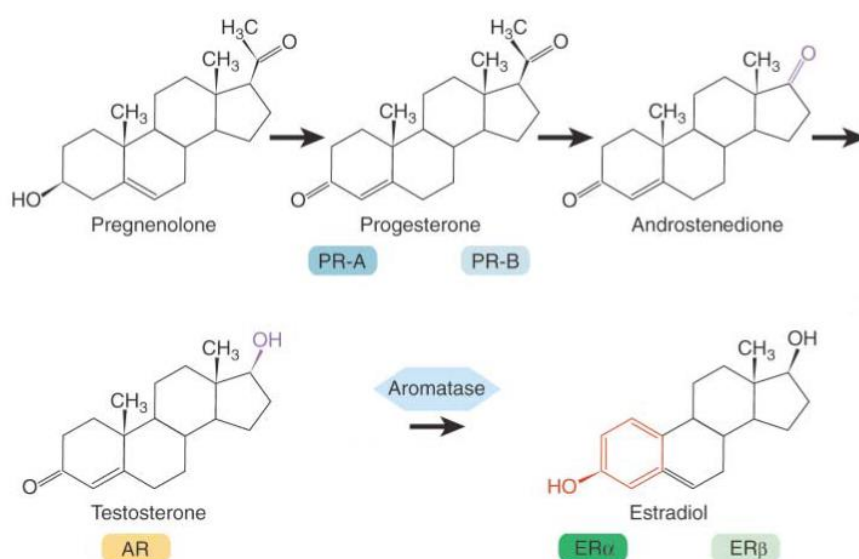


Figure 1: Synthesis of sex steroid hormones estradiol, testosterone and progesterone from pregnenolone; progesterone is the first hormone synthesized from pregnenolone and acts as an intermediate for synthesis of estrogen and androgens; estrogen develops from androgen via catalysation by enzyme aromatase (Cyp19), which forms an aromatic A ring. Sex steroid hormones bind to specific receptors; those receptors as well as the enzyme aromatase are expressed in cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells. Progesterone receptors are progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B), testosterone binds to androgen receptor (AR), and estrogen bind to estrogen receptor α and β (ER α , β) (adapted from Mendelsohn and Karas, 2005).

Nevertheless, intervention studies like the Women's Health Initiative (WHI) and the Estrogen/Progestin Replacement Study (HERS) could not prove the assumed beneficial effects of estrogen treatment. Rather these studies showed bivalent effects of hormone replacement therapy (HRT) or menopausal hormone treatment (MHT) on CVD. This led to the formulation of the timing hypothesis, which states that the timing of HRT initiation and differences in pre-existing atherosclerosis are very important factors for

effects of HRT on CVDs: it suggests that if started to late after menopause, HRT increases the risk of CVD, whereas it decreases CVD risk when started during the peri-menopausal period (Badeau et al., 2010; Mendelsohn and Karas, 2005; Reslan and Khalil, 2012; Turgeon et al., 2004). Known protective effects on the cardiovascular system of estrogen used in HRT comprise a decrease of LDL-C and lipoprotein a (Lp(a)) (Christodoulakos et al., 2004; Ushioda et al., 2006), an increase of HDL-C, a decrease of lipid peroxidation (Ayres et al., 1998), a decrease of insulin resistance (Sumino et al., 2003), a decrease of vascular smooth muscle cell growth and proliferation, an inhibition of intravascular accumulation of collagen, changes in blood coagulation, and direct vasodilation of blood vessel cells (Orshal and Khalil, 2004). However, HRT can become pro-atherogenic when administered to women with already advanced atherosclerotic plaque in later menopause (Figure 2).

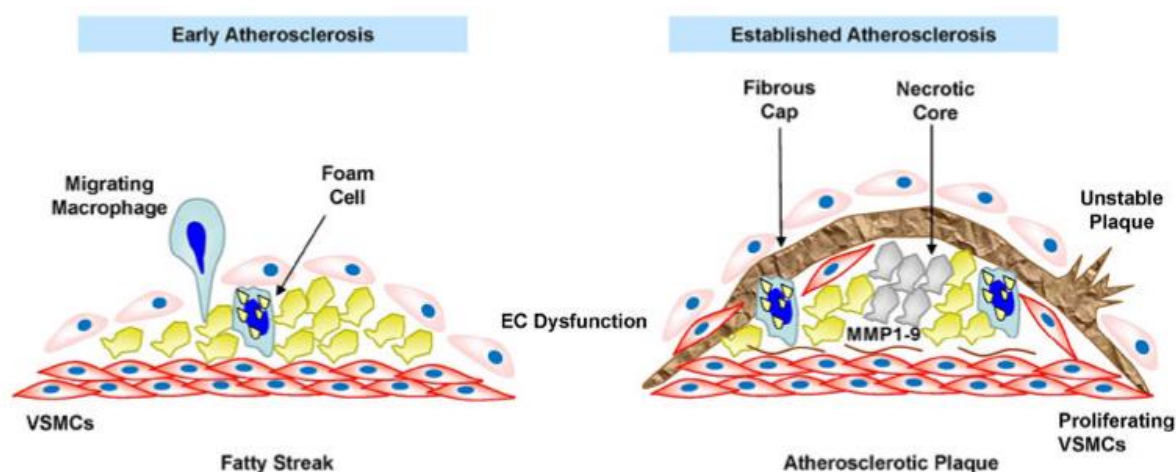


Figure 2: Disparity of HRT in early and established atherosclerosis; estrogen treatment can help maintaining the endothelial cell integrity, decrease pro-inflammatory molecules and thereby decrease lesion progression in early atherosclerosis(fatty streak); in established atherosclerosis, estrogenic HRT can lead to increased metalloproteinase expression, which causes instable fibrous caps and rupture of the plaque; VSMCs, vascular smooth muscle cells; EC, endothelial cells; (adapted from Reslan and Khalil, 2012).

Estrogenic HRT affects early atherogenesis in a protective manner by changing the plasma lipid profile, maintaining endothelial cell integrity, and decreasing the expression of pro-inflammatory molecules. These combined effects lead to a decrease in lesion progression. On the other hand estrogenic HRT in the presence of advanced atherosclerosis enhances the inflammatory process, and increases matrix

metalloproteinase expression, which causes instability of the fibrous cap and rupture of the plaque. Additionally, estrogen receptor function, expression and distribution change with onset of menopause, in a direction, which potentiates atherosclerosis risk (Reslan and Khalil, 2012).

Effects of testosterone and androgens were long thought to be pro-atherogenic due to studies showing an association of excessive exogenous testosterone doses and sudden cardiac death in men (Liu et al., 2003) or elevated testosterone concentrations causing atherogenic, metabolic changes in women suffering from polycystic ovary syndrome (Christakou and Diamanti-Kandarakis, 2008). In contrast to these earlier assumptions, several recent studies revealed an increased atherosclerosis risk in elderly men with low endogenous androgen levels (Traish and Kypreos, 2011; Wu and von Eckardstein, 2003).

Similarly, the effects of androgens on cholesterol metabolism are rather controversial: whereas one study claims an accumulation of CE and uptake of ac-LDL in male but not female human macrophages (McCrohon et al., 2000), another study shows a decrease of foam cell formation in murine macrophages, which was associated with a reduced expression of scavenger receptor lectin-type oxidized LDL receptor 1 (LOX-1) (Qiu et al., 2010). Catalano et al. investigated the cholesterol acceptor capacity of HDL and serum of healthy men and women with matched HDL-C concentrations (Catalano et al., 2008). They showed a slightly, but still significantly higher cholesterol efflux to serum via scavenger receptor class B type 1 (SR-B1) in women. This effect was explained by a higher concentration of mature plasma HDL and a higher acceptor capacity of HDL particles. In contrast they found a higher cholesterol acceptor capacity of serum from men via ABCA1 (Catalano et al. 2008).

1.2.2 Transgender patients: effects of cross gender hormone therapy on atherosclerosis

People with a marked incongruence between their experienced or expressed gender and their gender assigned at birth, are diagnosed with transsexualism (ICD-10) (www.who.int, 2013b) or gender dysphoria (DSM-V) (APA, 2013). The current

management of transsexualism includes cross gender hormone therapy, psychotherapy, and as an optional final step gender reassignment surgery (Dhejne et al., 2011). Transgender patients on cross gender hormone therapy are an interesting model for exogenous estrogen and testosterone effects on cholesterol metabolism, due to the drastic and rapid change of their hormone status. A long-term study from the Netherlands investigating the effects of long-term cross-sex hormone administration to transsexuals revealed no significantly increased mortality of female to male (FtoM) transsexuals and stated that increased mortality in hormone-treated male to female (MtoF) transsexuals was mainly due to hormone unrelated causes; ethinyl estradiol, which no longer is used in cross-gender hormone therapy, however increased the risk of cardiovascular death (Asscheman et al., 2011). Elamin and colleagues reviewed and summarized 16 studies on cardiovascular effects of cross-gender steroid use in transsexuals; with low quality evidence they suggest an increase of serum triglycerides in MtoF and FtoM, and a negligible effect on HDL-C and systolic blood pressure in FtoM (Elamin et al., 2010).

1.3 Atherosclerosis

Atherosclerotic processes start early in life developing from childhood onwards. Known cardiovascular risk factors influencing the progression of atherogenesis include smoking, physical inactivity, high fat diet, hypertension, diabetes, genetic factors, and dyslipidemia. Atherosclerosis can be defined roughly as a chronic inflammation of the arterial wall, caused by interactions of monocyte-derived macrophages, modified lipoproteins, T-cells, and cellular components of the arterial wall (Glass and Witztum, 2001; Navab et al., 1996; Ross, 1999).

The formation of fatty streaks, the initial stage of atherosclerotic lesions, starts with the adhesion of T-cells and monocytes to the endothelium of the arterial wall. This binding is initialized by adhesion factors like VCAM-1, which can be induced by oxidized low density lipoproteins (ox-LDL). After attaching to the surface of the arterial wall, T-cells and monocytes migrate into the subendothelial space. Monocytes differentiate towards

macrophages under the influence of macrophage colony-stimulating factor (M-CSF). Subsequently uptake of ox-LDL via scavenger receptor A (SR-A) or cluster of differentiation 36 (CD36), a member of the scavenger receptor B family, leads to foam cell formation (Steinberg, 2009). Cholesterol of ox-LDL is then esterified and stored in lipid droplets, converted to more soluble forms, or transferred to nascent or mature HDL via the membrane transporters ATP binding cassettes A1 (ABCA1), and G1 (ABCG1) or SR-B1. Although the uptake of modified LDL is initially anti-atherogenic, by removing pro-inflammatory and cytotoxic ox-LDL, the amassment of macrophages and their progressive uptake of ox-LDL eventually lead to atherosclerotic lesions (Fan and Watanabe, 2003; Glass and Witztum, 2001; Ross, 1999) (Figure 3).

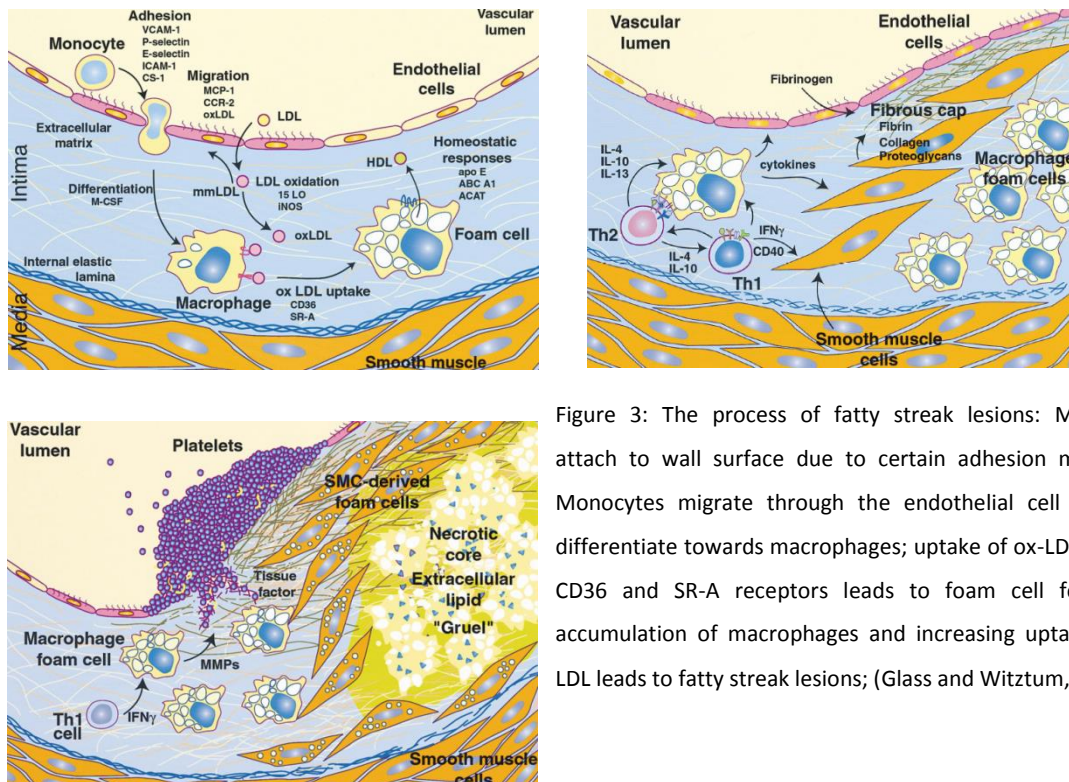


Figure 3: The process of fatty streak lesions: Monocytes attach to wall surface due to certain adhesion molecules. Monocytes migrate through the endothelial cell wall and differentiate towards macrophages; uptake of ox-LDL through CD36 and SR-A receptors leads to foam cell formation; accumulation of macrophages and increasing uptake of ox-LDL leads to fatty streak lesions; (Glass and Witztum, 2001)

1.3.1 Reverse cholesterol transport and HDL metabolism

Cholesterol is an important component of the cell membrane and also a precursor of vitamin D, bile acids, and steroid hormones. Cholesterol homeostasis is influenced by

dietary uptake, *de novo* synthesis of cholesterol (approximately 9 mg/kg bodyweight per day), and also by the process of reverse cholesterol transport (RCT) mediating the transport of excess cholesterol to the liver and the bile as well as by the direct excretion via the intestine, also known as TICE, the transintestinal cholesterol excretion (von Eckardstein et al., 2001; Hersberger and von Eckardstein, 2003; Lecerf and Lorgeril, 2011; Le May et al., 2013; van der Velde et al., 2007).

The term reverse cholesterol transport was first introduced by Glomset in 1968 and denotes the process by which peripheral (extrahepatic) cholesterol is transported back to the liver mainly by HDL particles for excretion in the bile and finally the feces (Cuchel and Rader, 2006; Glomset, 1968; Khara and Rader, 2013; Tall, 2008). This pathway for excretion of excess cholesterol is crucial to maintain the whole body steady-state of cholesterol metabolism (Cuchel and Rader, 2006).

HDL plays an important role in reverse cholesterol transport (Figure 4). HDL particles are very heterogeneous in their structure, size, metabolism, and biological function; they consist of apoproteins, apoA-I representing the majority followed by apoA-II, phospholipids, triglycerides, free cholesterol as well as cholesterol ester (Cuchel and Rader, 2006; Forti and Diament, 2006; Joy and Hegele, 2008). HDL can be subdivided by different methods into several fractions; such as HDL1, HDL2, and HDL3 by ultracentrifugation or α , pre- α , pre- β -1, and pre- β -2 fractions by electrophoresis. Interestingly pre- β HDLs do not contain apoA-II (Joy and Hegele, 2008). ApoA-I is synthesized and secreted by the liver and the intestine.

Although lipidation of lipid-poor apoA-I, or nascent HDL particles, is mostly performed by the liver and the intestine via ABCA1 mediated lipid efflux, in the context of RCT peripheral cells like macrophages also contribute to lipidation via ABCA1. In contrast to ABCA1, SR-B1 and ABCG1 promote cholesterol efflux to mature HDL particles. The plasma enzyme lecithin:cholesterol acyltransferase (LCAT) esterifies free cholesterol; the resulting CE then creates a hydrophobic core, which leads the formation of spherical mature HDL particles. Cholesterol ester transfer protein (CETP) allows CE from HDL particles to be transferred to apoB containing lipoproteins, such as VLDL or LDL, in exchange for triglycerides. Those lipoproteins then can transfer excess cholesterol to the

liver via LDL receptors (LDL-R), a pathway which significantly contributes to the overall RCT. The enzyme CETP is not present in rodents, which must be considered in the interpretation of animal studies (Cuchel and Rader, 2006; Tall, 2008). Phospholipid transfer protein (PLTP) can transfer phospholipids between HDL and LDL or triglyceride-rich lipoproteins; this contributes to the maturation of HDL particles (Huuskonen et al., 2001). Another function of PLTP is to mimic HDL particles in their ability to remove cholesterol and phospholipids from cells via ABCA1 (Oram et al., 2008). SR-B1 is responsible for FC and CE uptake by hepatocytes, remodeling HDL particles to a smaller form. Remodeling of mature HDL particles can also be achieved by hepatic lipase through hydrolysis of HDL triglycerides or endothelial lipase, through hydrolysis of HDL phospholipids (PL) (Cuchel and Rader, 2006). Finally cholesterol taken up by SR-B1 or LDL-R in the liver can then be excreted into the bile as unesterified cholesterol or bile acids (BA) via ABCG5/8- and ABCB11- mediated pathways (Cuchel and Rader, 2006; Tall, 2008) (Figure 4)-

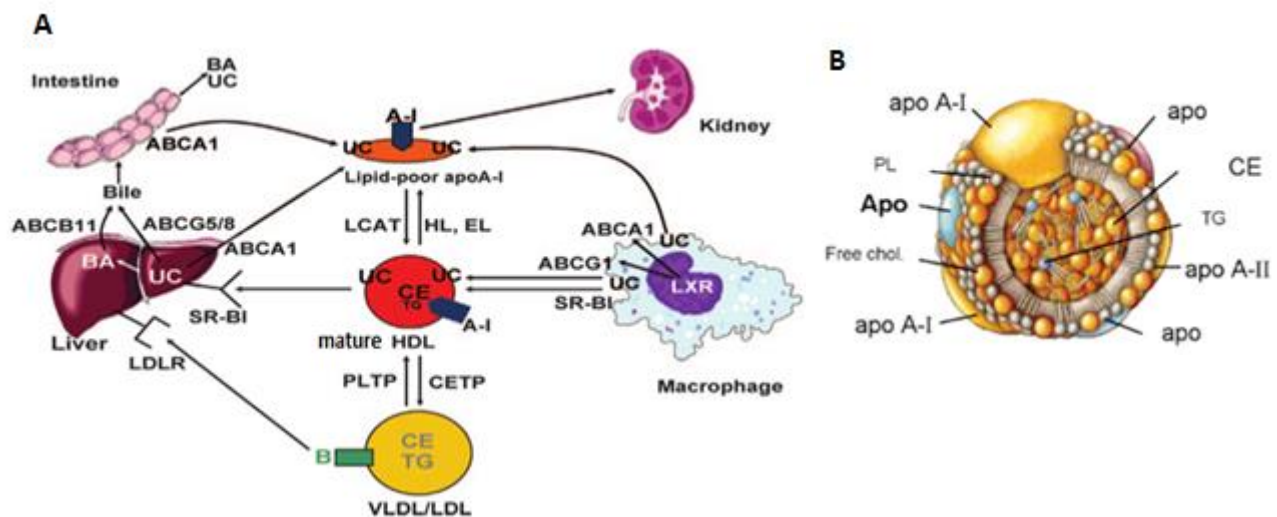


Figure 4: HDL metabolism (panel A) and composition (panel B) and reverse cholesterol transport (panel A); BA, bile acids; UC, unesterified cholesterol (free cholesterol); apoA-1, apoprotein A-I; LCAT, lecithin:cholesterol acyltransferase; HL, hepatic lipase; PLTP, phospholipid transfer protein; CETP, cholesterol ester transfer protein; TG, triglycerides; LDLR, LDL receptor; LXR, liver X receptor; (panel A adapted from Cuchel and Rader, 2006; panel B adapted from Forti and Diament, 2006).

1.3.1.1 Macrophage cholesterol efflux

Macrophages are phagocytes derived from monocytes. There are two major population of macrophages, M1 or classically activated macrophages and M2 also known as alternatively activated macrophages (Chinetti-Gbaguidi and Staels, 2011, Maiuri et al., 2013). Cholesterol accumulation in macrophages can occur via phagocytic or pinocytic uptake of cell debris or via uptake of modified lipoproteins through scavenger receptors (Hersberger and von Eckardstein, 2003).

The accumulation of monocyte derived, lipid-loaded macrophages, called foam cells, in lesions plays a central role in the atherogenic process and links macrophage cholesterol efflux most directly to atherosclerosis (Cuchel and Rader, 2006; Hersberger and von Eckardstein, 2003; Mairuri et al., 2012). Animal studies showed reduced development of atherosclerosis in mice genetically deficient in M-CSF- derived macrophages, emphasizing again the importance of macrophages in atherosclerosis (Smith et al., 1995).

As free (unesterified) cholesterol is toxic to macrophages and can ultimately lead to apoptosis, macrophages developed self-protective responses against free cholesterol toxicity. Hepatic and steroidogenic tissues, which can convert cholesterol to steroid hormones, are the only exception and are able to metabolize cholesterol (Cuchel and Rader, 2006). The first step is the esterification of free cholesterol, which can then be stored in lipid droplets due to its hydrophobic properties. The accumulation of cholesterol ester in cells leads to foam cell formation, which would eventually again lead to apoptosis. Macrophages, however, are able to export excess cholesterol to nascent or mature HDL particles, to maintain cellular cholesterol balance, which is referred to as the macrophage cholesterol efflux (Cuchel and Rader, 2006; Hersberger and von Eckardstein, 2003).

Cholesterol transporters and transfer pathways in macrophage cholesterol efflux

There are several ways for macrophages to reduce or limit the size of the cellular cholesterol pool (Figure 5). Although most efflux is dependent on extracellular acceptors, two exceptions exist: on the one hand macrophages can release cholesterol by secretion of lipid-rich apoE-containing particles or on the other hand by secretion of 27-hydroxycholesterol after cholesterol oxidation by the cytochrome P450 oxygenase CYP27 (Hersberger and von Eckardstein, 2003).

Nonetheless cholesterol efflux dependent on HDL is regarded to be the major pathway for excretion. Aqueous diffusion and microsolvubilization are autonomous from cellular proteins and are considered slow compared to pathways dependent on cellular proteins for secretion. Aqueous diffusion is the passive process in which free cholesterol desorbs from the plasma membrane and diffuses to extracellular lipoproteins, such as HDL, across a concentration gradient; this pathway can be reinforced by the esterification of cholesterol via LCAT, ensuring the functioning of the gradient between the cell and the lipoprotein (Rothblat et al., 1999).

Another receptor-independent pathway is the process of microsolvubilization, where lipid-poor apoproteins A-I, A-II, A-IV, C, and E associate spontaneously with phospholipids of the cell membrane and draw them into the extracellular compartment (Gillotte et al., 1999; Rothblat et al., 1999, Yancey et al., 1995). This is accompanied or followed by cholesterol efflux, but does not lead to formation of nascent HDL particle as this would require interaction of carboxy-terminal and amino-terminal domains of apoA-I (Chroni et al., 2003).

The major pathways for cholesterol efflux dependent on cellular proteins are mediated by ABCA1, ABCG1, or SR-B1. In contrast to ABCA1 and ABCG1, SR-B1 facilitates a bidirectional flux of free cholesterol between lipoproteins and cells (de La Llera-Moya, et al., 2001; Yancey et al., 2004). SR-B1 is a cell surface protein, which binds to a variety of ligands, such as modified lipoproteins (ox-LDL, and ac-LDL), and native lipoproteins like HDL, LDL, and VLDL, linking SR-B1 to RCT (Rigotti et al., 2003). Studies showed varying effects of SR-B1 expression in different cell types; whereas SR-B1 expression enhances

HDL-mediated cholesterol efflux in macrophages, it mediates the influx of cholesterol in liver or steroidogenic cells (Ji et al., 1997; Liu et al., 1999; de La Llera-Moya et al., 1999; Silver and Tall, 2001). Both SR-B1-mediated cholesterol efflux in macrophages and SR-B1-mediated uptake of lipids in hepatic cells are considered anti-atherogenic (Hersberger and von Eckardstein, 2003). This was confirmed in mouse models, showing that inactivation of SR-B1 in mice increased atherosclerosis, despite increasing HDL-C (Braun et al., 2002; Trigatti et al., 1999) and overexpression of SR-B1 in mice decreased atherosclerosis despite decreasing HDL-C (Arai et al., 1999; Kozarsky et al., 2000).

ABCA1 and ABCG1 belong to the large family of adenosinetriphosphate (ATP)-dependent transporters, which share common structural motifs for the active transport of a broad spectrum of substrates (Dean et al., 2001). The discovery that mutations of ABCA1 are responsible for Tangier disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999), a rare disease which is characterized by accumulations of cholesterol and cholesterol esters and macrophage foam cells in various tissues, peripheral neuropathy and increase in atherosclerosis, was a major breakthrough in the field of HDL research (Tall, 2008). ABCA1 and ABCG1 are ATPases which facilitate unidirectional cholesterol efflux to lipid-poor helical apolipoproteins and in case of ABCG1 to lipoprotein particles (Wang et al., 2006). In macrophages, ABCA1 promotes cholesterol efflux to lipid-poor apoA-I, whereas ABCG1, a half-transporter, which acts as a homodimer, promotes cholesterol efflux to HDL particles; this can happen in a sequential and synergistic manner, with ABCA1 constituting nascent HDL particles, which are then promoted to mature HDL particles by ABCG1 (Gelissen et al., 2006). Studies using genetic knockdown models indicate that ABCA1 and ABCG1 account for about 60-70 % of the cholesterol efflux to serum or HDL from cholesterol loaded and liver-X-receptor (LXR)-induced macrophages (Yvan-Charvet et al., 2007) Furthermore selective knock out of ABCA1 in apoE- or LDL-R-deficient mice increases atherosclerotic development significantly (Aiello et al., 2002). ABCG1 activity in macrophages depends on their lipid loading and LXR activation status. While ABCG1 is located predominately intracellular and is relatively inactive in non-cholesterol loaded macrophages, its expression increases in LXR-

activated and cholesterol loaded macrophages and it can be detected in the plasma membrane (Tall, 2008).

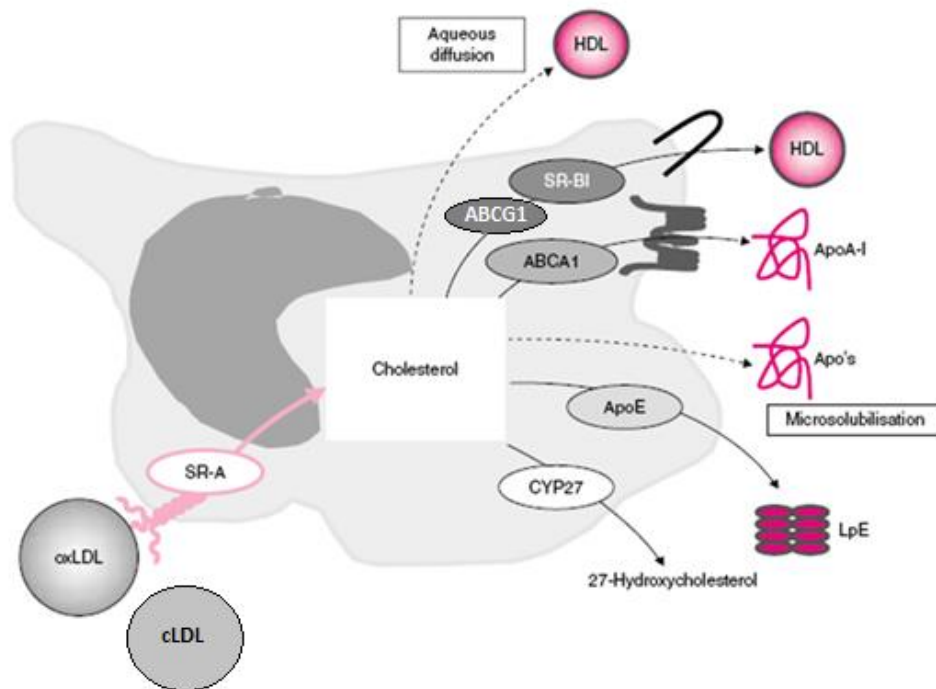


Figure 5: Macrophage cholesterol efflux; ABCA1, ATP binding cassette transporter A1; Apo, apolipoprotein; CYP27, cholesterol-27-hydroxylase; HDL, high-density lipoprotein; LpE, particles containing only apoE; ox-LDL, oxidized low-density lipoprotein; SR-A, scavenger receptor; c-LDL, carbamylated low-density-lipoprotein (adapted from Hersberger and von Eckardstein, 2003)

LXRs (α and β) play an important part in RCT by regulating the expression of ABCA1 and ABCG1 as well as several other genes involved in lipid metabolism (Zhao and Dahlman-Wright, 2010) (Figure 6). There are various ligands for LXRs such as oxysterols, which are oxidized derivatives of cholesterol (Costet et al., 2000; Zhao and Dahlman-Wright, 2010). In macrophages, 27-hydroxycholesterol is the most likely ligand, because the levels of 27-hydroxycholesterol correlate with cholesterol levels in the cell. Increasing levels of cellular cholesterol lead to generation of 27-hydroxycholesterol by the cytochrome P450 enzyme 27-cholesterol hydroxylase (CYP27) (Cavalier et al., 2006). 27-hydroxycholesterol binds to LXRs thereby inducing ABCA1 and subsequent cholesterol efflux (Fu et al., 2001). Cyclic adenosinmonophosphate (cAMP) also induces ABCA1 expression as well as phosphorylation (Haider et al., 2002; Haider et al., 2004;

Oram et al., 2000), but does not affect ABCG1 expression (Suzuki et al., 2004). T0901317, also used in this study, is a synthetic and commonly used inducer of LXR (Repa et al., 2007; Schulz et al., 2000).

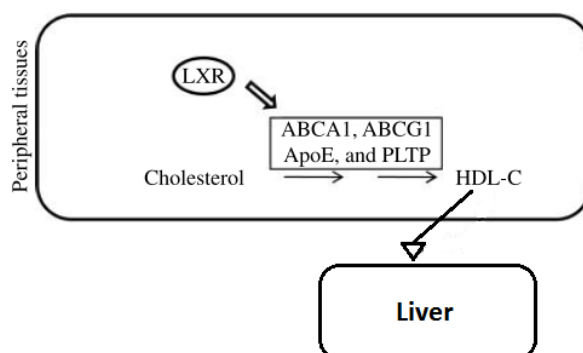


Figure 6: LXR induced cholesterol efflux in peripheral tissues, e.g. macrophages; LXR, liver X receptor increases cholesterol efflux in peripheral tissue to the liver by inducing ABCA1, ABCG1, Apo E, and PLTP; ABCA1/G1, ATP binding cassette A1/G1; Apo E, apoprotein E; PLTP, phospholipid transfer phosphatase (adapted from Zhao and Dahlman-Wright, 2010)

Macrophage cholesterol efflux in renal transplant recipients and transgender patients on cross gender hormone therapy

Although the plasma lipid levels improve in renal transplant recipients compared to patients suffering from CKD and patients on hemodialysis, post-transplantation dyslipidemia associated with low HDL-C, increased TG and increased LDL-C are common complications, also suggesting an impaired RCT (Ostovan et al., 2006). However, data on macrophage cholesterol efflux in renal transplant recipients are not available to the best of our knowledge. A recent study, performed in this laboratory by Meier et al. (submitted), showed substantial changes of serum cholesterol acceptor capacity and macrophage cholesterol export capacity in hemodialysis patients compared to matched controls as well as patients with CKD stage 3-5. Whereas cholesterol efflux of CKD patients was only slightly changed, analysis of serum from hemodialysis patients revealed a significantly reduced cholesterol acceptor capacity. This effect was linked to reduced serum apoA-I concentrations. Interestingly, the cholesterol export capacity of HMDM isolated from hemodialysis patients was nearly twice as high as in matched

controls, which may represent an adaptive response to the decreased cholesterol acceptor capacity (CAC).

In the circulation, estrogen is partly carried on LDL and HDL, in form of estrogen fatty acyl esters, whereas free estrogen only accounts for a minority of lipoprotein associated estrogen (Badeau et al., 2007; Badeau et al., 2009; Badeau et al., 2013). Nevertheless, unesterified 17 β -estradiol is used in most studies to investigate the effect of estrogen on lipid and lipoprotein metabolism (Helisten et al., 2001; Janocko and Hochberg, 1983; Vihma et al., 2003).

Both androgens and estrogens can affect cellular cholesterol metabolism as well as the formation of foam cells (Ng et al., 2001). 17 β -estradiol can affect macrophage cholesterol balance by a decrease of cholesterol ester accumulation via acetylated LDL and a reduction of SR-A expression as well as a reduced hydrolysis of cholesterol ester (Napolitano et al., 2001; Tomita et al., 1996) in macrophages of women but not men (Sulistiyani and St Clair, 1997). In 2009, Badeau and colleagues published a small study (only 4 normolipidemic premenopausal women and four age-matched men) suggesting an enhancement of macrophage cholesterol efflux by HDL-associated 17 β -estradiol fatty acyl esters. However, they were not able to confirm an effect of estrogen on macrophage cholesterol efflux in a larger study only a few years later (Badeau et al., 2013).

1.4 Aims

The aims of this study were to determine (i) the effect of renal transplantation on the cholesterol acceptor capacity of both HDL and serum from renal transplant recipients, (ii) the effect of renal transplantation on the cholesterol export capacity of human monocyte derived macrophages (HMDM) from renal transplant recipients, and (iii) the effects of cross gender hormone therapy on the cholesterol acceptor capacity of serum from transgender patients. Cholesterol acceptor capacity experiments were performed using the human cell line THP-1 as well as the murine cell model RAW 264.7.

2 Material and Methods

2.1 Patients

2.1.1 Renal transplant recipients

Renal transplant recipients were recruited using the exclusion criteria below in collaboration with Dr. Säemann, Mag. Kopecky, and Dr. Haidinger (Medical University of Vienna, Department of Internal Medicine III/, Division of Nephrology and Dialysis). Age- and sex-matched controls were recruited and approximately 45 mL of venous blood were collected after an overnight fast.

Exclusion criteria: diabetes mellitus, major infections for at least 6 months, mTOR inhibitors, BMI < 30 kg/m², liver diseases, lipid lowering drugs, and other secondary dyslipidaemias (e.g. Down syndrome).

Treatment: Renal transplant recipients received tacrolimus, mycophenolic acid, ciclosporin A, and low-dose prednisone for immunosuppression. Antihypertensive medication included: angiotensin converting enzyme inhibitors, angiotensin-II-receptor antagonists, calcium channel blockers, and beta blockers. Additionally, antiplatelet and urate-lowering drugs were administered.

2.1.2 Transgender patients

Transgender patients on cross gender hormone therapy were recruited strictly according to the following exclusion criteria by Dr. Ulrike Kaufmann (AKH Vienna, Universitätsklinik für Frauenheilkunde). Samples were collected after an overnight fast before and after at least six months of hormone treatment.

Exclusion criteria: diabetes mellitus, major infections, liver diseases, lipid lowering drugs, other secondary dyslipidaemias (e.g. Down syndrome), and other hormone treatments (e.g. thyroid hormones, etc.).

Treatment: MtoF patients received transdermal 17 β -estradiol (2x100 μ g/week) or oral 17 β -estradiol (2-4 mg/day), oral cyproterone acetate (50 mg/day), and oral finasteride (5 mg every other day). After sex-reassignment surgery hormone treatment as reduced to transdermal and oral 17 β -estradiol to 2x100 μ g/week or 2-4 mg/day (Ott et al., 2011).

FtoM patients received intramuscular testosterone undecanoate (1000 mg every 12 weeks) and oral lynestrenole (5 mg/day). After sex-reassignment surgery hormone treatment was reduced to testosterone undecanoate (1000 mg every 12 weeks) (Ott et al., 2011).

2.1.3 Ethical committee approvals

Both studies were approved by the Ethics Committee of the Medical University of Vienna. Protocol # 511/2007 applies to renal transplant recipients; protocol # 264/2011 applies for cross gender hormone therapy patients.

2.3 Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.00 (GraphPad Software, SanDiego, CA, USA) and SPSS 11.5.1.0 (IBM, Somers, NY, USA). Data from renal transplant patients and matched controls were analyzed using Mann-Whitney tests. Data from transgender samples before and after cross gender hormone treatment were analyzed using Wilcoxon matched pairs tests.

Lipid, lipoprotein cholesterol, and apolipoprotein analyses were contributed by Tatjana Stojacovic (Department of Laboratory Medicine - Medical University of Graz).

2.4 Lipoprotein isolation

Venous blood samples donated by healthy fasting volunteers were collected in 0.18 M EDTA (ethylene diamine tetra-acetic acid) containing tubes. After a centrifugation step (620 x g for 10 min, at 18 °C, brake 0) the upper phase, containing plasma was used for lipoprotein isolation. Lipoproteins were isolated by sequential flotation ultracentrifugation (Havel et al. 1955). To separate the lipoprotein fractions VLDL, LDL, and HDL density was adjusted using potassium bromide (KBr); the amount of KBr needed was calculated using Equation 1.

$$gKBr = \frac{V_{sample} * (\rho_{final} - \rho_{initial})}{1 - (0.321 * \rho_{final})}$$

Equation 1: Formula to calculate amount of KBr needed to adjust density of plasma.

ρ Plasma = 1.006 mg/mL; ρ VLDL= 1.02 mg/mL; ρ LDL= 1.07 mg/mL; ρ HDL= 1.22 mg/mL
Lipoproteins were separated starting with VLDL, as it represents the fraction with the lowest density. Tubes (Quick-Seal™ centrifuge tubes, Beckman Instruments Inc, Palo Alto, USA) were centrifuged at 50,000 rpm at 4 °C for 21 h using a 50.3 Ti (patient samples) or a 55.2 Ti (pooled control HDL) rotor in an Optima L-100 XP ultracentrifuge. VLDL was collected (upper fraction) and the procedure was repeated to gain LDL and repeated twice for the HDL fraction (only once for patient samples). All lipoproteins were subjected to dialysis (dialysis buffer: 154 mM NaCl, 34 mM EDTA, pH 7.4; 4 °C for 24 h) for KBr removal. Protein content was determined by Bradford assay.

2.4.1 Lipoprotein modification

Acetylated LDL was prepared according to the method of Basu et al. (1976). Therefore LDL (c= 2 mg/ mL) and saturated $C_2H_3NaO_2$ in the relation 1:1 were carefully mixed on ice while adding 0.1 % acetic anhydride every 10 min for five times. Then the mixture

was stirred for 30 min. Ac-LDL was subjected to dialysis (4 °C for 24 h). Protein content was assessed by a Bradford assay. Ac-LDL was sterilized by filtration before used in cell culture.

2.5 Protein content determination

To determine protein concentrations the Bio-Rad Protein Assay based on the Protein assay developed by Bradford (1976) was used. The procedure was performed in a 96-well plate; 1-50 µL of sample were mixed with 20 µL Bradford reagent and filled up with ddH₂O to a volume of 100 µL. After 10- 30 min absorbance was measured at 595 nm using a Bio-RadiMarkTM microplate reader. Bovine serum albumin (BSA) was used to create a standard curve of known protein concentrations; unknown sample protein concentrations were determined by interpolation.

2.6 Cell culture

2.6.1 THP-1 cells

The human acute monocytic leukemia cell line (THP-1) (ATTC TIB-202, LGC Standards, Teddington, UK) was cultured in cell culture flasks (Greiner Bio One, Kremsmünster, Austria) at 37 °C under 5 % CO₂ atmosphere in RPMI 1640 complemented with 10 % fetal bovine serum (FBS) (heat inactivated FBS, Gibco, Life Technologies Corporation, Carlsbad, USA), 2 mM L-Glutamine, penicillin (100 units/ml) and streptomycin (0.1 mg/mL, PAA, Pasching, Austria). A CASY[®] Cell Counter + Analyser System Model TT (Schärfe Systeme, Reuttligen, Germany) was used to determine cell number and viability. Every second day cells were diluted with fresh medium to a cell density of $\sim 0.7 \times 10^6$ cells/ mL.

Differentiation of THP-1 monocytes towards macrophages was induced by addition of PMA (phorbol 12-myristate 13-acetate). 0.5×10^6 cells/ mL with a viability higher than 95 % were seeded in multiwell plates (Greiner Bio One, Kremsmünster, Austria), volumes according to Table 1, and incubated with 160 nM PMA at 37°C for 72 h.

Table 1: Plate size and seeding volume for THP-1 differentiation towards macrophages.

Wells per plate	Seeding volume [mL]
6	3
12	1.2
24	0.6

2.6.2 RAW 264.7 cells

The mouse leukemic monocyte macrophage cell line (RAW 264.7) was cultured in cell culture flasks (Greiner Bio One, Kremsmünster, Austria) at 37 °C under 5 % CO₂ atmosphere in DMEM high glucose complemented with 10 % FBS (heat inactivated FBS, Gibco, Life Technologies Corporation, Carlsbad, USA), 2 mM L-Glutamine, penicillin (100 units/ml) and streptomycin (0.1 mg/mL) (PAA, Pasching, Austria). Every second day cells were diluted with fresh medium. 0.1×10^6 cells/mL with a viability higher than 95 % were seeded in 24 well plates, in a volume of 0.6 mL.

2.6.3 Human monocyte derived macrophages

Venous blood was collected from patients and controls, after an overnight fast using the Vacuette® blood collection set, Vacuette® EDTA tubes and Vacuette® serum tubes (9 mL tubes, Greiner Bio One, Kremsmünster, Austria). To isolate serum, serum tubes were centrifuged at 1500 x g, at 18°C for 10 min, brake 9. The amount of serum needed for differentiation towards macrophages was stored at 4°C; residual serum was stored at -

20°C. EDTA tubes were centrifuged at 620 x g, at 18 °C for 10 min without brake. The upper fraction contained plasma and was used for lipoprotein isolation. The bottom fraction was replenished with RPMI-1640 to approximately 8 mL and mixed carefully. To isolate peripheral blood mononuclear cells (PBMC), the RPMI-1640/blood mixture was very carefully layered on top of a Ficoll-Paque™ PREMIUM phase (GE Healthcare, Buckinghamshire, UK), (blood to ficoll ratio 1:1) and centrifuged at 320 x g, at 18 °C, for 30 min without brake. After centrifugation four layers were distinguishable; the top layer consisted of medium, remaining plasma and platelets, the second layer was the white PBMC ring, beneath it a ficoll containing layer was visible. Erythrocytes and granulocytes made up the bottom layer.

The PBMC ring was collected and transferred into a new 50 mL tissue culture tube containing ~ 30 mL RPMI-1640, and centrifuged at 320 x g and 18 °C for 10 min. The supernatant was discarded and the pellet was suspended in ~26 mL RPMI-1640; then the centrifugation step was repeated. The pellet was resuspended in ~ 2mL RPMI-1640 complemented with 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (0.1 mg/mL), and 10 % autologous serum, and filled up to the required volume, Table 2. Nine mL of EDTA blood was required to seed one well of a 6-well plate or two wells of a 24 well plate. After two hours of incubation at 37°C, medium was replaced, volume see Table 2. Medium was changed every second day. One week after seeding differentiation towards macrophages was completed.

Table 2: Type of tissue culture plate, volumes of seeding and medium change for HMDM

Wells per plate	Seeding volume [mL/well]	Medium volume during differentiation [μL/well]
24	1	350
12	2	750
6	4	2000

2.7 Cholesterol acceptor capacity of serum and HDL

0.5×10^6 THP-1 cells or 0.1×10^6 RAW 264.7 cells were seeded on 24 well plates and differentiated towards macrophages. THP-1 and RAW 264.7 cells were loaded with ac-LDL ($50 \mu\text{g}/\text{mL}$). THP-1 derived macrophages and RAW 264.7 cells were incubated with $0.5 \mu\text{Ci}$ [^3H]-cholesterol/ well, $50 \text{ ng}/\text{mL}$ ac-LDL, $3 \mu\text{M}$ LXR agonist, T0901317, one day prior to the efflux experiment. Cells were incubated for 24 h at 37°C .

To measure the cholesterol acceptor capacity, cells were washed twice with warm (37°C) PBS. $250 \mu\text{L}$ / well RPMI-FBS were added for equilibration of cells. After two hours incubation at 37°C , cells were washed again twice with PBS. Cholesterol acceptor capacity was measured towards RPMI w/o FBS or DMEM high glucose w/o FBS supplemented with HDL ($10 \mu\text{g}/\text{mL}$) or serum (1%); non-supplemented RPMI w/o FBS or DMEM high glucose w/o FBS was used as a blank and negative control. For samples from transgender patients, media without phenol red was used because phenol red can display estrogenic properties (Berthois et al., 1986). Each sample was tested in triplicate sets of wells. After two hours (RAW 264.7) or six hours (THP-1) incubation at 37°C , the supernatant was transferred to tubes and centrifuged for 10 min, at 1750 rpm, at 4°C (Eppendorf Centrifuge 5403). In the meantime cells were washed twice with warm PBS and then lysed by adding $250 \mu\text{L}$ 0.1 M NaOH; after one hour incubation at RT, lysed cells were transferred to 1.5 mL reaction vials. $150 \mu\text{L}$ of either supernatant or cell lysates were added to $\sim 6 \text{ mL}$ scintillation fluid each. After thorough mixing, disintegrations per minute (dpm) were measured using a β -counter (Tricarb 2800 TR Liquid Scintillation Analyzer, PerkinElmer). The protein content of cell lysates was measured by Bradford assay.

Cholesterol acceptor capacity was expressed as percentage radioactivity in the supernatant divided by the radioactivity of cell lysates and supernatant (Equation 2). To eliminate unspecific signals from medium, the cholesterol acceptor capacity of medium only was subtracted from respective measurements.

$$Efflux \% = \frac{100 * dpm(supernatant)}{dpm(lysates + supernatant)}$$

Equation 2: Formula to calculate the efflux expressed in percentage.

2.8 Cholesterol export capacity of HMDM

Isolated human monocytes were seeded on 24 well plates and differentiated towards macrophages for one week under autologous serum. HMDM were incubated with 0.25/(1.5µL) µCi [³H]-cholesterol/ well one day prior to the efflux experiment. Cells were incubated for 24 h at 37°C.

To measure the cholesterol export capacity, cells were washed twice with warm (37°C) PBS. 250 µL/ well RPMI w/o FBS were added for equilibration of cells. After two hours incubation at 37°C, cells were washed again twice with PBS. Efflux was measured towards RPMI w/o FBS supplemented with pooled control HDL (10 µg/ mL) or serum (1%); non-supplemented RPMI w/o FBS was used as a blank and negative control. Each individual sample was tested in duplicate sets of wells. Supernatant was transferred to tubes after six hours incubation at 37°C and centrifuged for 10 min, at 1750 rpm, at 4°C (Eppendorf Centrifuge 5403). Meanwhile cells were washed twice with warm PBS and then lysed by adding 250 µL 0.1 M NaOH; lysed cells were transferred to 1.5 mL reaction vials after one hour incubation at RT. 150 µL of either supernatant or cell lysates were added to ~6 mL scintillation fluid each. After thorough mixing, disintegrations per minute (dpm) were measured using a β-counter (Tricarb 2800 TR Liquid Scintillation Analyzer, PerkinElmer). Protein content of residual cell lysates was measured by Bradford assay. Equation 2 was used for calculation of cholesterol export capacity of HMDM.

3 Results

3.1 Effects of renal transplantation on macrophage cholesterol efflux

Clinical characteristics of study patients and controls

Renal transplant recipients were recruited in collaboration with the (Medical University of Vienna, Department of Internal Medicine III/Division of Nephrology and Dialysis); patients were selected strictly according to the exclusion criteria in 2.1. Controls and patients were matched carefully for sex and age. Results of lipid, apoproteins analyses and clinical characteristics of renal transplant recipients and matched controls are listed in

Table 3.

Statistical analyses revealed significant differences in serum creatinine and apoA-II levels between patients and controls. Serum creatinine was higher in renal transplant recipients (1.2 ± 0.4 mg/dL compared to $0.9 \pm 0.2^{**}$ mg/dL in controls, $p = 0.0021$); the reference range for serum creatinine in healthy male adults is 0.6-1.2 mg/dL and 0.5-1.1 mg/dL for females, depending on age, diet, muscle mass, etc. (www.medicinenet.com, 2013). ApoA-II was decreased in renal recipients compared to the control group (39.5 ± 6.8 mg/dL vs. $43.7 \pm 3.3^{**}$ mg/dL, $p = 0.0083$).

Table 3: Clinical characteristics, lipid and apoprotein data of renal transplant recipients and matched controls; Data were analyzed using Mann-Whitney tests, and are given as means $\pm$ SD.; patients vs. controls *p<0.05, ** p<0.01, *** p<0.001.

	Patients	Controls
N	15	15
<i>Clinical characteristics</i>		
Gender (f/m)	(7/8)	(7/8)
Age (years)	48 $\pm$ 6	47 $\pm$ 5
BMI [kg/m ²]	25.1 $\pm$ 3.5	26.5 $\pm$ 4.7
Smoking (yes/no)	(7/8)	(4/11)
Dialysis duration (months)	17.4	-
GFR [mL/min/1.73 m ²]	65 $\pm$ 13	-
Creatinine [mg/dL]	1.2 $\pm$ 0.36	0.9 $\pm$ 0.2**
Albumin [g/dL]	4.5 $\pm$ 0.35	4.7 $\pm$ 0.28
<i>Serum lipids</i>		
Cholesterol [mg/dL]	222 $\pm$ 51.3	213 $\pm$ 32.3
Triglycerides [mg/dL]	154 $\pm$ 80.2	122 $\pm$ 66.3
LDL-C [mg/dL]	136 $\pm$ 12	133.5 $\pm$ 8.5
HDL -C [mg/dL]	55.5 $\pm$ 4.7	55.1 $\pm$ 3.05
FC [mg/dL]	69.9 $\pm$ 18.6	59.6 $\pm$ 10.4
Phospholipids [mg/dL]	250 $\pm$ 43.9	240 $\pm$ 29
FFA [mmol/L]	0.6 $\pm$ 0.3	0.7 $\pm$ 0.3
<i>Serum apoproteins</i>		
Apo A-I [mg/dL]	160 $\pm$ 37.4	156 $\pm$ 20.9
Apo A-II [mg/dL]	39.5 $\pm$ 6.8	43.7 $\pm$ 3.3**
Apo B [mg/dL]	103 $\pm$ 31.6	103 $\pm$ 20.5
Apo C-II [mg/dL]	5.5 $\pm$ 2.7	5.2 $\pm$ 1.9
Apo C-III [mg/dL]	15 $\pm$ 7.7	11.9 $\pm$ 3.9
Apo C-II/Apo C-III [mg/dL]	0.38 $\pm$ 0.02	0.44 $\pm$ 0.02
Apo E [mg/dL]	12.2 $\pm$ 3.1	11.1 $\pm$ 1.8
Lp (a) [mg/dL]	23 $\pm$ 21.3	16.5 $\pm$ 16.8

Abbreviations: GFR, glomerular filtration rate; LDL-C, low-density lipoprotein cholesterol, calculated according to Equation 3; HDL-C, high-density lipoprotein cholesterol; FC, free cholesterol; FFA, free fatty acids; Apo, apolipoprotein; Lp(a), lipoprotein(a).

$$LDL - C = TC - (HDL - C + \frac{TG}{5})$$

Equation 3: LDL-C calculation according to Friedewald's formula; TC, total cholesterol; TG, Triglycerides; mg/dL;

3.1.1 Effects of renal transplantation on the cholesterol acceptor capacity of serum

To determine the effects of renal transplantation on the cholesterol acceptor capacity of serum, either THP-1 cells differentiated towards macrophages using PMA, or RAW macrophages were used. Cells were loaded with cholesterol using acetylated LDL and trace labelled with [³H]-cholesterol and incubated with a LXR agonist for maximal expression of ABCA1 and ABCG1; this assures that cholesterol efflux depends mainly on the cholesterol acceptor capacity of serum and HDL and also provides stable experimental conditions. Cholesterol acceptor capacity of 1 % serum was measured for 6 h in THP-1 and for 2 h in RAW cells after 24 h incubation with ac- LDL, [³H]-cholesterol, and LXR agonist.

The cholesterol acceptor capacity of serum from renal transplant patients compared to controls was neither altered in the THP-1 nor in the RAW cell model, (Figure 7). Cholesterol acceptor capacity of serum from renal transplant recipients was 36.1 ± 3.4 % vs. 38.2 ± 3.3 % in controls using the THP-1 cell model. Cholesterol efflux was clearly lower in RAW cells, amounting to about 25 % of the values measured in THP-1 cells. Nevertheless the unaltered cholesterol acceptor capacity of renal transplant patient's serum could be confirmed in this second cell line (9.9 ± 2.2 % vs. 7.7 ± 2 %, n.s.)

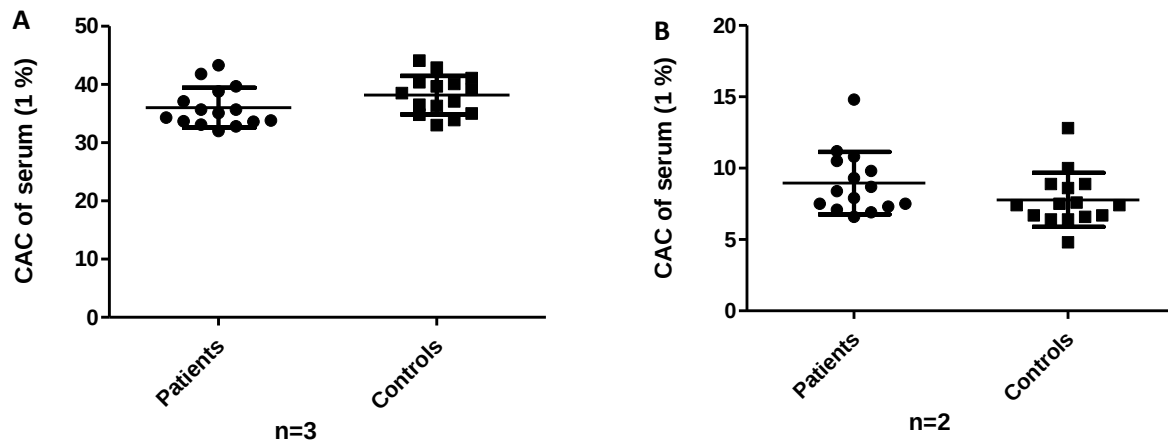


Figure 7: Cholesterol acceptor capacity of serum from renal transplant recipients vs. controls (1 %) measured in THP-1 cells (panel A, three independent experiments) and RAW cells (panel B, two independent experiments); Cholesterol acceptor capacity is expressed in %; Data were analyzed using Mann-Whitney tests and are given in mean $\pm$ SD.

3.1.2 Effects of renal transplantation on the cholesterol acceptor capacity of HDL

To measure the cholesterol acceptor capacity of HDL from renal transplant recipients compared to controls, THP-1 cells, differentiated towards macrophages, or RAW macrophages were incubated with ac-LDL, LXR agonist and [3 H]-cholesterol one day prior to the efflux experiments as described above. Efflux experiments were conducted using 10 μ g/mL of HDL as cholesterol acceptor for 6 h in THP-1 cells and for 2 h in RAW cells.

HDL cholesterol acceptor capacity of renal transplant patients did not differ from control in THP-1 cells (patients 18 ± 5 vs. controls 16.9 ± 4 %; n.s.). Like in experiments using serum, efflux percentages measured using RAW cells (patients 1.8 ± 0.5 % vs. controls 2 ± 0.4 %; n.s.) were significantly lower compared to THP-1 cells, but the result of an unchanged cholesterol acceptor capacity of HDL could be confirmed in this second cell line (Figure 8).

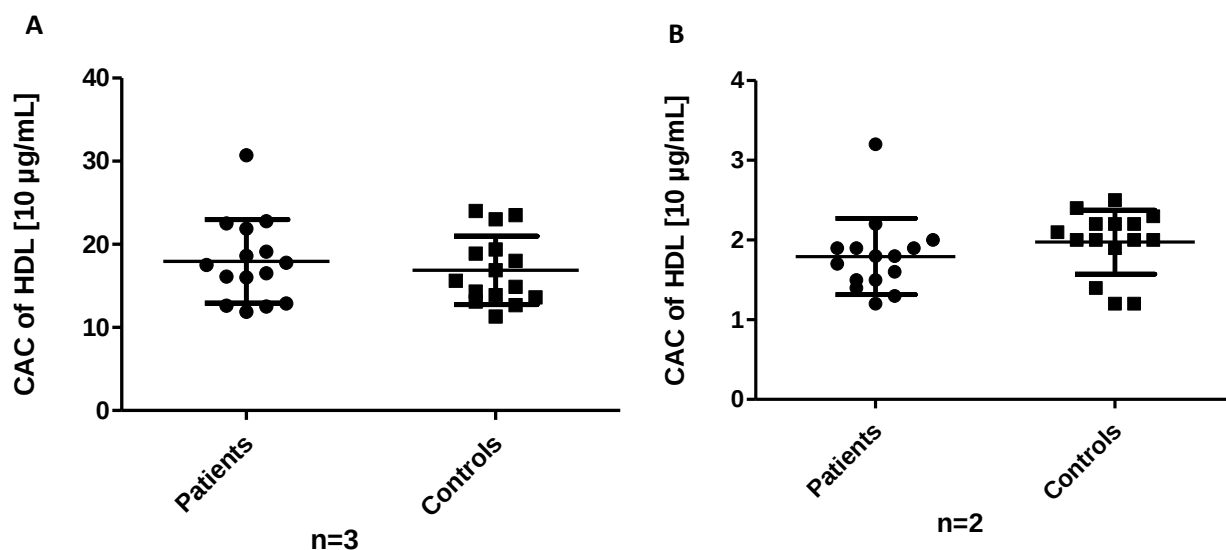


Figure 8: Cholesterol acceptor capacity of HDL from renal transplant recipients vs. controls [10 µg/mL] measured in THP-1 cells (panel A, three independent experiments) and RAW cells (panel B, two independent experiments); Cholesterol acceptor capacity is expressed in %; Data were analyzed using Mann-Whitney tests and are given in mean $\pm$ SD.

3.1.3 Effects of renal transplantation on the cholesterol export capacity of HMDM

To analyze the contribution of the cells to the cholesterol efflux process in addition to the role played by cholesterol acceptors, the cholesterol export capacity of human monocyte derived macrophages was assayed. Monocytes were isolated from venous blood and differentiated towards macrophages under autologous serum for a week. One day prior to efflux they were incubated with tritium labelled cholesterol. Cellular cholesterol export capacity was measured using pooled control serum 1 % or HDL [10 µg/mL] for 6 h (Figure 9).

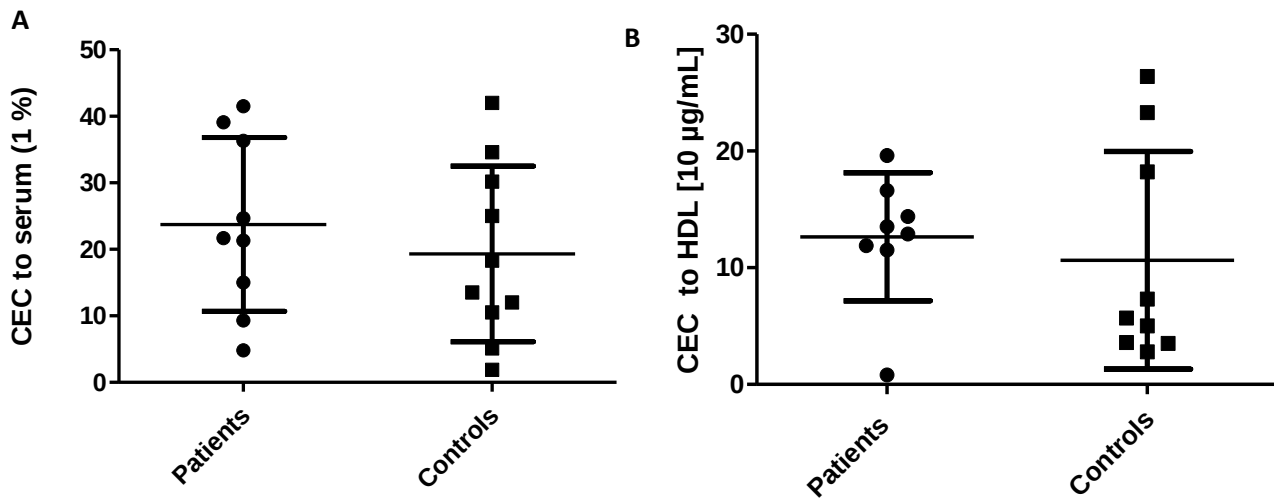


Figure 9: Cholesterol export capacity of human monocyte derived macrophages from renal transplant recipients vs. controls towards pooled control serum (1 %) (panel A, measured in triplicates) and pooled control HDL [10 µg/mL] (panel B, measured in triplicates); Cholesterol export capacity is expressed in %; Data were analyzed using Mann-Whitney tests and are given in mean ± SD.

Cholesterol export of HMDM to pooled control serum showed no significant differences between patients and controls but was about twice as high as the cholesterol export to pooled control HDL; patient's HMDM cholesterol export to pooled control serum was unchanged compared to the control group (n=9; 23.7±13.1 % vs. n= 10; 19.3±13.2 %; n.s.; 7 matched pairs). Similarly the cholesterol export capacity of patient's HMDM to pooled control HDL did not differ from control (n=8; 12.7±5.5 % vs. n=9; 10.6±9.3 %; n.s.; 5 matched pairs).

3.1.4 Correlation of cholesterol acceptor and export capacities

In earlier studies the cholesterol export capacity of HMDM from dialysis patients was inversely correlated to cholesterol acceptor capacity of serum and HDL (Meier et al., submitted). Therefore the cholesterol export capacity of HMDM from renal transplant

recipients and controls and the cholesterol acceptor capacity of their serum and HDL for correlations were analyzed, Figure 10. The cholesterol export capacity of patients and matched controls did not correlate with the cholesterol acceptor capacity of patients and matched controls neither for HDL ($r=-0.03199$, $p=0.8977$) nor for serum ($r=-0.2817$, $p=0.2426$).

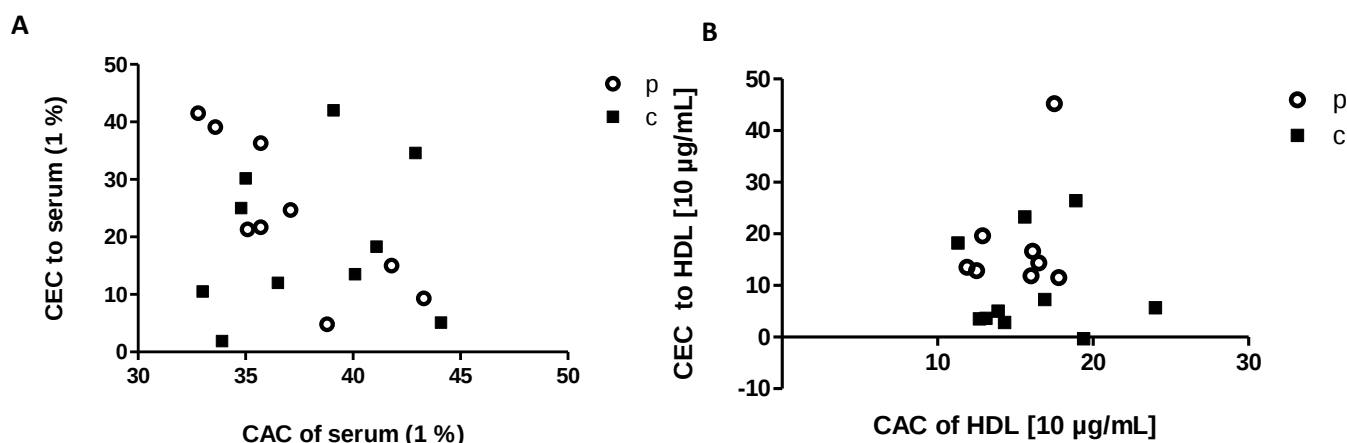


Figure 10: Correlations between CAC of serum or HDL and CEC of HMDMs from renal transplant recipients vs. controls towards pooled control HDL and serum; (A) Data of CAC and CEC towards serum from patients and matched controls, $r=0.2817$, $p=0.2426$; (B) Data of CAC and CEC of HDL from patients and matched controls, $r=-0.03199$, $p=0.8977$;

3.1.5 Cholesterol acceptor capacity of pooled sera of chronic kidney disease, hemodialysis, and renal transplant patients in RAW 264.7 cells

The next objective was to compare the data of renal transplant recipients to patients in other stages of chronic kidney disease (CKD) studied earlier. Therefore pooled serum samples collected in earlier studies from patients with CKD stage 3-5 and from patients on hemodialysis, as well as the pooled sera of renal transplant recipients and matched controls collected in the present study were analyzed for their cholesterol acceptor capacity in RAW cells (Figure 11). In these earlier studies (Meier et al., submitted), performed in THP-1 cells, the cholesterol acceptor capacity of dialysis patient's serum

was reduced significantly ($p < 0.001$) likely due to low apoA-I levels. In contrast there were no obvious differences between all three groups and their matched controls in the RAW cell model used in the present study. Pooled serum of renal transplant patients showed a CAC of 24.6 ± 2.81 %, which did not differ from the matched control group (23.4 ± 2.12 %); likewise the hemodialysis group (patients 24.4 ± 3.3 % vs. controls 25 ± 0.82 %) and the CKD group (patients 23.9 ± 3.54 % vs. controls 26 ± 3.01 %) showed no drastic changes of CAC of pooled serum in the RAW cell model.

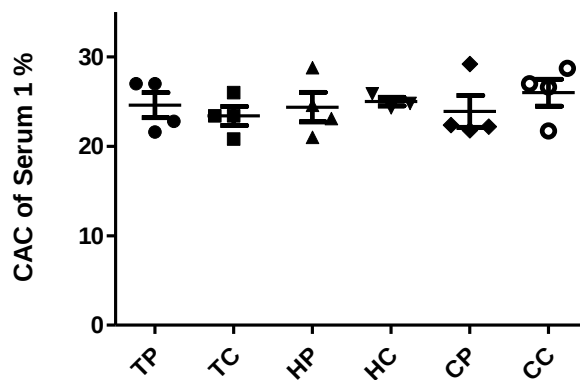


Figure 11: Cholesterol acceptor capacity of pooled serum of renal transplant, dialysis, and CKD patients and their matched controls in RAW cell model; TP, transplant patients; TC, transplant controls; HP, hemodialysis patients; HC, hemodialysis controls; CP, CKD patients; CC, CKD controls; Data were analyzed using One way ANOVA, Kruskal-Wallis test, non-parametric and are given in mean $\pm$ SD.

3.2 Effects of cross gender hormone therapy on macrophage cholesterol efflux

Male to female

Cross gender hormone therapy resulted in several changes in the plasma apoprotein pattern, plasma phospholipids, and creatinine concentrations, and produced the expected alterations in hormone levels. Results of lipid, apoproteins analyses, clinical characteristics and hormone status of transgender patients are listed in

Table 4.

ApoA-I (before 163 ± 8.7 mg/dL vs. after $200 \pm 7.1^{**}$ mg/dL, $p=0.0039$) and A-II (before 42.6 ± 1.9 mg/dL vs. after $48.5 \pm 2.6^{**}$ mg/dL, $p=0.0078$) increased after hormone therapy. The ratio of apo C-II/C-III decreased after hormone treatment before (0.45 ± 0.022 mg/dL vs. after $0.36 \pm 0.023^{**}$ mg/dL, $p=0.0039$).

Surprisingly serum lipoprotein and lipids showed no significant changes after hormone treatment except for an increase in serum phospholipids (before 214 ± 13.6 mg/dL vs. after $244 \pm 7.7^*$ mg/dL, $p=0.012$). Plasma serum creatinine was decreased after hormone therapy (1 ± 0.03 mg/dL vs. $0.9 \pm 0.03^*$ mg/dL, $p=0.0322$).

As expected, serum estradiol and SHBG levels increased from 33.4 ± 5.7 pg/mL to $276 \pm 127.8^{**}$ pg/mL with $p=0.002$ and 46.4 ± 4.1 nmol/L to $92.3 \pm 14.24^*$ nmol/L with $p=0.0195$, respectively. Testosterone decreased from 6 ± 0.5 ng/mL to $0.23 \pm 0.06^{**}$ ng/mL with $p=0.002$.

Table 4: Clinical characteristics, lipid and apoproteins data, and hormone status of transgender patients before and after cross gender hormone therapy. Data were analyzed using Wilcoxon tests. Data are given as means $\pm$ SD.; before vs. after; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

	M-F		F-M	
N	10		15	
Clinical characteristics				
Age (years)	42±14		33±6	
BMI [kg/m ²]	23.8±2.7		24.3±5.1	
Smoking (yes/no)	(3/7)		(7/8)	
	<i>before</i>	<i>after</i>	<i>before</i>	<i>after</i>
Albumin [g/dL]	5.1±0.4	5.1±0.4	5.4±0.49	5.1±0.69
Creatinine [mg/dL]	1±0.08	0.9±0.1*	0.95±0.123	1.05±0.108*
Serum lipids				
Cholesterol [mg/dL]	191±32.8	205±22.1	199±34.1	201±42.8
Triglycerides [mg/dL]	87±37	89±40.6	73.9±33	87.5±52.2
LDL-C [mg/dL]	109±28.4	109±20.8	114±34.6	124±34.2
HDL-C [mg/dL]	64±10.8	78.3±14.8	70.13±11.7	59.9±15.7
FC [mg/dL]	50.7±8.6	54±6.5	51.7±9.3	53.3±12.1
Phospholipids [mg/dL]	214±40.9	244±24.5*	224±30.2	218±41.9
FFA [mmol/L]	0.84±0.43	0.8±0.29	0.8±0.4	0.7±0.51
Serum apoproteins				
Apo A-I [mg/dL]	163±26	200±22.5**	177±25.2	154±34.1*
Apo A-II [mg/dL]	42.6±5.7	48.5±8.3**	43.9±5.2	41.5±7.7
Apo B [mg/dL]	83.6±18.1	84±16.8	87.5±22	94.4±25.5
Apo C-II [mg/dL]	4.4±1.6	4.4±1.3	3.7±1.1	4.8±2*
Apo C-III [mg/dL]	9.9±3.2	12.1±2.8	10±1.9	12±3.7
Apo C-II/Apo C-III [mg/dL]	0.45±0.07	0.36±0.07**	0.36±0.062	0.39±0.068
Apo E [mg/dL]	12.4±2.8	13.1±2.6	12.5±2.5	11.9±2.1
Lp (a) [mg/dL]	20±22.5	22.8±28.3	27.8±28.8	25±27.7
Hormone status				
Estradiol [pg/mL]	33.4±18	277±727.4**	134±111.4	84.3±151*
LH [mU/mL]	10.7±14.7	14.5±24.6	7.9±7.5	26.3±30.2
SHBG [nmol/L]	46.4±13	92.3±45*	58.4±28.8	30.3±10.2***
FHS [mU/mL]	5.8±5	17.57±27	5.8±2.7	46.3±55.6*
Testosterone [ng/mL]	6±1.6	0.23±0.18**	0.48±0.2	6±3***

Abbreviations: M-F, male to female; F-M, female to male; LDL-C, low-density lipoprotein cholesterol, calculated according to Equation 3; HDL-C, high-density lipoprotein cholesterol; FC, free cholesterol; FFA, free fatty acids; Apo, apolipoprotein; Lp(a), lipoprotein(a); LH, luteinizing hormone; SHBG, sexual hormone binding globulin; FSH, follicle stimulating hormone.

Female to male

In contrast to male to female patients, serum apoA-I levels decreased in the case of female to male patients after hormone therapy (177 ± 6.5 mg/dL vs. $154 \pm 8.8^*$ mg/dL, $p=0.0353$). In serum from these patients apoC-II increased from 3.7 ± 0.3 mg/dL to $4.8 \pm 0.5^*$ mg/dL ($p=0.011$), and creatinine levels increased (0.95 ± 0.03 mg/dL vs. $1.05 \pm 0.03^*$ mg/dL, $p=0.0135$) (Table 4).

Serum lipids and lipoproteins of female to male patients did not differ from baseline values after hormone treatment.

Female to male patient's hormone levels also changed drastically with decreased estradiol (134 ± 28.8 pg/mL to $84.3 \pm 39^*$ pg/mL, $p=0.0494$) and SHBG levels (58.4 ± 7.44 nmol/L to $30.3 \pm 2.64^{***}$ nmol/L, $p=0.0002$). Female to male patient's testosterone levels increased from 0.48 ± 0.053 ng/mL to $6 \pm 0.8^{***}$ ng/mL ($p<0.0001$).

3.2.1 Effects of cross gender hormone therapy on cholesterol acceptor capacity of serum

The effects of cross gender hormone therapy on the cholesterol acceptor capacity of serum were determined using either THP-1 cells, which were differentiated towards macrophages using PMA, or RAW 264.7 macrophages. Macrophages were incubated with acetylated LDL for cholesterol loading, trace-labelled with [3 H]-cholesterol, and treated with a LXR agonist for maximal expression of ABCA1 and ABCG1. Efflux experiments were performed the next day; Cholesterol acceptor capacity of serum (1 %) was measured in THP-1 and RAW 264.7 cells, which were incubated for 6h and for 2 h, respectively.

Male to female

The hormone treatment resulted in significant changes of cholesterol acceptor capacity of serum measured in the THP-1 cell line (Figure 12). Unexpectedly, cholesterol acceptor capacity was reduced from 29.6 ± 1.2 % to $26 \pm 1.1^{**}$ % ($p=0.0059$) after male to female hormone therapy. Although a slight decrease could also be observed in RAW 264.7 cells, there were no significant changes after hormone therapy (23.1 ± 1 % vs. 21.6 ± 0.9 %; n.s.).

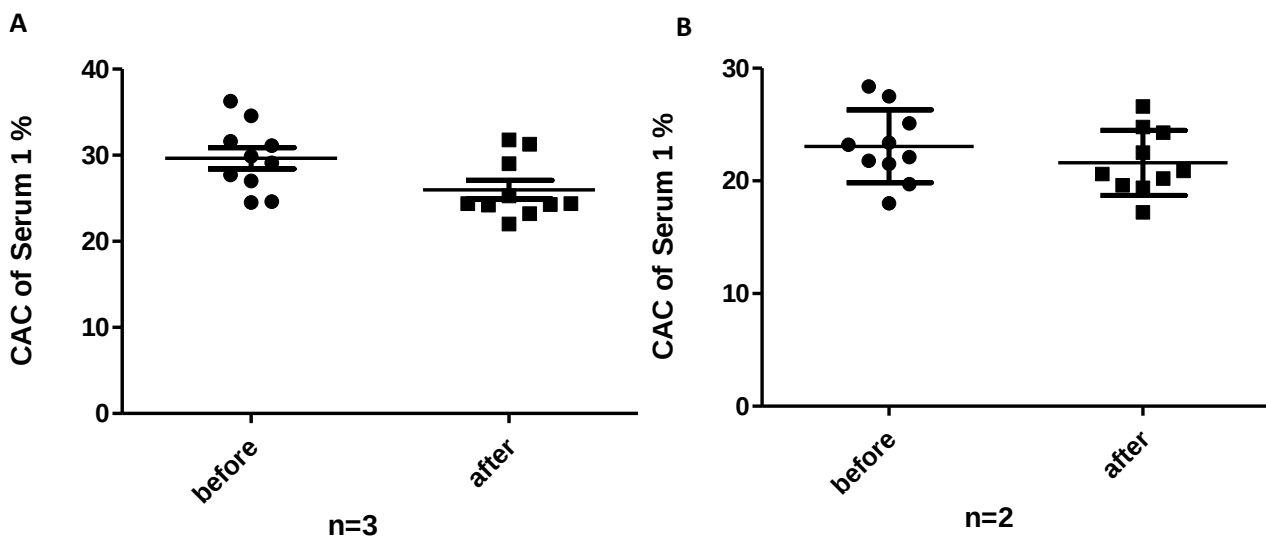


Figure 12: Effects of cross gender hormone therapy on the cholesterol acceptor capacity of serum in male to female transsexuals before and after hormone treatment measured in THP-1 (panel A, three independent experiments) and using RAW 264.7 macrophages (panel B, two independent experiments); Data were analyzed using Wilcoxon tests and are given in mean $\pm$ SD.

Female to male

Despite the drastic changes in hormone status of female to male patients, no significant changes on cholesterol acceptor capacity of serum could be observed using THP-1 cells ($25 \pm 0.9\%$ vs. $22.8 \pm 0.9\%$; n.s.) (Figure 13). This could be confirmed in RAW 264.7 cells, the second cell line ($12.5 \pm 0.44\%$ vs. $12.9 \pm 1.2\%$; n.s.).

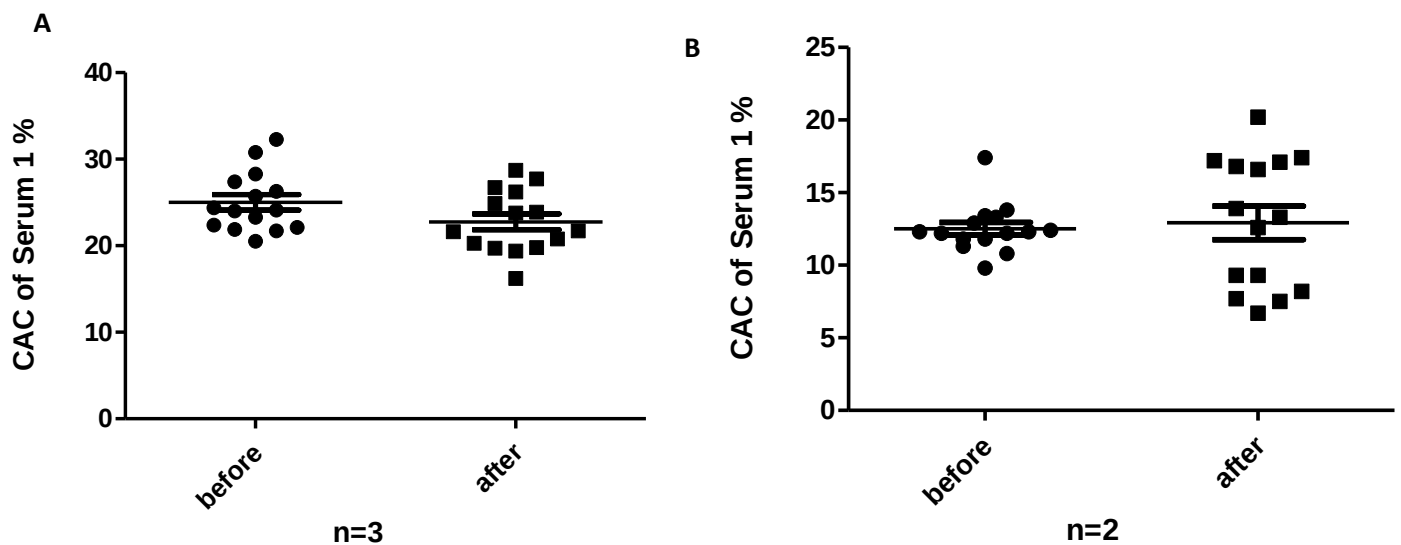


Figure 13: Effects of cross gender hormone therapy on the cholesterol acceptor capacity of serum in female to male transsexuals before and after hormone treatment measured in THP-1 cells (panel A, three independent experiments) and using RAW 264.7 cells (panel B, two independent experiments); Data were analyzed using Wilcoxon signed rank tests and are given in mean $\pm$ SD.

4 Discussion

CVDs are a rising cause of death worldwide. CKD patients on hemodialysis, but also renal transplant recipients as well as men and postmenopausal women are more prone to CVDs than the general population (Badeau et al., 2009; Badeau et al., 2013; Jardine et al., 2011; Mendelsohn and Karas, 2005; Ostovan et al., 2006; Reslan and Khalil, Rossi et al., 2002; 2012; Vitale et al., 2009; Vitale et al., 2010; Watorek et al., 2011).

Atherosclerosis, the main underlying cause of CVDs is influenced by many different factors, such as genetical predisposition, smoking, dyslipidemia, hypertension, high fat diet, diabetes, and physical inactivity. There are also specific somatic pathways, which can influence the outcome of atherosclerosis to a certain degree; one of these pathways is the RCT, an anti-atherogenic pathway for excretion of excessive cholesterol from cholesterol loaded macrophages (foam cells) in arterial walls via HDL particles, other lipoproteins or apoproteins back to the liver for excretion in the bile and finally the feces. The very first step of the RCT, macrophage cholesterol efflux presents the crucial link to atherosclerosis and is defined by the cholesterol efflux from macrophages, turned into foam cells, to HDL particles or apoA-I proteins (Cuchel and Rader, 2006; Hersberger and von Eckardstein, 2003; Mairuri et al., 2012; Tall, 2008).

4.1 Renal transplant recipients

Several studies have addressed the effects of CKD and hemodialysis on macrophage cholesterol efflux and especially on HDL function (Holzer et al., 2011; Meier et al., submitted; Weichhart et al., 2012). Although there are many studies concerning adverse effects of immunosuppressants and change on HDL particle composition after renal transplantation, no research on the effects of renal transplantation on macrophage cholesterol efflux has been published, to the best of our knowledge. Thus, the one main

aim of this thesis was to answer the question whether renal transplantation improves macrophage cholesterol efflux.

The first goal was to determine the cholesterol acceptor capacity of serum and HDL from renal transplant recipients. These results showed that the cholesterol acceptor capacities of serum and HDL from renal transplant recipients were unaltered compared to matched controls in both cell lines used.

Previous work from this laboratory (Meier et al. submitted) showed a slight increase of cholesterol acceptor capacity of serum from patients with CKD stage 3-5. Serum from hemodialysis patients, however, had a significantly decreased cholesterol acceptor capacity, which was linked to decreased apoA-I concentrations. ApoA-I levels in renal transplant recipients did not differ from matched controls. This was not a longitudinal study, and the number of participants in this study was relatively low (patients n=15; controls n=15). However, controls were matched carefully and patients and controls were recruited using strict exclusion criteria. Therefore the unchanged apoA-I levels could indicate an improvement after successful renal transplantation.

Despite an improvement of lipid pattern and macrophage cholesterol efflux after successful kidney transplantation, renal transplant recipients show a higher atherosclerosis risk compared to general population. One possible explanation may be an altered composition of HDL-C in renal transplant patients leading to impaired anti-atherogenic or even pro-atherogenic properties of HDL independent from macrophage cholesterol efflux (Kimak et al., 2010; Kimak et al., 2011).

HDL displays various other anti-atherogenic properties such as antioxidant and anti-inflammatory properties, mediated for example by the HDL-associated enzymes platelet activating factor acetylhydrolase (PAF), paraoxonase, and glutathione peroxidase. However, those anti-atherogenic properties are lost in the dysfunctional plasma HDL of patients suffering from systemic inflammation, coronary heart disease, diabetes, and chronic renal disease (Scanu and Edelstein, 2008).

In 2011, Holzer et al. published a proteomic and lipid study of HDL from CKD patients on maintenance hemodialysis revealing a protein and lipid composition, which differs

significantly from healthy controls. Not only were the HDL particles composed of clearly increased serum amyloid A (SAA), apoC-III, albumin, lipoprotein-associated phospholipase A2, triglycerides, and lysophospholipid, but also contained distinctly decreased phospholipids; thereby impairing HDL's feature to facilitate cholesterol efflux from macrophages (Holzer et al., 2011).

One year later, Weichhart et al. (2012) also investigated the impairment of HDL in CKD patients and could link the loss of the anti-inflammatory role of HDL to a protein, namely serum amyloid A, which is enriched in HDL of CKD patients (Weichhart et al., 2012).

There are no functional studies of HDL from renal transplant recipients, but a research group from Poland investigated the relationships between lipoproteins, serum lipids, triglyceride-rich lipoproteins and HDL in post-renal transplant patients. They could show that post-renal transplant patients displayed disturbed composition, concentration and metabolism of triglyceride-rich lipoproteins and HDL particles. Those findings led them to suggest changes in the HDL subclasses and their distribution into smaller size particles (Kimak et al., 2010).

ApoA-II levels in the present study were decreased significantly in patients compared to the control group. ApoA-I/apoA-II ratios were also higher compared to hemodialyzed patients (Meier et al., submitted). The reduced serum apoA-II levels could be explained by a shift of HDL subclasses, to either HDL₂, which are rich in apoA-I, or pre- β particles, containing apoA-I only. According to Patsch et al. (1987), HDL₂ consists of 80.1 % apoA-I and 12.0 % apoA-II with a particle size of 8.8-12.9 nm and HDL₃ of 65.7 % apoA-I and 25.7 % apoA-II with a particle size of 7.2-8.8 nm (Patsch et al., 1987). Pre- β - particles do not contain apoA-II and are assumed to be the most effective acceptors of cellular cholesterol via ABCA1, and are therefore anti-atherogenic (Barrans et al., 1996; Castro and Fielding, 1988; Huang et al., 1993; Joy and Hegele, 2008). Recently Kimak et al. observed a trend towards smaller HDL particles in renal transplant recipients compared to healthy controls. Taken together this suggests that in renal transplant recipients studied here, HDL particles may shift to pre- β -HDL rather than HDL₂ after renal transplantation (Kimak et al., 2010; Kimak et al., 2012; Patsch et al., 1987). This could explain the improved cholesterol acceptor capacity of HDL and serum from renal

transplant recipients from this study compared to hemodialyzed patients studied earlier (Meier et al., submitted).

In addition to the experiments investigating the cholesterol acceptor capacity of serum from newly recruited renal transplant recipients and matched controls described above, an attempt was made to reproduce earlier findings on cholesterol acceptor capacity of hemodialyzed and CKD patients obtained in THP-1 cells (Meier et al., submitted), in RAW 264.7 cells. Therefore the cholesterol acceptor capacity of pooled serum of CKD and hemodialysis patients and matched controls was determined in RAW 264.7 cells. Interestingly, the earlier results showing a decreased cholesterol acceptor capacity of serum from hemodialysis patients (Meier et al., submitted) could not be confirmed in this second cell line. This might be caused by differences in cellular cholesterol metabolism.

RAW 264.7 were established in 1978 by Raschke et al., two years later, in 1980, Tsuchiya et al. first mentioned the human acute monocytic leukemia cell line THP-1 and the additional treatment with PMA for macrophage differentiation (Qin, 2012; Raschke et al., 1978; Tsuchiya et al., 1980). Both cell lines are frequently used to analyze macrophage functions. The immortalized cell lines possess advantages in comparison to primary cells, for example storage, handling and availability, but the fact that they stem from tumors must be considered in the analysis of results (Qin, 2012).

The mobilization of cholesterol is a subject of intensive study. Only unesterified or free cholesterol can be exported from cells to extracellular cholesterol acceptors. Therefore hydrolysis of cholesterol ester by cholesterol ester hydrolases (CEH) is the first intracellular step of cholesterol export efflux from macrophages (Brown et al., 1980). Expression of these CEH differs between THP-1 and RAW cells. The exact identity of CEH has not been clarified yet, but there are likely candidates (Ouimet and Marcel, 2012). The carboxylesterase 1 (CES1), or cholesteryl ester hydrolase is expressed in human, but not in murine macrophages. Overexpression of CES1 in macrophages decreases CEH mass and increases cholesterol efflux (reviewed in Ouimet and Marcel, 2012). The hormone sensitive lipase (HSL) became an enzyme of interest because it is activated by cAMP, which increases efflux of FC from macrophages (Goldberg and Khoo, 1990; Small

et al., 1989). Although the expression of HSL in murine macrophages was demonstrated by Khoo et al. (1993), expression of HSL in human THP-1 cells remains debatable. Gosh et al. (2010) were not able to detect expression of HSL in THP-1 macrophages or in HMDMs (Gosh et al., 2010; Li and Hui, 1997). The role of HSL in CE hydrolysis remains unclear, because effects of HSL deficiency on atherosclerosis and the necessary interaction of HSL with lipid droplets, the natural form of CE in foam cells, has not been studied efficiently (Gosh et al., 2010; Ouimet and Marcel, 2012). The third enzyme regarded as a possible candidate is KIAA1363, with the murine ortholog neutral cholesterol ester hydrolase 1 (Nceh1). Knockout of this enzyme in macrophages leads to an increase of cellular CE and to a reduced cholesterol efflux (Ouimet and Marcel, 2012).

Kritharides et al. (1998) investigated the cholesterol efflux and metabolism in THP-1 macrophages and reported that THP-1 macrophages do not accumulate esterified cholesterol as much and as fast as RAW 264.7 macrophages when stimulated with ac-LDL. Also hydrolysis of cytosolic esterified cholesterol is much higher in RAW 264.7 macrophages than in THP-1 macrophages (Brown et al., 1979; Kritharides et al., 1998). This is mirrored in the efflux incubation protocols for RAW 264.7 and THP-1 cells, where incubation with cholesterol acceptors is clearly longer for THP-1 macrophages (6 h) compared to RAW 264.7 cells (2 h). The incubation times were selected based on earlier time course experiments (Meier et al., unpublished) to ensure measurements during the linear phase of cholesterol efflux experiments. Other cell types, like Fu5AH hepatoma cells, and different subclasses of HDL particles (Lagrost et al., 1995) need different incubation periods.

In this study THP-1 cells were chosen, because they stem from a human donor and are relatively well studied. Qin (2012) reviewed data on PMA treated THP-1 cells reporting a down-regulation of LDL receptor expression and an up-regulation of scavenger receptors. This allows PMA treated THP-1 macrophages to take up modified lipoproteins and turn into foam cells which provide a reasonable model for monocyte-macrophages in the arterial wall during atherogenesis (reviewed by Qin, 2012). THP-1 macrophages express similar gene patterns compared to primary macrophages (reviewed in Qin, 2012). However several differences in basal and stimulated THP-1 cells exist (Kohro et

al., 2004). Primary cells would be the optimal cell model to study changes of macrophage cholesterol efflux in response to certain treatments, but primary cells, like HMDMs present many obstacles. HMDM account for only 3-9 % of all leukocytes, therefore their availability is limited. Also donor accessibility, isolation and differentiation of monocytes towards macrophages and their short survival in cell culture presents challenges (Qin, 2012). Unfortunately data on a direct comparison of cholesterol efflux of RAW 264.7, THP-1, and HMDM do not exist, to the best of our knowledge.

The second goal was to analyze cholesterol export capacity of HMDM isolated from renal transplant recipients. Cellular cholesterol efflux does not only depend on the capacity of the acceptors to take up cholesterol, but also on the export capacity of macrophages. Therefore HMDM were differentiated under autologous serum without cytokines to closely reflect the metabolic state of the patients.

Cholesterol export capacity of HMDM did not display any significant changes between renal transplant recipients and controls; this is in contrast to earlier observations in hemodialyzed patients, where cholesterol export capacity was increased nearly twofold compared to matched control, which might function as an adaptive response to the decreased cholesterol acceptor capacity of serum (Meier et al., submitted). The unaltered cholesterol export capacity in renal transplant recipients could indicate that cellular cholesterol metabolism can be normalized after successful renal transplantation.

Serum creatinine levels of renal transplant recipients were significantly higher compared to control creatinine levels. However, both groups lie within the range of normal creatinine levels. Creatinine, a breakdown product of creatine, is transported to the kidney for excretion via urine; therefore high creatinine levels can indicate kidney dysfunction (www.medicinenet.com, 2013).

4.2 Transgender patients

CVD risk is different between men pre-menopausal and menopausal women (Badeau et al., 2009; Badeau et al., 2013; Jardine et al., 2011; Mendelsohn and Karas, 2005; Ostovan et al., 2006; Reslan and Khalil; Watorek et al., 2011). Because women develop heart disease later in life than men, sex steroids, especially estrogen, were long thought to be responsible for this difference. Intervention studies like the Estrogen/progestin Replacement Study (HERS) and the Women's Health Initiative (WHI) failed to show the expected beneficial effects of estrogen on CVDs during HRT (Mendelsohn and Karas, 2005; Reslan and Khalil, 2012; Turgeon et al., 2004). Although estrogen indeed can display anti-atherogenic properties, these intervention studies showed that estrogen can also act pro-atherogenic. The timing of HRT as well as pre-existing atherosclerotic plaques play an important role for a beneficial effect of HRT; if administered too late after onset of menopause to women with already advanced atherosclerotic plaques, HRT can be pro-atherogenic (Mendelsohn and Karas, 2005; Reslan and Khalil, 2012). In concurrence with these results, Wang et al. (2011) recently reviewed data on sex differences in lipid and lipoprotein metabolism and supported the thesis that the cardio-protective lipid profile of premenopausal women is not entirely caused by the differences in sex steroids (Wang et al., 2011).

Only a few studies have addressed the effects of sex steroids on macrophage cholesterol efflux: Badeau et al. proposed an enhancement of macrophage cholesterol efflux by HDL-associated 17 β -estradiol fatty acyl esters; however, the authors were not able to duplicate these results in a larger study (Badeau et al., 2009; Badeau et al., 2013). Another study investigated cholesterol efflux capacity of serum and HDL of men and women with comparable HDL-C levels, showing a higher SR-B1-associated cholesterol efflux capacity in women; this effect was explained by a higher concentration of mature plasma HDL and their higher cholesterol acceptor capacity. Serum from men, however, showed a higher cholesterol efflux capacity via ABCA1 (Catalano et al., 2008). Interestingly, Darabi et al. proposed an increase of ABCA1 gene expression in leukocytes of post-menopausal women after HRT (Darabi et al., 2010).

Transgender patients on cross gender hormone therapy present an interesting model to study effects of exogenous sex steroids, due to the rapid and drastic changes of their hormone status. Indeed, there are several studies using transgender patients on cross gender hormone therapy to investigate hormonal effects, for instance, on the serum lipid profile (Ott et al., 2011; Elamin et al., 2010), fat cell size (Elbers et al., 1999), insulin resistance (Cupisti et al., 2010; Elbers et al., 2003), body composition (Berra et al., 2006; Mueller et al., 2011) or serum leptin levels (Elbers et al., 2009). To the best of our knowledge however, there are no studies on possible changes in macrophage cholesterol efflux after cross gender hormone treatment.

Therefore the third aim of this thesis was to investigate whether the cholesterol acceptor capacity of serum from MtoF and FtoM transsexuals, who underwent cross gender hormone therapy for at least six months, changed.

The investigation of effects of cross gender hormone therapy on the cholesterol acceptor capacity of serum revealed unexpected results. As mentioned before, estrogen can display a variety of beneficial anti-atherogenic properties *in vivo* and *in vitro* (Ayres et al., 1998; Christodoulakos et al., 2004; Orshal and Khalil, 2004; Sumino et al., 2003; Ushioda et al., 2006). Based on these findings and also on the increased apoA-I concentration the cholesterol acceptor capacity of serum from MtoF transsexuals was expected to be higher after hormone treatment. Contrary to this expectation, the cholesterol acceptor capacity of serum was reduced significantly after MtoF hormone therapy as determined in THP-1 cells. A similar trend was observed in RAW 264.7 cells, but did not reach significance. In these patients, however, the serum apoA-I/apoA-II ratio remained unchanged, which may explain at least in part why CAC did not increase. Lagrost et al. (1995) studied the changes of cholesterol efflux after altering the HDL₃ particle composition using Fu5AH hepatoma cells; they reported a decrease of cellular cholesterol efflux after substitution of apoA-II for apoA-I in HDL₃ particles without changing the other lipoprotein components (Lagrost et al., 1995). Still the reason for a decrease of cholesterol acceptor capacity of serum remains unclear. Further studies investigating a larger group of patients in a prospective manner and measuring the CAC of isolated HDL particles in addition to serum will be required to address this question.

Nevertheless, these data of a decreased cholesterol acceptor capacity are in line with the similarly unexpected findings of an unaltered or even increased atherosclerosis risk in male to female transsexuals (Asscheman et al., 2011).

In FtoM transsexuals no significant changes of the cholesterol acceptor capacity of serum after cross gender hormone treatment could be observed using THP-1 or RAW cells. This was unexpected, because serum apoA-I levels were lower after hormone treatment, which usually is associated with lower cholesterol efflux capacity. However, Khera et al. (2011), recently published a study on serum cholesterol acceptor capacity in a relatively large group of coronary patients and controls and reported that, although apoA-I and HDL levels were significant determinants of cholesterol efflux capacity, they only accounted for about 40 % of the observed variations in efflux (Khera et al., 2011). This leads to the question, whether cholesterol acceptors other than HDL and apoA-I can account for the unchanged cholesterol efflux in this study. Albumin is a known cholesterol acceptor, but serum albumin levels were not changed in the patients studied here and thus cannot be held responsible for the unaltered cholesterol efflux in FtoM transsexuals in the presence of reduced apoA-I. The finding of unchanged serum cholesterol acceptor capacity is consistent with the unaltered atherosclerosis risk of FtoM transsexuals (Asscheman et al., 2011; Maiga et al., 2013).

Surprisingly, serum lipids and lipoproteins did not change significantly during hormone treatment except for an increase of serum phospholipids in MtoF patients. In MtoF the ratio of apoC-II/apoC-III decreased during hormone therapy, whereas in FtoM apoC-II levels increased. These findings concur with data presented by Ashavaid et al. (2005) who reported a decreased ratio of apoC-II/apoC-III in women and higher levels of apoC-II in healthy Indian men (Ashavaida et al., 2005).

Elamin et al. reviewed and summarized 16 studies on cardiovascular effects of cross gender hormone therapy on transsexuals; they report that cross gender hormone therapy leads to an increase of plasma triglycerides in MtoF and FtoM and has a negligible effect on HDL-C and systolic blood pressure in FtoM. Due to methodical

limitations, imprecision and heterogeneity, however, those studies present very low quality evidence (Elamin et al., 2010). A retrospective cohort study of 171 transsexuals published by Ott et al. (2011), showed increased plasma triglycerides and total cholesterol in both MtoF and FtoM. LDL was increased in FtoM transsexuals, whereas HDL was increased in MtoF transsexuals, but decreased in FtoM (Ott et al., 2011). In the present study based on a small number of patients, TG levels in both MtoF and FtoM tended to increase but did not reach significance.

Creatinine levels decreased in MtoF and increased in FtoM during hormone therapy. This is consistent with the data from the body composition studies of MtoF transsexuals by Mueller et al. (2011), showing a decrease of lean body mass (Mueller et al., 2011) and of Berra et al. (2006) who found an increase of lean body mass in FtoM transsexuals (Berra et al., 2006)

In conclusion, data of renal transplant recipients indicate an improvement of macrophage cholesterol efflux, both in terms of cellular export and serum acceptor capacity, compared to hemodialysis patients. This is consistent with the reduced atherosclerosis risk in renal transplant recipients compared to hemodialysis patients (Watorek et al., 2011).

The data of transgender patients are contrary to the traditional view of the beneficial effects of estrogen and the detrimental effects of testosterone on the lipoprotein metabolism. This is consistent with other studies, showing that sex differences in lipid metabolism and ultimately development of atherosclerosis cannot be entirely explained by different hormone status of men and women (Mendelsohn and Karas, 2005; Wang et al., 2011).

5 Abstract

CKD patients on hemodialysis but also renal transplant recipients have an increased risk of atherosclerosis, compared to the general population. The transport of excess cholesterol from macrophages to HDL and apoA-I is the first step of reverse cholesterol transport and crucial for the atheroprotective effects of HDL. Earlier studies showed a decrease of serum cholesterol acceptor capacity (CAC) and an increase of cholesterol export capacity (CEC) of human monocytes derived macrophages (HMDM) in hemodialysis patients. Data on CEC and CAC in renal transplant recipients are not available.

Men and postmenopausal women have a higher atherosclerosis risk than premenopausal women. These differences were often attributed to sex steroids, but the role of estrogen and testosterone in atherosclerosis appears to be more complex than anticipated. Transsexuals on cross gender hormone therapy are an interesting model, which has been used to study hormonal effects on serum lipid profile, insulin resistance, and body composition. However, studies on macrophage cholesterol efflux are missing. In this thesis the effects of renal transplantation on CAC of serum and HDL, and the CEC of HMDM as well as the effects of cross gender hormone therapy on the CAC of serum from transsexuals were analyzed.

15 renal transplant recipients (age 48 ± 6 yrs., > 6 months after transplantation, $\text{GFR} < 60 \text{ ml/min/1.73m}^2$), 15 matched controls (age 47 ± 5 yrs.), 10 male to female (age 42 ± 14 yrs.) and 15 female to male (age 33 ± 6 yrs.) transsexuals were recruited using strict exclusion criteria to avoid confounding factors. [^3H]-CAC of serum and HDL was determined using cholesterol loaded THP-1 macrophages and RAW 264.7 cells. [^3H]-CEC was studied in HMDM differentiated towards macrophages under autologous serum using normal serum or HDL as acceptors.

Both CAC of HDL and of serum and CEC of HMDM were similar in renal transplant recipients and controls. Serum lipids, lipoproteins and apolipoproteins did not differ between renal transplant recipients and controls except for lower apoA-II levels in renal transplant recipients. This may indicate a shift of HDL subclasses towards pre-beta HDL

particles, which could explain the improved CAC of renal transplant recipients compared to hemodialyzed patients.

Unexpectedly, CAC of serum from male to female transsexuals were not increased but significantly decreased after hormone therapy as measured in THP-1 cells. Serum from female to male transsexuals showed no changes in CAC. These data are not in line with the traditional view that estrogen displays beneficial and testosterone detrimental effects on lipoprotein metabolism and ultimately atherosclerosis.

6 Zusammenfassung

Patienten mit chronischem Nierenversagen weisen vor allem während einer Hemodialysebehandlung aber auch noch nach einer Nierentransplantation ein deutlich höheres Atheroskleroserisiko auf, als die allgemeine Bevölkerung. Der Transport von überschüssigem Cholesterin von Makrophagen zu HDL und apoA-I ist der erste Schritt des reversen Cholesterin Transports und entscheidend für die antiatherogene Wirkung von HDL. Frühere Studien haben eine Verringerung der Cholesterin Akzeptor Kapazität von Serum und eine Erhöhung der Cholesterin Export Kapazität von HMDM von Dialysepatienten gezeigt. Daten bzgl. Cholesterin Export Kapazität und Cholesterin Akzeptor Kapazität von Nierentransplantierten Patienten sind nicht verfügbar.

Männer und postmenopausale Frauen weisen ein höheres Atheroskleroserisiko auf als premenopausale Frauen. Diese Differenzen werden oft den Effekten von Geschlechtssteroiden zugeschrieben, jedoch scheinen die Rollen von Östrogen und Testosteron in der Atherosklerose komplizierter zu sein als bisher angenommen. Transsexuelle, die sich einer gegengeschlechtlichen Hormontherapie unterziehen, sind ein interessantes Modell, welches benutzt wurde um hormonelle Effekte z.B. auf das Serumlipidprofil, die Insulinresistenz, und die Körperzusammensetzung zu studieren. Studien über den Makrophagen Cholesterin Efflux gibt es jedoch nicht.

In der vorliegenden Masterarbeit wurden die Auswirkungen von Nierentransplantation auf die Cholesterin Akzeptor Kapazität von Serum und HDL und die Cholesterin Export Kapazität von HMDM, sowie die Auswirkungen von gegengeschlechtlicher Hormontherapie auf die Cholesterin Akzeptor Kapazität von Serum von Transsexuellen analysiert.

15 Nierentransplantierte Patienten (Alter 48 ± 6 J., > 6 Monate nach der Transplantation, $GFR < 60 \text{ ml/min/1.73m}^2$) und 15 Kontrollen (Alter 47 ± 5 J.), sowie 10 Mann zu Frau (Alter 42 ± 14 J.) und 15 Frau zu Mann Transsexuelle (Alter 33 ± 6 J.) wurden nach strengen Ausschlusskriterien rekrutiert um Störfaktoren zu vermeiden. Die [^3H]-Cholesterin Akzeptor Kapazität von Serum und HDL wurde mittels Cholesterin beladenen THP-1 Makrophagen und RAW 264.7 Zellen bestimmt. Die

[³H]-Cholesterin Export Kapazität wurde in mit autologen Serum differenzierten HMDM gemessen, dabei wurden normales Serum und HDL wurden als Akzeptoren verwendet.

Sowohl die Cholesterin Akzeptor Kapazität von HDL und Serum als auch die Cholesterin Export Kapazität von HMDMs waren bei Nierentransplantierten Patienten im Vergleich zu Kontrollen unverändert. Auch die Serum Lipide, Lipoproteine und Apolipoproteine unterschieden sich nicht, mit der Ausnahme von niedrigeren ApoA-II Konzentrationen bei Nierentransplantierten Patienten. Dies könnte auf eine Verschiebung zu Gunsten von pre-β HDL Partikeln hinweisen, was wiederum die verbesserte Cholesterin Akzeptor Kapazität von Nierentransplantierten Patienten gegenüber Dialysepatienten erklären könnte.

Unerwarteterweise war die Cholesterin Akzeptor Kapazität von Serum von Mann zu Frau Transsexuellen, in THP-1 Zellen gemessen, nach der gegengeschlechtlichen Hormontherapie nicht erhöht, sondern signifikant erniedrigt. Serum von Frau zu Mann Transsexuellen zeigte keine Änderung der Cholesterin Akzeptor Kapazität. Diese Daten argumentieren gegen die traditionelle Ansicht, dass Östrogen positive und Testosteron negative Effekte auf den Lipoprotein Metabolismus und letztlich auf die Entstehung von Atherosklerose ausübt.

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