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Causa latet, vis est notissima.

(Ovid)

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List of Abbrevations

ABCA1	ATP binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
acLDL	acetylated LDL
Apo	apoprotein
BSA	bovine serum albumin
CAC	cholesterol acceptor capacity
CA-IMT	carotid artery intima media thickness
CD36	cluster of differentiation 36
CEC	cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CKD	chronic kidney disease
CRF	chronic renal failure
CVD	cardiovascular disease
cLDL	carbamylated LDL
EC	endothelial cell
ESRD	end stage renal disease
FC	free cholesterol
FCS	fetal calf serum
FFA	free fatty acids
GFR	glomerular filtration rate
HCAEC	human coronary endothelial cell
HDL	high density lipoprotein
HD	hemodialysis
HMDM	human monocyte-derived macrophages
ICAM-1	intercellular adhesion molecule 1
IDL	intermediate density lipoprotein
IL	interleukine
IFN-γ	interferon-γ
LCAT	lecithin:cholesterin acyltransferase
LDL	low density lipoprotein
LDLR	LDL receptor

LPL	lipoprotein lipase
M-CSF	macrophage colony-stimulating factor
mmLDL	minimally oxidized LDL
MPO	myeloperoxidase
nLDL	native LDL
oxLDL	oxidized LDL
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PPAR-γ	peroxisome proliferator activated receptor γ
RCT	reverse cholesterol transport
rpm	revolutions per minute
SDS-PAGE	sodium dodecylsulfate polyacrylamid gel electrophoresis
SMC	smooth muscle cell
SR-A	scavenger receptor class A
SR-B1	scavenger receptor class B type 1
TNF-α	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low density lipoprotein

1 Introduction

Young adults suffering from chronic kidney disease (CKD) have an excessively elevated risk of developing atherosclerosis and cardiovasclular disease (CVD). High density lipoproteins (HDL) have antiatherogenic potential, mainly through reverse cholesterol transport (RCT) from peripheral tissues to the liver. In CKD the composition of the HDL particles is abnormal, which might reduce their capacity as an acceptor for cellular cholesterol and thus impair macrophage cholesterol efflux and RCT. It is speculated that the alterations in HDL composition and function present in CKD patients may contribute to CVD development as they may counteract the antiatherogenic properties of HDL. Still, the potential influence of CKD on lipoprotein metabolism and RCT is incompletely understood (Holzer et al., 2011b).

The aim of this study was to determine

- the cholesterol acceptor capacity of serum and HDL, obtained from adult and pediatric CKD patients, dialysis patients and from healthy, matched controls, and

- the cholesterol efflux capacity of human monocyte-derived macrophages (HMDM) isolated from the study participants and from healthy, matched controls.

1.1 Atherosclerosis

1.1.1 Epidemiology of atherosclerosis

Cardiovascular disease is the major cause of deaths in industrialized societies (Wang and Tall, 2003; Basnakian et al., 2010) and responsible for 30% of all deaths worldwide in 2005 (WHO, 2008) and 50% of all deaths in Western societies (Lusis, 2000).

Atherosclerosis, the underlying cause of CVD, is a chronic, progressive disease that is characterized by lipid accumulations and chronic inflammation in the intima of the arterial wall (Lundberg and Hansson, 2010; Andersson et al., 2010). It is associated with of a combination of exogenous and endogenous risk factors (Chade et al., 2005; Cooney et al., 2009) which include hypertension, diabetes, dyslipidemia (e.g. high (LDL), low (HDL)), high fat diet, smoking, increased oxidative stress and genetic predisposition (Glass and Witztum, 2001; Chade et al., 2005; Menon et al., 2005). Progressing from early lesions

of the arterial wall to fibrous plaque formation (Navab et al., 1996; Ross, 1999), it can lead to acute clinical events, like myocardial infarction or stroke eventually (Lusis, 2000; McGill et al., 2000). Prevention of atherosclerosis should begin in adolescence by attempting to control the known risk factors (McGill et al., 2000).

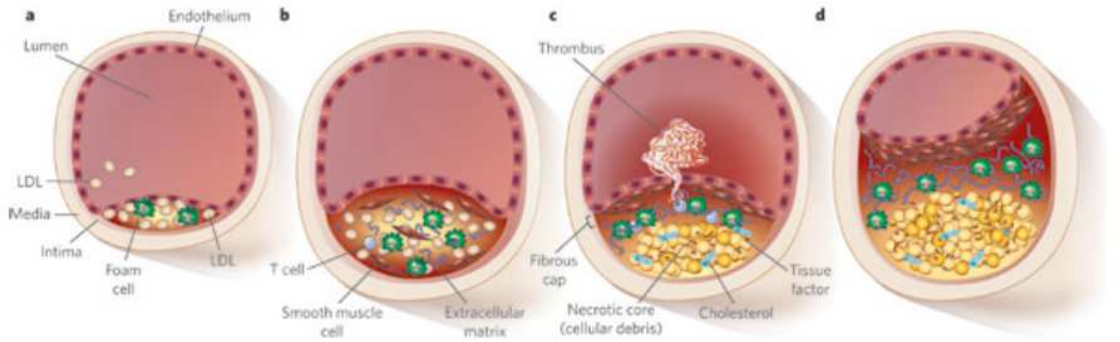
1.1.2 Pathogenesis of atherosclerosis

Development of fatty streaks

The development of atherosclerosis starts in childhood by formation of so called fatty streaks, which represent the earliest type of atherosclerotic lesion (McGill et al., 2000; Napoli et al., 1997). One of the primary events of atherogenesis is the accumulation of LDL in the subendothelial matrix (Fig. 1.1 a). In areas where the endothelium is subject to constant shear stress it becomes dysfunctional. Furthermore, ROS can be held resposible for initiating key events in atherosclerosis, namely inactivation of nitric oxide (NO) and oxidation of LDL, which leads to a cycle in which oxidized LDL (oxLDL) in turn stimulates the production of reactive oxygen species (ROS) (Chade et al., 2002; Amann et al., 2002; Chade et al., 2003). LDL passes through endothelial cell (EC) junctions by passive diffusion and is then oxidized, which is initiated by exposure to 'oxidative waste of vascular cells' (Lusis et al., 2004). The accumulation of modified LDL stimulates ECs to produce adhesion molecules, like vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which are indispensable for the adhesion of monocytes to activated vascular ECs (Apostolov et al., 2007b, a), as well as growth factors, like macrophage colony-stimulating factor (M -CSF) (Lusis, 2000). Recruitment of monocytes and lymphocytes to the intima of the artery wall is a main characteristic of atherosclerotic disease (Apostolov et al., 2007b, a).

P- and E- selectins mediate the first step of the binding process, the leukocyte 'rolling' along the endothelial surface. Adhesion molecules and chemotactic factors mediate the entry of certain leukocytes, in particular monocytes, in the arterial wall (Lusis, 2000).

Fig. 1.1: Atherosclerosis progression (Rader and Daugherty, 2008)



Monocytes adhere to endothelial cells and migrate into the vessel wall where they differentiate to macrophages (Lusis, 2000; Apostolov et al., 2007b). Macrophages play a key role in atherogenesis, as they take up and store cholesterol from modified LDL via scavenger receptors, like scavenger receptor class A (SR-A) and cluster of differentiation 36 (CD36) (Tontonoz et al., 1998; Lusis, 2000). Peroxisome proliferator activated receptor-γ (PPAR-γ) and cytokines (like tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)) regulate the expression of these scavenger receptors. In contrast to the LDL receptor (LDLR), SR-A and CD36 are not down-regulated when the cholesterol content of the macrophages increases, which causes unregulated cholesterol uptake and leads to foam cell formation (Llaverias et al., 2005). The resulting early lesions, so called fatty streaks, consist primarily of lipid-laden macrophages, and are not clinically significant, but in part progress to the more advanced fibrous lesions (Lusis, 2000).

Development of advanced lesions

monocyte-derived macrophages and T-lymphocytes present in the atherosclerotic plaque can produce several cytokines (like interleukin-6 (IL-6) or IL-18), chemokines (like monocyte chemoattractant protein-1 (MCP-1)), growth factors, enzymes (like myeloperoxidase (MPO)), and disintegrins which activate ECs and smooth muscle cells (SMCs) (Fig. 1.1 b)(Koenig and Khuseyinova, 2007). SMCs are caused to migrate to the intima from the medial layer and accumulate in fatty streaks where they secrete collagen which leads to the formation of a fibrous cap (Fig. 1.1 c). The process continues by further migration of mononuclear cells from the blood (Lusis, 2000; Lusis et al., 2004). As the atherosclerotic plaque increases in size, it starts to narrow the vessel lumen. Ulceration of the plaque results in thrombosis, intramural bleeding, calcification and complications like aneurysma, restriction or occlusion of the vessel-lumen and embolism (Fig. 1.1 c, d) (Lippi et al., 2011).

1.2 Chronic kidney disease

1.2.1 Epidemiology of chronic kidney disease

Chronic kidney disease, like atherosclerosis, is a worldwide health problem (Sarnak et al., 2003; Chade et al., 2005; Foundation, 2002) that affects approximately 11% of the adult population (Levey et al., 2003). In the past decade, incidence and prevalence of CKD have been rising and are expected to increase further (The National Kidney Foundation, 2002; Levey et al., 2003), while the population with end-stage renal disease (ESRD) has more than doubled (Chade et al., 2005).

Cardiovascular disease (CVD) contributes to the development and progression of renal injury through pathogenic mechanisms such as endothelial dysfunction, and inflammation (Chade et al., 2005).

CVD risk factors, such as older age, diabetes mellitus, systolic hypertension, dyslipidemia, hyperhomocysteinemia, oxidative stress and elevated inflammatory markers are also prevalent in CKD (The National Kidney Foundation, 2002). In the US >65% of all cases of ESRD are caused by diabetes and hypertension. For prevention and treatment of CKD it is crucial to recognize and treat the risk factors at an early stage of the disease (Chade et al., 2005).

The National Kidney Foundation defined CKD as:

"1. kidney damage for >3 months, as defined by structural or functinonal abnormalities of the kidney, with or without decreased glomerular filtration rate (GFR),

manifest by either:

- pathological abnormalities, or
- markers of kidney damage, including abnormalities in the composition of the blood or urine, or abnormalities in imaging tests.

2. GFR<60 mL/min/1.73 m^2 for >3 months, with or without kidney damage".

Based on measured or estimated (from serum creatinine) glomerular filtration rate (GFR; reference values: 90-120 ml/min/1.73m^2; see table 1) the 'Stages of chronic kidney disease' were established in the K/DOQI CKD Guidelines (Kidney Disease Outcomes Quality Initiative; see table 1) (The National Kidney Foundation, 2002).

Tab. 2: Stages of Chronic Kidney Disease

Stage	Description	GFR (ml/min/1.73m^2)
1	Kidney damage with normal or ↑ GFR	≥90
2	Kidney damage with mild ↓ GFR	60-89
3	Moderate ↓ GFR	30-59
4	Severe ↓ GFR	15-29
5	Kidney failure	<15 or dialysis

(The National Kidney Foundation, 2002)

Albuminuria, that is, presence of abnormal quantities of albumin in the urine, may precede functional impairment, and may be accompanied by a lowered GFR and therefore is a possibility to identify renal disease and an independent marker of CV risk (Schiffrin et al., 2007).

1.2.2 Atherogenic alterations of lipoprotein metabolism in chronic kidney disease

CKD is known to cause oxidative stress, inflammation, lipoprotein oxidation, impaired lipoprotein metabolism and to enhance atherosclerosis (Moradi et al., 2009b; Sarnak et al., 2003). An inflammatory response may be triggered by changes in plasma components, as well as by alteration of endothelial structure and function (Schiffrin et al., 2007). The proceeding impairment of renal function in CKD, leading to dyslipidemia and/or accumulation of uremic toxins, may play a role in the progression of endothelial dysfunction and atherogenesis (Schiffrin et al., 2007; Kwan et al., 2007). The higher amount of reactive oxygen species (ROS) present in CKD increases the oxidation of lipids, carbohydrates and protein, and rises as the disease progresses, which again can favor atherogenesis (Chade et al., 2002; Amann et al., 2002; Chade et al., 2003).

In CKD patients, total and LDL-cholesterol levels are often normal, however, LDL are more sensitive to oxidation (Maggi et al., 1994) and subject to significantly increased carbamylation (Apostolov et al., 2005). The resulting proatherogenic effect is comprised of EC activation by modified LDL, and thus enhanced ICAM-1 and VCAM-1 expression, followed by monocyte adhesion (Apostolov et al., 2007a).

Due to accumulation of intermediate density lipoproteins (IDL), which contain very low density lipoprotein (VLDL) and chylomicron remnants, plasma triglyceride levels are

increased. Moreover, HDL maturation is impaired in CRF. As a result, plasma HDL-levels are diminished and HDL composition is altered (Schiffrin et al., 2007; Kwan et al., 2007). Also, apolipoprotein-AI (apoA-I) synthesis is decreased, which, in addition to the low plasma-HDL, leads to a diminished potential of HDL to act as an antioxidant and protector of the endothelium (Chade et al., 2005; Kwan et al., 2007). Therefore, endothelial function is impaired, which promotes atherosclerosis and enhances the CVD risk (Schiffrin et al., 2007; Kwan et al., 2007).

In 2009 Moradi et al. showed that besides diminished plasma HDL levels, also HDL quality was impaired due to enhanced oxidation in hemodialysis patients, which resulted in diminished atheroprotective capacity (Moradi et al., 2009a). Moreover, Weichhart et al. (2012) demonstrated that the composition of HDL is altered in uremia and that therefore its anti-atherogenic function is impaired. Hence, HDL quality might be critical for atherosclerosis progression during CRF (Weichhart et al., 2012).

Recently, Moradi et al. showed that artificially implemented CRF by 5/6 nephrectomy in a murine model resulted in aggravated aortic lipid accumulation through enhanced lipid influx. It was the result of an upregulation of scavenger receptor A1 (SR-A1) and lectin-like oxidized LDL receptor 1 (LOX-1), which mediate cholesterol influx, as well as lecithin:cholesterin acyltransferase (LCAT) - which esterifies the effluxed cholesterol - deficiency. In this model, the lipid accumulation occurred despite compensatory upregulation of liver X receptor (LXR), ATP binding cassette (ABC) transporter A1 (ABCA1) and ABCG1, which mediate cholesterol efflux (described in detail in section 1.3.4) (Moradi et al., 2009b).

There are studies that point out an inverse relationship between lipid profile and mortality in dialysis, in which lower levels of total cholesterol are associated with increased mortality (Liu et al., 2004; Iseki et al., 2002). In 2002 Iseki et al. found the highest 10-year mortality rate in subjects with less than 140 mg/dl serum cholesterol at baseline, and the lowest in subjects with a baseline level between 200 and 219 mg/dl (Iseki et al., 2002). In 2007 Kilpatrick et al. published a study for which a large national database of maintenance hemodialysis patients (82,958 subjects) in the United States had been researched, and reported a phenomenon referred to as 'reverse epidemiology', which describes an association between total hypercholesterolemia (>200 mg/dl), LDL hypercholesterolemia as well as hypertriglyeridemia and better survival of hemodialysis patients (Kilpatrick et al., 2007). However, this is discussed controversially as, among others, Levin et al. (2007) stated that metabolic outcomes in hemodialysis patients, like changes in cholesterol level, need to be interpreted differently in a hemodialysis cohort than in the general population (Levin et al., 2007).

1.2.3 The association of chronic kidney disease and atherosclerosis

Oxidative stress, inflammation, hypertension and dyslipidemia are regarded to be paramount among the risk factors that contribute to the development of atherosclerosis and CVD in CKD (Moradi et al., 2009b). CKD appears to be a risk factor for CVD and patients suffering from CKD are more likely to die from CVD than to develop kidney failure (Sarnak et al., 2003; Tonelli et al., 2006; Schiffrin et al., 2007).

The prevalence of CVD in ESRD patients is remarkably increased, compared to healty controls (Chade et al., 2005; Sarnak et al., 2003) and about 50% of ESRD patients die from a cardiovascular event (Schiffrin et al., 2007), therefore CVD is the leading cause of death in patients with ESRD on chronic dialysis (Krediet and Balafa, 2010; Ponda and Barash, 2008), which is even more distinct in the age group from 25 to 30 years (Schiffrin et al., 2007).

In children and young adults suffering from ESRD, there is a high prevalence of coronary and carotid atherosclerosis and the CVD mortality is increased up to 700-fold as the development of atherosclerosis starts early in these patients (Oh et al., 2002; Schiffrin et al., 2007). The younger the person studied, the higher the relative risk of CVD in comparison to the general population (see table 2) (Schiffrin et al., 2007).

Tab. 3: CV risk according to stages of CKD

CV risk according to stages of CKD	
stage	CV risk (odds ratio, univariate)
1	Depending on degree of proteinurea
2	1.5
3	2-4
4	4-10
5	10-50
ESRD	20-1000
The increase in risk in comparison with people free of CKD depends on the age of the population studied: The younger the person, the higher the relative risk. Microalbuminurea increases the CV risk 2- to 4-fold.	

(modified after Schiffrin et al., 2007)

Atherosclerosis on top of pre-existing renal injury may, as mentioned before, aggravate

CKD progression via similar pathophysiological pathways (Chade et al., 2005). Glomerular cells and cells of the vessel wall share some of their characteristics (Ross, 1999; Abrass, 2004), so atherosclerosis and glomerulosclerosis can be seen as comparable processes (Chade et al., 2005). In both, atherogenic lipoproteins can induce cell injury and trigger growth factor-secretion leading to vascular hypercellularity and proliferation of extracellular matrix (Ross, 1999). One of the primary events that lead to atherosclerosis is the deterioration of endothelial function, which is present in renal disease and has been proposed as the major pathophysiological mechanism involved in the association between reduced GFR and the increased risk of CV events. The acceleration of atherosclerosis may be due to a combination of endothelial dysfunction, low-grade inflammation, dyslipidemia and renal disease, and, furthermore, together with hypertension explain the elevated prevalence of CV events in CKD (Schiffrin et al., 2007).

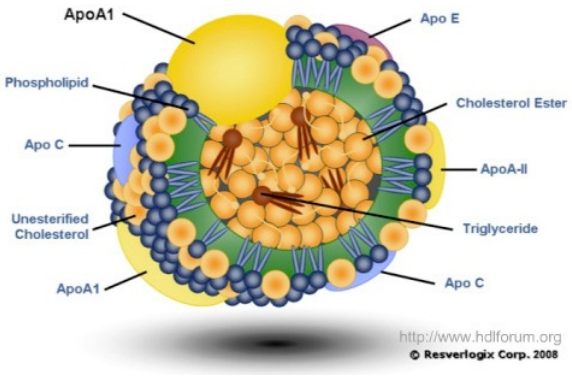
In 2002, Shoji et al. reported that CKD as well as alterations secondary to CKD, but not hemodialysis (HD) or HD duration, are responsible for atherosclerosis development by measuring the intima media thickness of carotid arteries (CA-IMT) with the use of high-resolution ultrasonography. Other studies however, did find a significant correlation between HD duration and CA-IMT (Burdick et al., 1994; Shoji et al., 2002).

1.3 Lipoproteins

1.3.1 Lipoprotein composition

For transportation in the blood, lipids have to bind to proteins and form the so called lipoproteins. Lipoproteins consist of a hydrophobic core containing triglycerides and cholesteryl-esters (CE), which is surrounded by an amphipathic monolayer of phospholipids and free cholesterol (FC) and apolipoproteins (for HDL composition see fig. 1.2) (Lusis et al., 2004).

Fig. 1.2: HDL composition

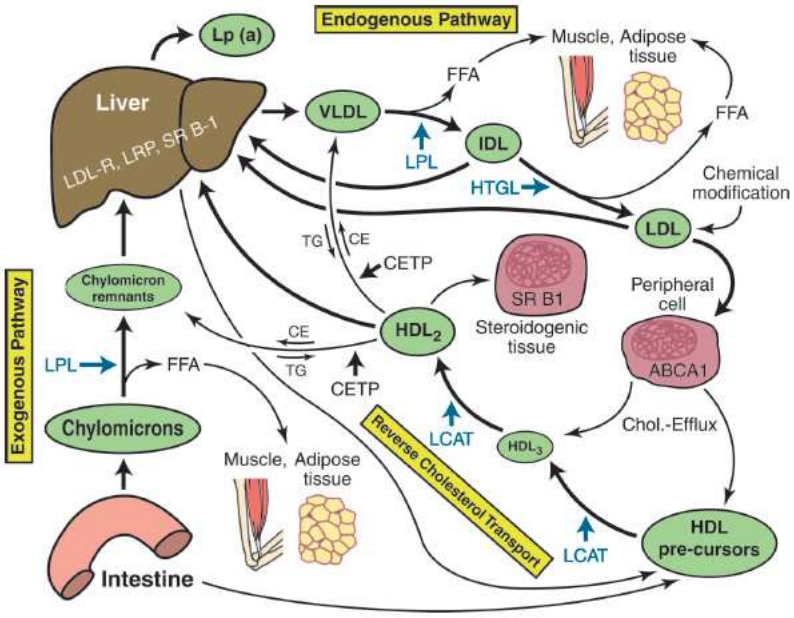


Lipoproteins are classified according to their specific densities and molecular sizes and possess different physiological functions, lipid composition and apolipoprotein content. The main lipoprotein classes are: high density lipoprotein (HDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicrons (Krieger, 1999; Kwan et al., 2007). They are usually seperated by sequential ultracentrifugation or electrophoresis.

1.3.2 Lipoprotein metabolism

There are two main pathways of lipoprotein metabolism - the endogenous pathway, in which lipoproteins originate in the liver and the exogenous pathway, in which lipoproteins mainly contain dietary fat (Kwan et al., 2007; see fig. 1.3).

Fig. 1.3: Lipoprotein metabolism (Kwan 2007)



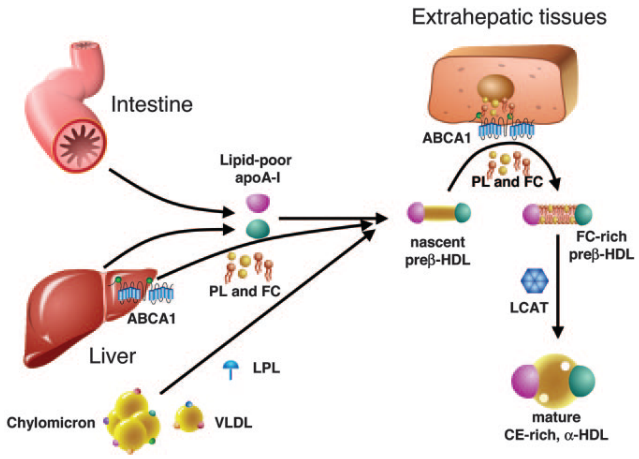
After absorption dietary lipids (triglycerides, cholesterol) are packaged in chylomicrons. Lipoprotein lipase (LPL) lipolyzes the chylomicrons and delivers the free fatty acids (FFA) to the tissues. The remaining chylomicron remnants are taken up by the liver via the LDL receptor (LDLR) and LDL receptor-related protein (LRP) mediation. In the liver, triglycerides and cholesterol are actively synthesized and packaged before they are secreted in VLDL along with lipids originally derived from the intestine. After lipolysis by LPL, the VLDL particles convert to IDL, half of which are removed from the circulation by hepatic receptors. The remaining half undergoes further lipolysis resulting in the formation of LDL. Several enzymes, such as cholesteryl ester transfer protein (CETP), function in the transfer of lipids between the different lipoproteins (Lusis et al., 2004). The removal of LDL is mediated by the LDLR, in the liver as well as in peripheral tissues (Lusis et al., 2004).

Chylomicron and VLDL surface remnants, together with precursors secreted by liver and intestine, form HDL particles (see fig. 1.4). HDL is capable of faciliating the removal of cellular cholesterol. After esterification of the cholesterol by lecithin:cholesterin acyltransferase (LCAT), it is transported to the liver via HDL and taken up via scavenger receptor class B type 1 (SR-B1), for processing and excretion (Lusis et al., 2004).

1.3.3 HDL in atherosclerosis and chronic kidney disease

High-density lipoprotein (HDL), one of the major carriers of cholesterol in the blood (Podrez, 2010), is a strong atheroprotective factor due to its anti-inflammatory, antioxidant, antiapoptotic, antithrombotic and NO-promoting effects and by enhancing endothelial function (Movva and Rader, 2008). Furthermore, it plays a pivotal role in reverse cholesterol transport (RCT - described later), which constitutes its strongest antiatherogenic property (Lusis, 2000; Cutri et al., 2006; Vergeer et al., 2010; Wang and Tall, 2003). It has been shown (*in vitro*) that HDL inhibits the expression of endothelial adhesion molecules, LDL-induced monocyte transmigration (Vergeer et al., 2010) and lipoprotein oxidation, which contributes to its antiatherogenic effect (Lusis, 2000).

Fig. 1.4: HDL metabolism (Lewis and Rader, 2005)



Low levels of HDL-cholesterol (<40 mg/dl) are an independent risk factor for CVD (Cho, 2009) and a rise in plasma HDL reduces the relative risk of coronary heart disease significantly (Wilson et al., 1998). However, not only quantity, but also quality of HDL is critical for cellular cholesterol efflux and prevention of cardiovascular events (Chirinos et al., 2005; Cho, 2009).

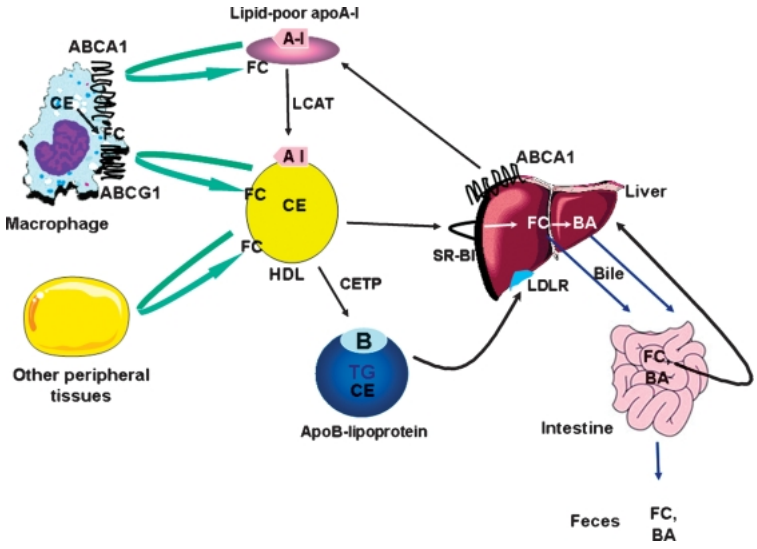
Approximately 70% of the total HDL protein content is constituted by apoA-I, which is synthesized by liver and intestine - other apolipoproteins associated with HDL are apoA-II (15-20%), A-IV, C-I, C-II, C-III and E (Oram, 2003; Movva and Rader, 2008; Podrez, 2010). The main properties of HDL, lipid binding and cellular cholesterol efflux as well as lecithin:cholesterol acyltransferase (LCAT) activation, are primarily due to apoA-I (Cho, 2009).

In CKD, the composition of HDL is altered and its anti-inflammatory properties are impaired (Kwan et al., 2007). Initial results in hemodialysis patients including diabetics suggest that the ability of HDL to promote cholesterol efflux may also be reduced (Marsche et al., 2008).The HDL plasma level is diminished in CKD as shown by Gonen et al. (1985).

1.3.4 Reverse cholesterol transport

Glomset (1968) was the first to propose a mechanism now referred to as reverse cholesterol transport, which describes the mobilization of excess cholesterol from peripheral tissues and its transport to the liver for disposal (see fig. 1.5) (Lusis, 2000; Cutri et al., 2006; Vergeer et al., 2010; Wang and Tall, 2003; Pagler et al., 2007; Glomset, 1968). To prevent foam cell formation and atherosclerosis progression, adequate cholesterol efflux from macrophages is crucial.

Fig. 1.5: Reverse cholesterol transport (Rader et al., 2009)



Cholesterol from modified LDL is taken up by macrophages mainly via SR-A1/2 and CD36. The expression of these scavenger receptors is increased in blood monocytes of CKD patients (Chmielewski et al., 2005).

The initial step of RCT, cellular cholesterol efflux to lipid-poor apoA-I, the main apolipoprotein of HDL, and mature HDL (Vergeer et al., 2010; Kwan et al., 2007), is mediated mainly by ABCA1 and ABCG1 (Klucken et al., 2000; Wang et al., 2004; Pagler et al., 2007) and SR-B1 (Pagler et al., 2006; Ji et al., 1997; Chinetti et al., 2000). The

cholesterol taken up by HDL is esterified by LCAT, which is activated by apoA-I, and transported to the core of the HDL particle, thereby creating the gradient required to maintain the cholesterol efflux from cells to HDL (Cutri et al., 2006; Kwan et al., 2007).

HDL delivers the cholesterol to the liver where it is taken up via SR-B1, a scavenger receptor with a high affinity for lipid-rich HDL (Cutri et al., 2006; Liadaki et al., 2000). Cholesterol can also be transferred to VLDL and LDL (apo-B containing lipoproteins), which is mediated by cholesteryl ester transfer protein (CETP) and followed by either uptake by the liver via LDLR or delivery to peripheral tissues (Cutri et al., 2006). The rate of RCT is not always associated with plasma levels of HDL and apoA-I (Movva and Rader, 2008).

$ApoE^{-}/^{-}$mice develop severe atherosclerotic lesions spontanously and therefore are used extensively in atherosclerosis research. Using a murine model, Singaraja et al. (2002) showed that increased ABCA1 activity leads to a decreased tendency to develop atherosclerotic lesions, as well as increased cholesterol efflux from macrophages (Singaraja et al., 2002).

Increasing cholesterol efflux from macrophages has been discussed as a possible treatment for atherosclerosis (Joseph et al., 2002). The effect of CKD/ESRD on cellular cholesterol efflux and the ability of uremic HDL to act as an acceptor for cellular cholesterol have not yet been studied sufficiently.

Membrane transporters and receptors relevant for reverse cholesterol transport

ATP binding cassette transporters A1 and G1

ABCA1 and ABCG1 are part of the ABC superfamily, a family of receptors that typically consist of four transmembrane domains and uses energy gained from ATP hydrolysis to transport specific substrates through a membrane against a concentration gradient (Higgins, 1992). The ABCA1, ABCG1 and apolipoprotein-mediated cholesterol efflux from macrophages plays an important role in protection against atherosclerosis and ABCA1 expression is up-regulated in cholesterol engorged macrophages of atherosclerotic lesions (Lusis et al., 2004; Kielar et al., 2001; Lawn et al., 2001).

ABCA1 has a pivotal role in cholesterol homeostasis and HDL metabolism as it mediates the cholesterol efflux to lipid poor forms of apoA-I, probably by direct binding of apoA-I to ABCA1 and direct transport of cholesterol and phospholipids as substrates (Oram, 2003; Wang and Tall, 2003; Wang et al., 2004).

ABCG1 stimulates cholesterol efflux to mature HDL and therefore take part in the regulation of lipid efflux. ABCG1 expression in HMDM is enhanced in response to acLDL loading (Nakamura et al., 2004; Klucken et al., 2000; Wang et al., 2004).

Activation of liver X receptor/retinoic acid receptor (LXR/RXR) results in an increase of ABCA1/ABCG1 expression (Oram, 2003; Wang and Tall, 2003; Wang et al., 2004) and thus enhances cholesterol efflux to apoA-I (Wang et al., 2004). It furthermore promotes other antiatherogenic mechanisms like bile acid synthesis and inhibition of intestinal cholesterol absorption. Synthetic LXR ligands, however, in a murine model also promoted lipogenesis and hypertriglyceridemia which in turn promotes atherosclerosis (Joseph et al., 2002).

The significance of ABCA1/ABCG1 for cellular cholesterol efflux probably depends on both: their relative cellular expression as well as the concentration of apolipoproteins and HDL (Nakamura et al., 2004) [for review see (Zannis et al., 2006)]. Incubation of human coronary artery endothelial cells (HCAECs) with uremic plasma led to decreased expression of these genes (Cardinal et al., 2007).

Scavenger receptor class B type 1

SR-B1 is a membrane glycoprotein of 82 kDa with one large extracellular domain and two transmembrane domains (Zannis et al., 2006) and acts antiatherogenically in two ways: it promotes cholesteryl ester (CE) uptake in the liver as well as cellular cholesterol efflux in peripheral tissues (Pagler et al., 2007).

HDL delivers CE to the liver, where SR-B1 mediates CE uptake by hepatocytes without degradation of the HDL particle (Krieger, 1999). SR-B1 is also present in macrophages of atherosclerotic lesions (Chinetti et al., 2000; Hirano et al., 1999), where it mediates cholesterol efflux from macrophages to mature HDL (Ji et al., 1997) and therefore contributes to protection against atherosclerosis (Zhang et al., 2003). According to Zhang et al. (2003), SR-B1 protects $ApoE^{-}/^{-}$mice from developing atherosclerotic lesions without enhancing macrophage cholesterol efflux to HDL or apoA-I or altering plasma lipid levels or HDL subpopulations. They suggest that the antiatherogenic properties of SR-B1 might not be related to its ability to efflux cellular cholesterol from macrophages (Zhang et al., 2003).

SR-B1 deficiency in mice is associated with an increased risk to develop atherosclerosis as it affects the lipid and apoprotein composition of HDL (Zannis et al., 2006) [for reviews on SR-B1 see (Krieger, 1999; Zannis et al., 2006)].

Duong et al. (2006) demonstrated that a large amount of cholesterol efflux from human WI38VA13 fibroblasts and mouse peritoneal macrophages to the plasma is mediated by a pathway independent from either ABCA1 and SR-B1 respectively. They showed, that even after inhibiting both, ABCA1 as well as SR-B1, still a remarkable amout of uninhibitable efflux occured and suggest this to be due to aqueous diffusion to phospholipid containing acceptors present in plasna and dependend on their concentration (Duong et al., 2006).

1.3.5 Modified lipropoteins

Modified LDL, namely carbamylated LDL (cLDL) and oxidized LDL (oxLDL), which are remarkably elevated in CKD patients (Apostolov et al., 2005), can induce endothelial dysfunction and injury (Chade et al., 2005), and show cytotoxicity against ECs, therefore acting proatherogenic (Ok et al., 2005). It is commonly accepted that the atherosclerotic process can be initiated by EC-injury due to modified LDL (Basnakian et al., 2010), as the expression of adhesion molecules for monocyte binding (e.g. ICAM-1) in ECs is increased under their influence (Kamanna et al., 1999).

Lipoprotein oxidation

Oxidatively modified LDL (oxLDL) is commonly divided in two groups: minimally modified LDL (mmLDL) and extensively oxidized LDL (oxLDL). While mmLDL, which is chemically different from native LDL, is not recognized by most scavenger receptors but by the LDL receptor, oxLDL is recognized by scavenger receptors, but not by the LDLR (Levitan et al., 2010). LDL oxidation is known to be a critical factor in atherosclerosis development. Macrophages are able to take up and degrade modified LDL, which results in cholesterol accumulation. Foam cell formation can be inhibited by the down-regulation of certain scavenger receptors (Levitan et al., 2010).

Treatment of ECs or macrophages with mmLDL results in induction of chemotactic and proinflammatory proteins (Levitan et al., 2010). It is suggested that mmLDL exerts its EC stimulating properties before fatty streak formation, since in established lesions LDL modification is advanced and monocyte attraction and migration is mediated by chemotactic factors derived fom macrophages and SMCs rather then LDL (Berliner et al., 1990).

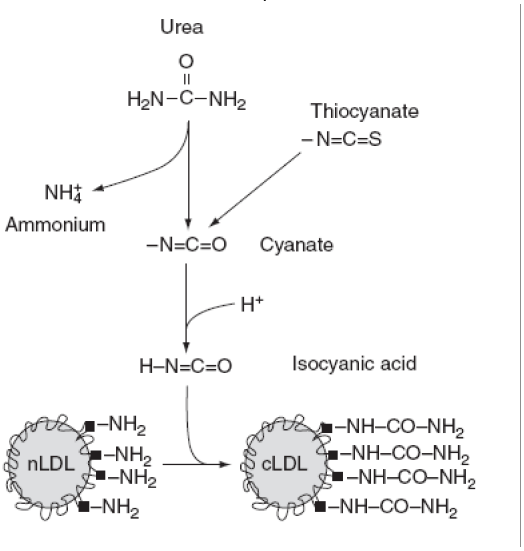
The presence of oxLDL in atherosclerotic lesions has been reviewed by Witztum and Steinberg (2001) and it is commonly accepted that oxidation takes place in the subendothelial matrix, not in the circulation due to the precence of antioxidants in the latter.

OxLDL has been shown to inhibit SR-B1 expression in Raw 264.7 macrophages and as a result to down-regulate cholesterol flux in a dose- and time-dependent manner (Han et al., 2001).

Lipoprotein carbamylation

Carbamylation is the nonenzymatic modification of protein by cyanate (OCN^-) (see fig.1.6). The spontanous dissociation of urea to cyanate and ammonia results in an elevation of cyanate. Isocyanic acid, the active isoform, irreversibly binds to the NH_2 and N-terminal groups of amino acids (Kraus and Kraus, 2001; Basnakian et al., 2010).

Fig. 1.6: LDL carbamylation (Basnakian et al., 2010)



Carbamylation is mostly associated with a partial or complete loss of protein function (Kraus and Kraus, 2001; Apostolov et al., 2005; Basnakian et al., 2010) and a significant elevation of protein carbamylation is present in uremia (281.5±46.9 mg/L cLDL in ESRD patients compared with 86.1±29.7 mg/L cLDL in controls; $p<0.001$) (Kraus and Kraus, 2001; Apostolov et al., 2005).

Gonen et al. (1985) observed altered LDL function in CKD subjects and suggested the possibility that LDL carbamylation might occur *in vivo*. In their study, uremic LDL and cLDL showed similar properties of interacting with fibroblasts. The diminished uptake of these particles by fibroblasts may increase the residence time within the subendothelial region of the vessel wall, and therefore exert the atherogenic properties of uremic LDL (Gonen et al., 1985).

It has been shown that cLDL induces various pathways of damaging endothelial and vascular SMCs (Ok et al., 2005; Apostolov et al., 2007a), and furthermore ICAM-1

and VCAM-1 expression of endothelial cells, which leads to attraction and adhesion of monocytes (Apostolov et al., 2007b; Basnakian et al., 2010; Thorne et al., 1996, a). Its cytotoxicity is similar to the one of uremic LDL, oxLDL and acLDL (Basnakian et al., 2010). Basnakian et al. (2010) suggested that carbamylated LDL (cLDL) highly contributes to the atherosclerotic process, especially in ESRD patients, as well as, cLDL being the cause of uremic atherosclerosis and the link to CKD (Basnakian et al., 2010).

2 Material and Methods

All reagents were purchased from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

All experiments (except cholesterol efflux) involving THP-1 cells and human peripheral blood monocytes were performed under sterile conditions using an air laminar flow (MWG AG Biotech, Ebersberg, Germany) and 24-well tissue culture plates (greiner-bio-one cellstar®).

For all THP-1 and monocyte/HMDM experiments RPMI-1640 (Roswell Park Memorial Institute cell culture medium, Biochrom AG, Berlin, Germany) was used as medium, either

- unsupplemented (RPMI-UK),
- supplemented with 100 U/ml penicillin, 0,1 mg/ml streptomycin (PAA, Pasching, Austria), 2mM glutamine (PAA, Pasching, Austria) (RPMI w/o FCS), or
- supplemented with 100 U/ml penicillin, 0,1 mg/ml streptomycin, 2mM glutamine and 10% FCS (fetal calf serum, GIBCO, Carlsbad, CA, USA) (RPMI compl.).

2.1 Patients and Controls

Samples (20-30 ml of venous blood) were obtained from patients with CKD, selected strictly according to the exclusion criteria shown in Table 3, after an overnight fast (pediatric subjects, adult and dialysis patients). Within 2h after blood collection monocytes were isolated under sterile conditions and treated as described under 3.2.1, serum was aliquoted and stored at -20°C, lipoproteins were fractioned and also stored at -20°C..

All patients as well as pediatric controls were recruited at the Vienna General Hospital (AKH Wien) in collaboration with the Dept. of Pediatrics of the Medical University of Vienna (Medizinische Universität Wien - MUW), the Dept. of Pediatric Surgery (MUW) and the Dept. of Internal Medicine III (MUW) per the following exclusion criteria for patients with CKD:

- Proteinuria $\geq$ 1g/l
- DOQI $<$ 3

- BMI $> 30 kg/m^2$

- Clinical instablility or infection during the last three months
- Diabetes mellitus, liver disease
- Other conditions associated with secondary dyslipoproteinemia (such as hypothyroidism, trisomy 21)
- Treatment with growth hormone, lipid lowering drugs or immunosuppression

Controls (healthy, fasting volunteers) were carefully chosen and matched by sex, age and BMI (see Table 3). Informed consent (see fig. 6.1 and 6.2) was obtained from all subjects. A specification of the clinical characteristics of patients and controls can be found in the addendum.

The study was approved by the Ethics Committee of the Medical University of Vienna (protocol #511/2007).

Tab. 4: Patients data

		sex		age		DOQI		Creatinin (mg/dl)		BMI	
		male	female								
		n	n	mean	sd	mean	sd	mean	sd	mean	sd
pediatric	patients	10	2	11.1	5.2	3.75	.75	3.21***	1.28	20.43	4.15
	controls	9	3	11.0	4.8	.	.	.48	.19	16.98	3.27
adult	patients	6	11	34.6	8.6	3.81	.75	3.79***	3.02	22.65	3.51
	controls	6	11	34.8	9.3	.	.	.78	.11	22.52	2.52
dialysis	patients	8	6	36.7	10.2	.	.	7.66***	4.09	25.26	4.77
	controls	8	6	35.5	9.5	.	.	.87	.14	24.20	3.10

2.2 Cell Culture

2.2.1 Monocyte isolation and differentiation

Reagents:

- 0.18 M EDTA (ethylene diamine tetra-acetate), pH 8
- Ficoll Paque (GE Healthcare, Uppsala, Sweden)
- RPMI-UK
- RPMI w/o FCS

Fasting venous blood samples for preparation of EDTA plasma and serum were obtained from adult subjects and were centrifuged at 620 g, 18°C, brake 0 for 10 minutes (Eppendorf centrifuge 5810 R). Plasma and serum were removed under sterile conditions in a laminar air flow (MWG AG Biotech, Ebersberg, Germany), aliquoted and stored at either +4°C or -20°C for further use. The plasma was replaced with RPMI-UK, blood and RPMI-UK were mixed carefully using a plastic pipette. Ficoll Paque (density 1.077g/l) was placed in a 15 or 50 ml tissue culture tube (depending on the sample volume; Ficoll Paque : blood-RPMI-mix = 1 : 1), the blood-RPMI-mix was then slowly layered above. After centrifugation at 320 g and 18°C for 30 minutes (brake 0) the upper layer, consisting of RPMI-UK and blood platelets, was removed and the buffy coat was collected and transferred in 10 or 50 ml tissue culture tubes containing 5 ml RPMI-UK per 4 ml of initial blood sample. This was followed by two washing steps. The first one was a centrifugation at 320 g, 18°C, 10 min, brake 9, after which the medium was removed using a Pasteur pipette. The cell pellet was resuspended in fresh RPMI-UK (5 ml RPMI-UK per 4 ml of initial blood sample) and, in the second washing step, again centrifuged at 320 g, 18°C, 10 min, brake 9. Then the cell pellet was resuspended in RPMI w/o FCS and the cell suspension was seeded in 6 wells (24-well plates, 1 ml cell suspension + 10% autologous serum/well). After 2h incubation at 37°C to allow the cells to adhere to the plate, medium was changed to 350 µl RPMI w/o FCS + 10% autologous serum/well.

For differentiation to macrophages medium was exchanged every 2-3 days (350 µl RPMI w/o FCS + 10% autologous serum/well) for 7 days, efflux experiments were performed on day 8 after the isolation process. The cells were kept at 37°C + 5% CO_2 in an CO_2 - incubator (C150, Binder, Tuttlingen, Germany).

2.2.2 THP-1 – cells

THP-1 cells (TIB-202; LGC Standards) are a human non-adhesive monocytic leukemia cell line and can be differentiated into macrophage-like cells with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA). This cell line is commonly used to study foam cell formation in atherosclerosis development (Auwerx, 1991).

Reagents:

- RPMI compl.
- 810 µM PMA (phorbol 12-myristate 13-acetate)

THP-1 cells were kept in suspension in RPMI compl. at a concentration below 1x10^6cells/ml. For the cholesterol efflux experiments, cells were seeded in 6 wells of a 24-well-plate at a concentration of 5*10^5cells/well and differentiated towards macrophages in 250 µl RPMI-compl. containing 160 nM PMA for 3 days.

2.3 Bradford protein assay (Bradford, 1976)

Reagents:

- Bradford protein assay (BioRad Laboratories, Hercules, CA, USA)
- Albumin, bovine serum, BSA (Sigma-Aldrich, St. Louis, MO, USA)

The Bradford protein assay is a method to determine the protein content of a sample (cell lysates, lipoproteins). By binding of Coomassie brilliant blue G-250 to protein a shift in the absorbance maximum from 465 nm to 595 nm is caused and monitored (Bradford, 1976). Therefore 20 µl of Bradford reagent were mixed with 10 µl (for THP-1 macrophages) or 30 µl (for HMDM) cell lysate and combined with distilled water to reach a final volume of 100 µl. Each time the Bradford assay was performed, a standard curve was prepared using 0, 1, 2, 4 or 6 µl BSA (c = 1mg/ml), 20 µl Bradford reagent and the amount of distilled water needed to reach a final volume of 100 µl. Absorbance was measured at 595 nm using a microplate reader. (BioRad iMark™ Microplate Reader).

2.4 Western blotting

Western blotting is a method for immunodetection of proteins with specific antibodies. For separation of the proteins the cell lysates are loaded onto SDS (sodium dodecyl sulfate) - polyacrylamide gels, and separation in the electric field occurs according to the molecular mass of the proteins. For immunodetection the proteins are blotted onto a nitrocellulose membrane after electrophoretic separation and detected by using specific antibodies.

For Western blotting of CD36 in monocyte/macrophage cell lysates, monocytes were isolated from 2 healthy volunteers and seeded on three 24 well plates, 2 wells per person on each plate, as described before under 2.2.1, and differentiated towards macrophages with RPMI w/o FCS containing 10% autologous serum.

2.4.1 Cell lysates

Reagents:

- PBS buffer
  - 137 mM NaCl
  - 2.7 mM KCl
  - 1.5 mM KH_2PO_4
  - 8.1 mM Na_2HPO_4x 12 H_2O pH 7.4
- Lysis buffer
  - 62,5 mM Tris
  - 8 M Urea
  - 20 mM EDTA
  - 20 mM EGTA (ethylene glycol tetra-acetate)
  - 10% Glycerol
  - 1% SDS
  - pH 6.8
- Protease inhibitor cocktail (1:100; Sigma-Aldrich, catalog number: P8340)

After a 2h incubation period the cells on plate 1 were washed 2 times with 300µl PBS (on ice) and solubilized with 100µl lysis buffer and 1 µl protease inhibitor cocktail/well. Then they were scraped with a rubber policeman, transfered to Eppendorf tubes (1.5 ml), sonified for 2 seconds, incubated on ice for 1h and stored at -20°C.

Plate 2 was subject to the same procedure after 7 days, plate 3 after 9 days of incubation during which medium (250 µl RPMI w/o FCS + 10% autologous Serum/well) was changed every 2-3 days.

2.4.2 Gel preparation and electrophoresis

Reagents:

- 30% acrylamid (BIS) solution, 29:1
- gel buffer (3 M Tris-HCl, 0.3% SDS, pH 8.45)
- 10% ammonium persulfate (APS, Pharma Biotech) in aqua dest.
- TEMED (N,N,N',N'-tetramethylethylenediamine)
- electrophoresis buffer (25 mM Tris, 200 mM glycine, 1% SDS, pH 8.0)
- sample buffer (4x)
  - 100 µl glycerol
  - 90 µl Tris buffer (0,267 µM Tris-HCl, 8% SDS, pH 6.8)
  - a dash of bromphenol blue (Amersham Biosciences, Little Chalfont, UK)
  - 10 µl β-mercapto-ethanol (Amersham Biosciences, Little Chalfont, UK)
  - sample buffer was always prepared just before use

The protein content of the samples was determined with the Bradford assay (Bradford, 1976) as described before, and the protein concentration of the samples was equaled by adding PBS buffer. To each sample 4 µl of sample buffer were added, the mixture was vortexed and incubated at 40°C for 30 minutes to denature the proteins.

Tab. 5: Protocol of SDS-page gel (0,75mm) preparation

	8%	4%
H_2O bidest.	1.6ml	1.1ml
30% Acrylamide/Bis Solution	1.1ml	0.3ml
3 M Tris-HCl, pH 8,45; 0.3% SDS	1.3ml	0.7ml
10% APS	30µl	20µl
TEMED	3µl	2µl

For gel electrophoresis the Mini-PROTEAN®3 Cell (Bio-Rad, Hercules, CA, USA) system was used. The corresponding glass plates (72 x 100 mm), suitable for a gel thickness of 0.75 mm, were washed with 70% ethanol prior to use and placed in the gel cassette according to the manufacturer's instructions.

Preparation of the running gel (8%) and the stacking gel (4%):

All of the components listed in table 5 were mixed in a clean tube, except for 10% APS and TEMED which were added just before the pouring of the gel. After adding the last two components the gel was poured immediately and covered with 30% isopropanol to prevent it from drying. 30 minutes later the isopropanol was removed, the stacking gel (prepared using the same mixing technique as for the running gel) was poured over the now polymerized running gel and the comb was inserted to form the slots. After additional 30 minutes the gel was ready for sample application.

Therefore the gel cassettes were moved to the electrophoresis chamber, which was filled with electrophoresis buffer. After that, the comb was removed and the slots rinsed using electrophoresis buffer. Equal amounts of the samples were loaded into the slots and a protein ladder (PageRuler™ Prestained Protein Ladder, Fermentas, Burlington, Canada) was added in a slot at the side of the gel. Electrophoresis was performed at 180 Volt and 4°C for 80 minutes.

2.4.3 Western blotting

Reagents:

- transfer buffer (25 mM Tris, 200 mM glycine, 1% SDS, 20% methanol, pH 8.3)
- Ponceau S stain (1% Ponceau, 30% trichloracetic acid, 30% sulfosalicylic acid)
- TBS buffer (200 mM Tris base, 1.45 M NaCl, pH 7.4)

- washing buffer (1 x TBS, 0.1 % TWEEN 20)

Tab. 6: Assembly of the transfer sandwich components

sponge
Whatman paper
nitrocellulose membrane
gel
Whatman paper
sponge

After electrophoresis the transfer sandwich was composed as described in table 6 and placed in a Mini-Trans-Blot®Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The Sponges, the Whatman filter paper, as well as the nitrocellulose membrane were steeped in transfer buffer before assembly to the sandwich. By rolling over the Whatman paper with a glass pipette the formation of air bubbles between the gel and the nitrocellulose membrane was avoided. The transfer sandwich, as well as a cooling block were then inserted in the Blotting apparatus. The transfer was conducted at a constant current of 0.4 Ampere for 45 minutes at 4°C. This was followed by staining of the nitrocellulose membrane with Ponceau S for 10 minutes, cutting of the nitrocellulose membrane according to the protein sizes and washing with washing buffer.

2.4.4 Immunodetection

Reagents:

- Washing buffer (1 x TBS buffer, 0,1% TWEEN 20)
- Blocking buffer (5% (w/v), non-fat, dry milk in washing buffer)
- Primary antibodies
  - anti-β-actin (Sigma): mouse IgG2A, 1:5000
  - anti-CD36 (Santa Cruz): mouse IgM, 1:150
- Secondary antibodies (diluted 1:10000 in blocking buffer)
  - Goat anti-mouse IgG-HRP conjugate

  - Rabbit anti-mouse Ig-HRP

- Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA)

For blocking of nonspecific sites the nitrocellulose membrane was incubated with blocking buffer at room temperature for 1 hour while shaking on a Belly Dancer™ undulating orbital shaker, then washed two times with washing buffer and incubated on a Belly Dancer™ undulating orbital shaker with primary antibody at 4°C overnight. An exception was the nitrocellulose for detecting CD36 which was blocked with 3% BSA in TBS-T.

The next morning the blots were washed 3 times (each time for 10 minutes) with washing buffer while shaking on a Belly Dancer™ undulating orbital shaker and then incubated with secondary antibody for 1 hour at room temperature - again on a Belly Dancer™ undulating orbital shaker. The same washing procedure (3 times, 10 minutes) was applied one more time, then the blots were ready for imaging.

Therefore, the Super Signal West Pico Chemiluminescent Substrate was applied to the nitrocellulose membrane according to the manufacturer's instructions, incubated for 5 minutes at room temperature and eventually placed in the Chemilmager™ 4400 (Biozym, Hannover, Germany) for detection. Quantification was carried out using the AlphaEase FC software (Alpha Innotech Corporation, CA, USA).

2.5 Lipoprotein isolation and fractionation

Reagents:

- Potassium bromide (KBr)

- Dialysis buffer (0.9 % NaCl, 0.1% EDTA, pH 7.4)

For separating the lipoprotein fractions (VLDL, LDL, HDL) according to density we used sequential flotation ultracentrifugation (Havel et al., 1955). Therefore the amount of KBr needed was calculated using the formula below:

$$gKBR = \frac{V(sample) * density\ final - density\ initial}{1 - (0{,}312 * density\ final)}$$

DPlasma = 1,006; DVLDL = 1,02; DLDL = 1,07; DHDL = 1,22 [mg/ml]

First, the density was adjusted for isolating VLDL and the samples centrifuged at 200,000xg at 4°C for 24h in a Optima L-100 XP ultracentrifuge using a 55.2 Ti rotor (Beckmann-Coulter, Brea, CA, USA). The VLDL fraction was removed, the density

of the remaining volume adjusted for LDL, and the ultracentrifugation was repeated. The procedure was repeated a third and fourth time for HDL. The lipoprotein fractions obtained were dialysed against (3x for 24h) using dialysis buffer (0.9 % NaCl, 0.1% EDTA, pH 7.4) for removing KBr. Afterwards the protein content of the lipoprotein fractions was determined with the Bradford Assay (described later).

2.6 Lipoprotein modification

Reagents:

- 2 mM copper sulfate ($CuSO_4$)
- 50 mM EDTA
- potassium cyanate (KCNO)
- phosphate (Na_2HPO_4)
- NICK-column (Sephadex G-50, Amersham Biosciences)
- saturated sodium-acetate
- acetic anhydride
- Dialysis buffer for acetylated LDL

Acetylated LDL

1 ml LDL (c = 2 mg/ml) and 1 ml saturated sodium-acetate were stirred on ice while adding 2 µl of acetic anhydride every 10 minutes (5 times) followed by additional 30 minutes of stirring on ice and 24 h dialysis at 4°C to remove excess acetic anhydride.

Carbamylated LDL

LDL (c = 2 mg/ml), 20 mg/ml KCNO and 50 mM $Na_2HPO_4^-$ were incubated for 4 h at 37°C, then passed over a NICK-column to remove KCNO (100 µl to column, rinsed with 400 µl PBS, eluted with 400 µl PBS). The concentration of LDL after this procedure was about 0.5 mg/ml.

Minimally modified LDL

LDL (c = 2 mg/ml) was passed over a NICK-column to remove EDTA which has the ability to build complexes with $CuSO_4$ and to inhibit LDL oxidation by $CuSO_4$. Afterwards, 5 µM $CuSO_4$ was added and LDL (c = 0.5 mg/ml) was incubated for 2 h at 37°C. Then 50 µM EDTA was added to stop the reaction of $CuSO_4$ and LDL.

Native LDL

Native LDL at a concentration of 2 mg/ml was passed over a NICK-column to ensure it had undergone the same procedure as the modified LDLs. Concentration of LDL after this procedure was about 0.5 mg/ml.

After modification the lipoproteins were passed through a 0.45µm sterile filter. In most cases preparations of 400µl per modification were sufficient for the experiments.

2.7 Cholesterol efflux Experiments

Reagents:

- [1,2-^{3}H(N)-] Cholesterol (1 µCi/µl, Perkin Elmer, Waltham, MA, USA)
- Ready Safe Scintillation cocktail (Beckman Coulter)
- Biofluor Plus (Perkin Elmer) (after the stop of production of Ready Safe Scintillation Cocktail)
- PBS
- RPMI-UK
- HDL
- Serum
- LXR-Agonist (10 mM Liver-X-Receptor agonist, T0901317)
- acLDL

24h prior to efflux experiments THP-1 macrophages as well as HMDM were trace-labeled with [^{3}H]-Cholesterol (0.25 µCi/ml, 2 µl/well for THP-1 cells, 1,5 µl/well for HMDM, in 24-well-plates). Simulatneously, acLDL (50 µg/ml) and LXR-agonist (3 µM) was added to load the cells with cholesterol and to maximize cholesterol efflux by upregulating ABCA-1 and ABCG-1 expression. This faciliates the detection of differences in the acceptor properites of serum or HDL from patients and controls.

To start the experiment the cells were washed twice with 300 µl PBS (37°C) and equilibrated with RPMI-UK (250µl/well) at 37°C for 2h. Then medium was removed and the cells were again washed twice with 300 µl PBS (37°C). Afterwards, the cells were incubated with medium (250 µl RPMI-UK/well) containing 10 µg/ml HDL, 1% serum, apoA-I at different concentrations, or no additional substances for 6h. After this period the supernatant was removed and centrifuged at 1750 rpm and 4°C for 10 minutes (Eppendorf centrifuge 5403). 150 µl of supernatant were mixed with 13 ml scintillation cocktail and counted in a beta counter (Tri-Carb 2800 TR; Perkin Elmer).

The remaining cells were washed twice with 300 µl PBS (37°C) and 250 µl NaOH (0,1 M) were added for cell lysis (room temperature, 45 min). 150 µl of cell lysates were mixed with 13 ml scintillation cocktail and counted in a beta counter (Tri-Carb 2800 TR; Perkin Elmer). The protein content of cell lysates was determined with the Bradford assay, as described before.

Efflux was calculated as percentage radioactivity in the media divided by the amount of radioactivity in cell lysates and media using following formula:

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

In every experiment, also efflux to medium only with no additional substances was measured, then substracted as a background from the counts for serum, HDL and apoA-I respectively, to exclude the influence of other factors probably present in the medium that might affect cellular cholesterol efflux.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA) and SPSS 11.5.1.0 (IBM, Somers, NY, USA). Data on serum lipids, serum apoproteins and HDL composition were provided by Tatjana Stojacovic (Dept. of Laboratory Medicine - Medical University of Graz), and are summarized in table 8 in detail.

3 Results

Macrophage cholesterol efflux is the first step of reverse cholesterol transport and essential for cholesterol homeostasis. HDL exerts its antiatherogenic properties mainly by acting as an acceptor for cellular cholesterol, which is exported from cells via several pathways. Among them are the ABCA1 mediated efflux to apoA-I, as well as the ABCG1 or SR-B1 mediated efflux to HDL, which constitute the primary step of the transport of cellular cholesterol - RCT - from peripheral tissues back to the liver for excretion via bile and feces (Vergeer et al., 2010).

In CKD the composition of HDL is altered and also HDL function and RCT may be affected, the risk of developing atherosclerosis is increased significantly and CVD mortality is excessively high (Kwan et al., 2007; Oh et al., 2002; Schiffrin et al., 2007).

The aim of this thesis was to study the cholesterol acceptor capacity of serum and HDL obtained from adult and pediatric CKD patients and dialysis patients, as well as the cholesterol efflux capacity of HMDM isolated from the study participants and healthy, matched controls.

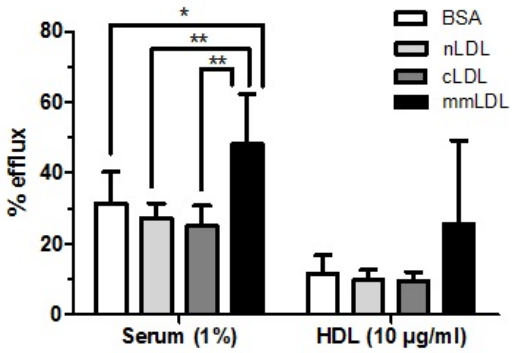
3.1 Cholesterol efflux from THP-1 macrophages

THP-1 cells can be differentiated into macrophage-like cells and therefore are commonly used to study foam cell formation in atherosclerosis development (Auwerx, 1991). THP-1 cells were treated as described in the material and methods section (see 2.2.2). 24h prior to the efflux experiments the THP-1 macrophages were incubated with acLDL (50 µg/ml) and trace-labeled with [^{3}H]-cholesterol (1µCi). A synthetic LXR-agonist (T0901317, 3 µM) was added to maximize cholesterol efflux by upregulating ABCA1 and ABCG1 expression. Efflux was performed for 6 hours to 1% serum and 10 µg/ml HDL.

3.1.1 Influence of *in vitro* modified LDL on the cholesterol efflux capacity of THP-1 macrophages

For measuring the influence of *in vitro* modified LDLs on cellular cholesterol efflux capacity the THP-1 cells were differentiated towards macrophages and preincubated with either bovine serum albumin (BSA), nLDL, cLDL or mmLDL (50 µg/ml) for 48h. 24h prior to the efflux experiment the THP-1 macrophages were trace-labeled with [^{3}H]-cholesterol (1µCi) and treated with LXR-agonist (T0901317, 3 µM). Efflux was measured to 1% serum or 10µg/ml HDL, obtained from healthy volonteers, after 6 hours.

Fig. 3.1: Influence of *in vitro* modified LDLs on the cholesterol efflux capacity of THP-1 macrophages



*THP-1-cells were seeded at a density of 5*10^5 cells/well, differentiated in 350 µl RPMI-compl. containing 160 nM PMA for 3 days and preincubated with either BSA, nLDL, cLDL or mmLDL 48h prior to the efflux experiments. 24h prior to efflux experiments cells were trace-labeled with [^{3}H]-Cholesterol (1µCi). Each bar represents the mean ± SD, n=6. The results are expressed as % efflux. Statistical significance was analyzed using an unpaired t-test (p<0.05 *, p<0.01 **).*

cholesterol efflux after preincubation with mmLDL was increased significantly compared to BSA (p<0.05), nLDL (p<0.01) and cLDL (p<0.01) and was significantly higher to serum than to HDL (p<0.001) except in mmLDL.

cholesterol efflux to serum was significantly increased after preincubation with mmLDL compared to BSA , nLDL and cLDL respectively (mmLDL: 48.5±14.1 compared to BSA (31.5±8.9; p<0.05), nLDL (27.3±4.2; p<0.01), cLDL (25.1±5.6; p<0.01)).

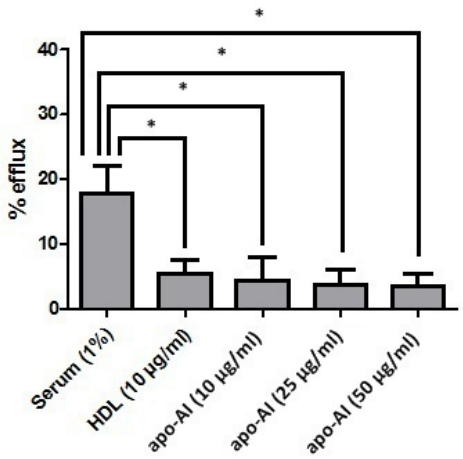
Svensson et al. (2005) reported SR-B1 to be upregulated under the influence of mmLDL, which might be an explanation for the enhanced cholesterol efflux to serum induced by mmLDL in this experiment.

The results on cholesterol efflux to HDL did not show a significant difference, probably due to the high standard deviation of the data obtained after mmLDL incubation.

3.1.2 Cholesterol acceptor capacity of apoA-I

To test the cholesterol acceptor capacity of apoA-I at different concentrations as well as the conditions for preincubation of THP-1 cells with human serum and to determine the cholesterol efflux capacity to apoA-I and HDL, THP-1 cells were differentiated towards macrophages and preincubated with 10% human serum obtained from healthy volunteers (1 male, 1 female) for 2 days. 24h prior to the efflux experiment the THP-1 macrophages were trace-labeled with [^{3}H]-cholesterol (1µCi). Efflux was measured to serum (1%) and HDL (10 µg/ml), obtained from healthy volunteers, or apoA-I (10, 25, 50 µg/ml).

Fig. 3.2: Cholesterol acceptor capacity of apoA-1



*THP-1-cells were seeded at a density of 5*10^{5}cells/well, differentiated in 350 µl RPMI compl. containing 160 nM PMA for 3 days, then preincubated with 10% human serum for 3 days. 24h prior to efflux experiments cells were trace-labeled with [^{3}H[-Cholesterol (1µCi). The bars for serum (1%), HDL (10 µg/ml) and apo-A1 (10, 25 µg/ml) represent the mean ± SD, n=8, for apoA-I (50 µg/ml) n=4. The results are expressed as % efflux.*

cholesterol efflux to serum was significantly higher compared to HDL and apoA-I at three different concentrations.

No difference could be observed in efflux to HDL and apoA-I , neither in THP-1 treated with male serum, nor in THP-1 treated with female serum, nor taken together. Also, the cholesterol efflux to the three different concentrations of apoA-I did not differ.

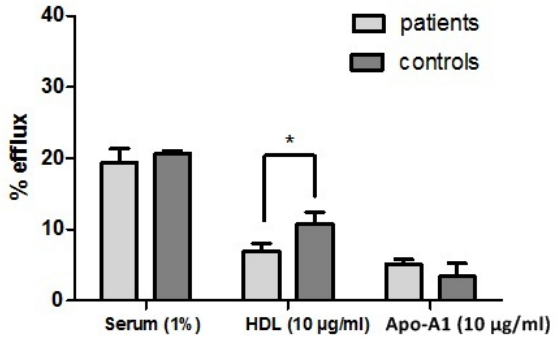
Similar findings were obtained in HMDM (see fig. 3.2.2).

3.1.3 Influence of uremic serum on cholesterol efflux capacity of THP-1 macrophages

To study the influence of uremic serum on the cholesterol efflux capacity of THP-1 macrophages, THP-1 cells were differentiated towards macrophages and incubated with 10% pooled serum from adult CKD patients or healthy, matched controls for 48h. Efflux was performed to control serum, HDL and apoA-I.

When cholesterol efflux to 3 different concentrations of apoA-I was measured in a previous experiment (see fig 3.2), no difference could be observed. Hence, for this experiment, it was decided to solely use the 10 µg/ml preparation.

Fig. 3.3: Influence of uremic serum on the cholesterol efflux capacity of THP-1 macrophages



*THP-1-cells were seeded in 24-well-plates at a densitiy of 5*10^{5}cells/well, differentiated towards macrophages and incubated with 10% pooled serum from uremic patients or controls, for 2 days. 24h prior to efflux experiments cells were trace-labeled with [^{3}H]-cholesterol (1µCi). Each bar represents the mean ± SD, n=3. The results are expressed as % efflux. Statistical significance was analyzed using an unpaired t-test (p<0.05 *).*

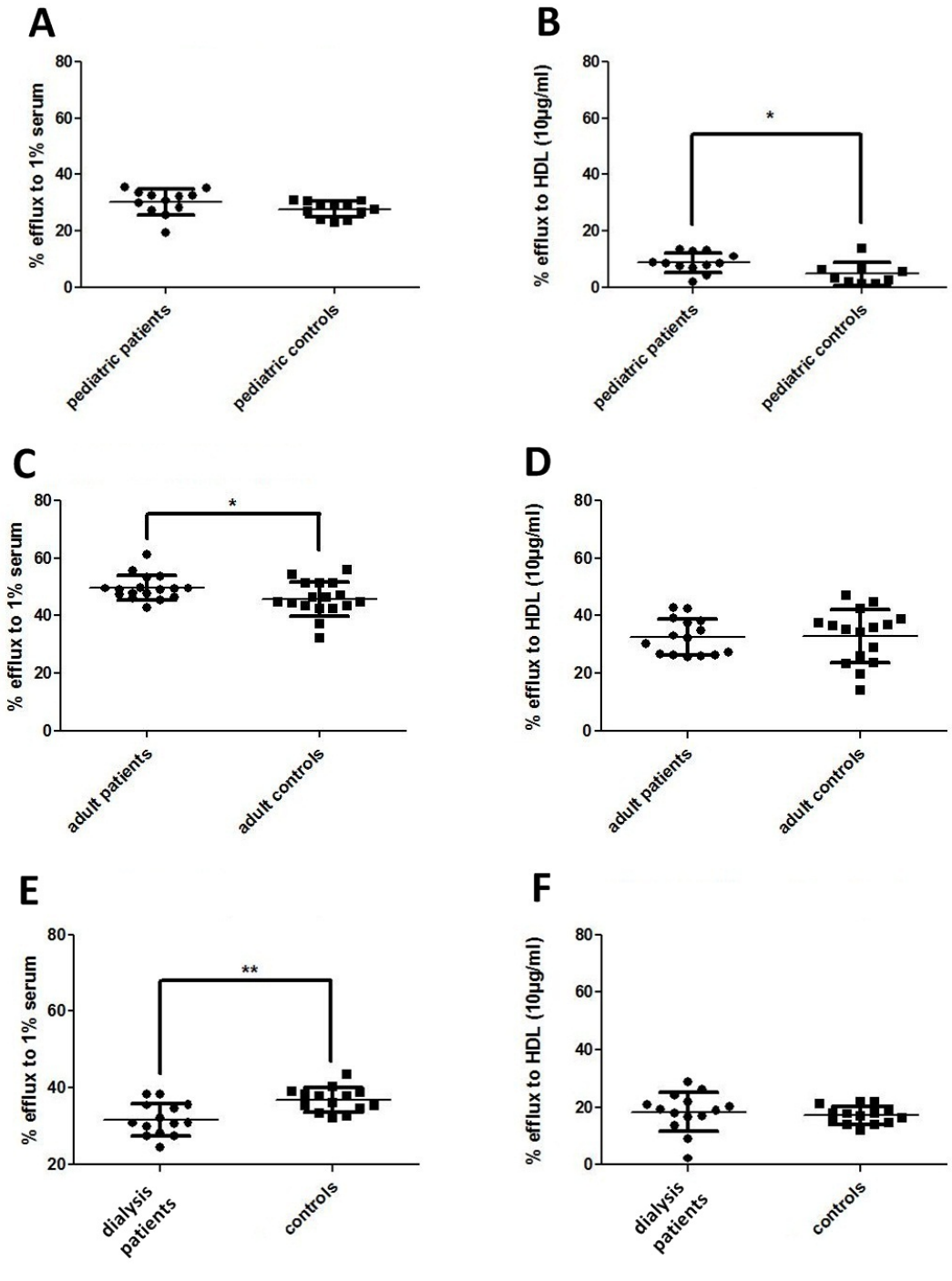
cholesterol efflux to HDL was significantly diminished in patients (p<0.05), efflux to serum and apoA-I was not altered.

The cholesterol efflux capacity of THP-1 macrophages incubated with serum obtained from adult CKD patients to HDL was decreased significantly ($p<0.05$; patients 7.00±1.04, controls 10.73±1.72) while efflux to serum and apoA-I did not differ between patients (serum 19.37±1.94; apoA-I 5.17±0.60) and controls (serum 20.67±0.38; apoA-I 3.40±1.85).

3.1.4 Cholesterol acceptor capacity of uremic serum and HDL for cellular cholesterol exported by THP-1 macrophages

Next, the influence of CKD on the cholesterol acceptor capacity of serum or HDL was studied. Therefore, THP-1 cells were differentiated towards macrophages and cholesterol efflux to 1% individual serum samples and 10 µg/ml individual HDL was measured. The patient's serum and HDL samples of adult CKD patients, dialysis patients and matched controls were analyzed individually.

Fig. 3.4: Cholesterol acceptor capacity of uremic serum and HDL for cellular cholesterol exported by THP-1 macrophages



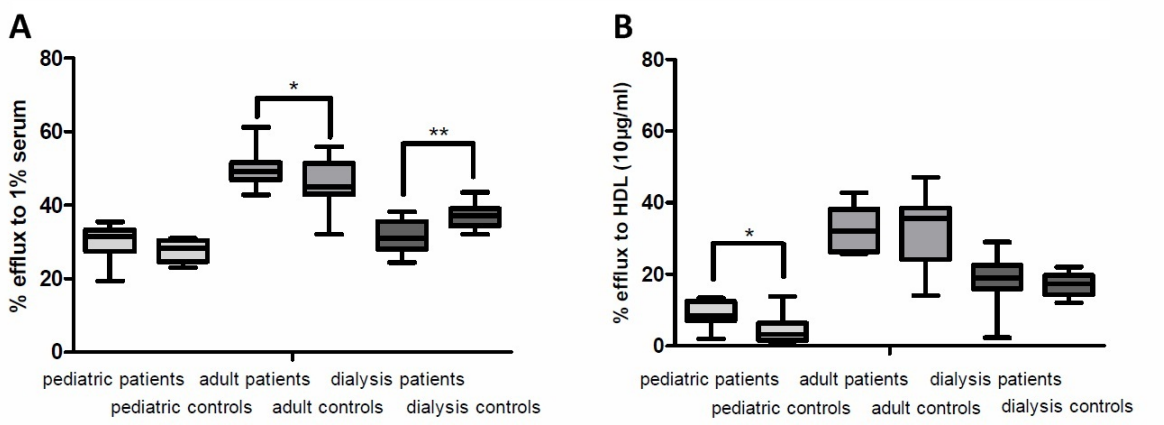
*THP-1-cells were seeded at a density of 5*10^{5}cells/well and differentiated in 250 µl RPMI-compl. containing 160 nM PMA for 3 days. 24h prior to efflux experiments cells were incubated with acLDL (50 µg/ml), LXR-agonist (T0901317, 3 µM) and trace-labeled with [^{3}H]-Cholesterol (1µCi). Statistical significance was analyzed using an unpaired t-test ($p<0.05$ *, $p<0.01$ **).*

cholesterol efflux to serum isolated from adult patients with CKD stage 3-5 was increased significantly ($p<0.05$; C) and diminished significantly in dialysis patients ($p<0.01$; E). Efflux to HDL was increased in pediatric patients ($p<0.05$; B) while it was not altered in adult CKD patients stage 3-5 or dialysis patients (D, F).

While it could be shown that the cholesterol acceptor capacity of serum of adult patients increases significantly (patients 49.68±4.34; controls 45.86±5.95; N=17; $p<0.05$; fig. 3.4, C), in dialysis patients the opposite occurred, namely a significant decrease (patients 31.73±4.22; controls 36.85±3.20; $p<0.01$; fig. 3.4, E). In efflux experiments to 10 µg/ml HDL no difference could be shown, in both, adult CKD patients stage 3-5 and dialysis patients, and controls respectively (fig. 3.4, D, F).

A significant increase in cholesterol efflux to HDL could be observed in pediatric patients (patients 8.71±3.52, N=12; controls 4.71±4.00; $p<0.05$, fig. 3.4, B), while in efflux to serum no difference was found in efflux to serum (fig. 3.4, A).

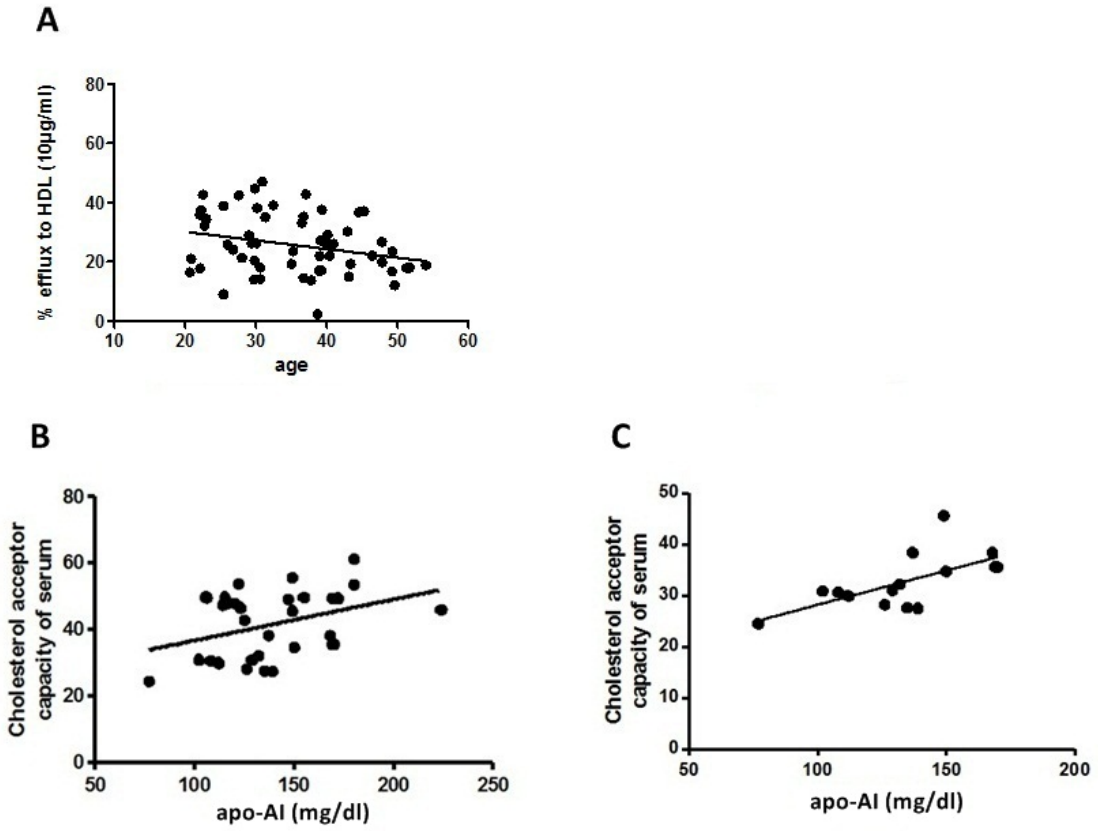
Fig. 3.5: Cholesterol acceptor capacity of uremic serum and HDL for cellular cholesterol exported by THP-1 macrophages-overview



*THP-1-cells were seeded at a concentration of $5*10^5$ cells/well and differentiated in 250 µl RPMI-compl. containing 160 nM PMA for 3 days. 24h prior to efflux experiments cells were incubated with acLDL (50 µg/ml), LXR-agonist (T0901317, 3 µM) and trace-labeled with [³H]-Cholesterol (1µCi). Statistical significance was analyzed using an unpaired t-test ($p<0.05$ *, $p<0.01$ **; pediatric patients/controls: n=12 per group, adult patients/controls: n=17 per group; dialysis patients/controls: n=14 per group).*

Whereas serum-HDL (mg/dl) was found to be diminished significantly in serum of pediatric patients and adult dialysis patients (pediatric controls 48.5±9.9, pediatric patients 32.2±13.2; $p<0.01$; dialysis controls 53.2±14.1, dialysis patients 36.8±9.3; $p<0.01$; tab. 8), the apoA-I serum levels (mg/dl) of pediatric patients were not altered. Contrarily, in adult hemodialysis patients apoA-I levels were decreased significantly (controls 166.6±29.8, patients 132.4±27.0; $p<0.01$; tab. 8). This might, at least to some extent, explain the significantly reduced cholesterol acceptor capacity of serum in dialysis patients (Fig. 3.5).

Fig. 3.6: Cholesterol efflux from THP-1 cells



cholesterol efflux from THP-1 cells to HDL of all adult subjects significantly correlates with age (A; R^2=0.068 ; p=0.0461). cholesterol efflux from THP-1 macrophages to serum isolated from adult CKD stage 3-5 and dialysis patients positively correlates with apoA-I levels (B; R^2=0.138; p=0.0398), especially in the group of dialysis patients (C; R^2=0.422; p=0.0088).

Cholesterol efflux from THP-1 macrophages to HDL of all adult subjects significantly correlated with age (R^2=0.068 ; $p<0.05$; fig.3.6, A).

cholesterol efflux to serum isolated from adult CKD and dialysis patients positively correlated with serum apoA-I levels (R^2=0.138; $p<0.05$; fig. 3.6, B), which was even more distinct in the group of dialysis patients (R^2=0.422; $p<0.01$; fig. 3.6, C).

3.2 Cholesterol efflux from human monocyte-derived macrophages (HMDM)

Macrophages play a major role in atherosclerosis development as they take up modified LDL via scavenger receptors, which leads to foam cell formation, subsequent formation of atherosclerotic lesions and CVD eventually (Lusis, 2000).

Much less is known about their ability to export cholesterol in this condition.

3.2.1 Western Blot Analysis

Cholesterol is taken up via SR-A1/2 and CD36 (Chmielewski et al., 2005). CD36 is not present in peripheral blood monocytes, but generated during differentiation towards macrophages. Thus, by scanning for the day during differetiation with the highest expression of CD36 it was possible to identify the most suitable time for [^{3}H]-cholesterol labelling and cholesterol efflux and, more importantly, to prove that the cells used for the subsequent experiments were fully differentiated human monocyte-derived macrophages.

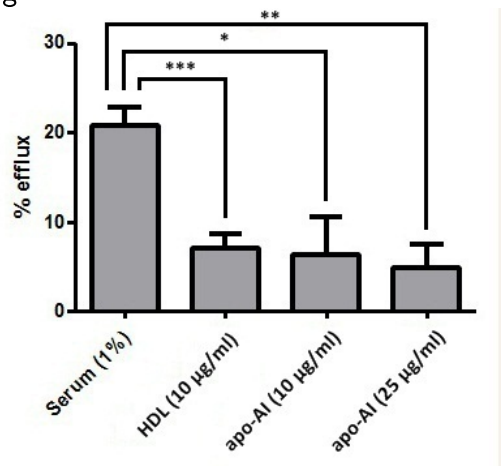
Monocytes were isolated as described before (see 2.2.1) and differentiated towards macrophages for 0, 7, or 9 days under 250 µl RPMI w/o FCS with 10% autologous serum, then Western Blot Analysis was performed. The highest expression of CD36, was observed on day 7 (data not shown). The cells were trace-labeled with [^{3}H]-Cholesterol (1µCi) on that day and efflux to serum (1%) or HDL (10µg/ml) was performed on day 8.

3.2.2 Cholesterol efflux from HMDM

To determine whether in contrast to the experiments in THP-1 macrophages in HMDM a difference can be detected in cholesterol export to HDL and apoA-I, cholesterol efflux from HMDM was measured to different acceptors (see section 3.1.2). Again, two concentrations of apoA-I (10 µg/ml and 25 µg/ml) were used according to the results of the experiment in THP-1 macrophages, which did not show a difference in efflux to the different concentrations of apoA-I (see fig. 3.2).

Monocytes were isolated from blood obtained from two healthy, fasting volonteers (one male, one female), as described before, and differentiated towards macrophages for 7 days under 250 µl RPMI w/o FCS containing 10% autologous serum. Efflux experiments were performed on day 8.

Fig. 3.7: Cholesterol efflux from HMDM



*Monocytes were isolated from blood samples obtained from two healthy, fasting volonteers (one male, one female), mixed and differentiated towards macrophages under RPMI w/o FCS with 10% pooled, autologous serum for 7 days. 24h prior to efflux experiment the cells were trace-labeled with [³H]-cholesterol (1µCi). cholesterol efflux was measured on day 8 to 1% healthy serum, 10 µg/ml healthy HDL or apoA-I (10 µg/ml, 25 µg/ml). Each bar represents the mean ± SD, n=4. The results are expressed in % efflux. Statistical significance was analyzed using an unpaired t-test ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).*

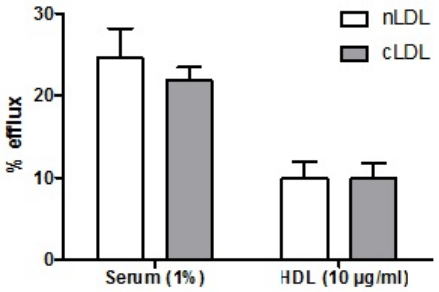
cholesterol efflux from HMDM to serum was significantly higher than to HDL ($p<0.0001$) and two different concentrations of apoA-I ($p<0.05$ to 10 µg/ml; $p<0.01$ to 25 µg/ml).

The results obtained in THP-1 cells could be confirmed in HMDM: there was no significant difference in efflux to HDL and apoA-I at both concentrations.

3.2.3 Influence of *in vitro* modified LDL on the cholesterol efflux capacity of HMDM

To investigate the influence of *in vitro* carbamylated LDL on cholesterol efflux from HMDM to control serum and HDL, blood samples were obtained from two healthy, fasting volonteers, monocytes were isolated as described before (see 2.2.1) and differentiated towards macrophages for 7 days under 250 µl RPMI w/o FCS containing 10% autologous serum. Two days before the efflux experiment the HMDM were preincubated either with nLDL or cLDL. 24h prior to the cholesterol efflux experiment the HMDM were trace-labeled with [³H]-cholesterol (1µCi) and efflux was performed on day 8 to 1% serum and 10 µg/ml HDL obtained from healthy volunteers.

Fig. 3.8: Influence of *in vitro* modified LDL on the cholesterol efflux capacity of HMDM



Monocytes were isolated from blood samples obtained from two healthy, fasting volonteers (one male, one female), mixed and differentiated towards macrophages under RPMI w/o FCS with 10% pooled, autologous serum for 7 days. The cells were preincubated with either nLDL or cLDL 48h prior to the efflux experiment. 24h prior to efflux experiment the cells were trace-labeled with [³H]-Cholesterol (1µCi). cholesterol efflux was measured on day 8 to 1% healthy serum or 10 µg/ml healthy HDL. Each bar represents the mean ± SD, n=6. The results are expressed in % efflux.

No alterations in cholesterol efflux to nLDL and cLDL could be observed.

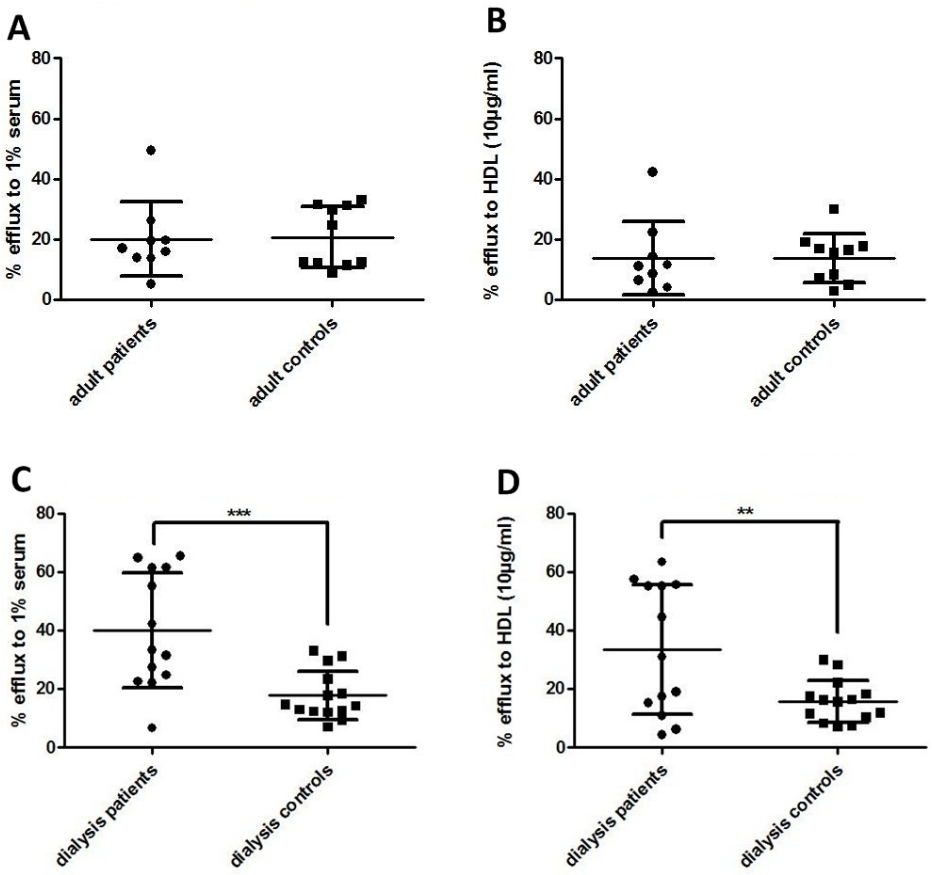
There was no significant difference in the cholesterol efflux capacity of HMDM treated with either nLDL or cLDL, neither to serum (nLDL 24.55±3.65; cLDL 21.78±1.68) nor HDL (nLDL 9.88±1.99; cLDL 9.83±1.97), although efflux to serum from HMDM incubated with cLDL was slightly lower. This is consistent with the results from previous experiments in THP-1 macrophages (see fig. 3.1), where efflux neither to serum nor to HDL was altered, no matter if the cells were incubated with nLDL or cLDL.

3.2.4 Influence of uremia on the cholesterol efflux capacity of HMDM

To compare the efflux capacity of HMDM of adult CKD patients, dialysis patients and healthy controls, blood samples were obtained from patients recruited at the Dept. of Internal Medicine III of the Medical University of Vienna (MUW) and controls carefully matched by sex and age (for details see 2.1 Patients and controls).

Monocytes were isolated within 2h after sample collection and differentiated towards macrophages with RPMI w/o FCS containing 10% autologous serum for 7 days (24-well-plates, 6 wells/sample). cholesterol efflux experiments were performed on day 8 using 1% healthy serum and 10 µg/ml HDL obtained from healthy donors.

Fig. 3.9: Influence of uremia on the cholesterol efflux capacity of HMDM



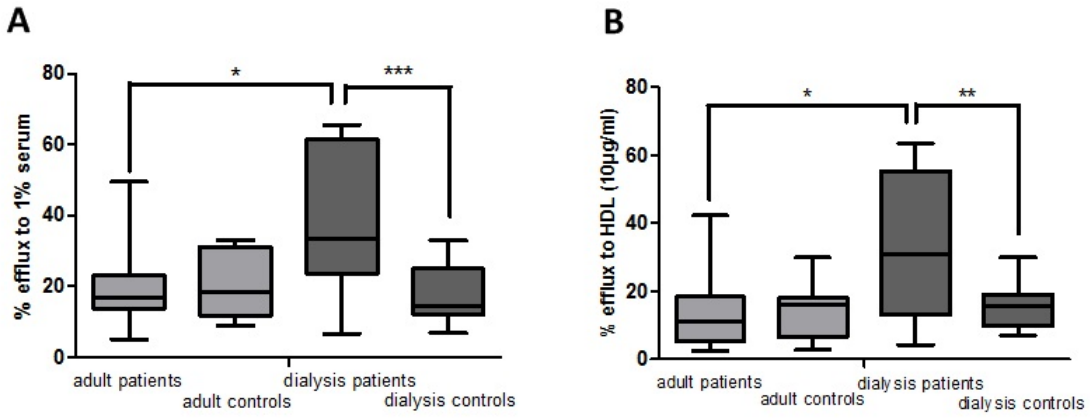
*Monocytes were isolated from venous blood samples and differentiated towards macrophages under RPMI w/o FCS with 10% autologous serum for 7 days. 24h prior to efflux experiments the cells were trace-labeled with [³H]-Cholesterol (1µCi). cholesterol efflux was measured on day 8 to 1% serum or 10 µg/ml HDL obtained from healthy volonteers. The results are expressed in % efflux. Statistical significance was analyzed using an unpaired t-test ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).*

In adult CKD patients, no difference in cholesterol efflux capacity of HMDM to control serum or HDL could be observed (fig. 3.9, A, B), whereas in dialysis patients a significant increase in efflux capacity to control serum as well as to control HDL could be shown (serum: patients 39.95±19.73; controls 17.74±8.36; $p<0.001$; fig. 3.9, C; HDL: patients 33.50±22.23; controls 15.74±7.20; $p<0.01$; fig. 3.9, D).

These observations are in contrast to the results obtained for cholesterol acceptor capacity of serum or HDL using THP-1 cells (see Fig. 3.5), where the acceptor capacity of serum of dialysis patients was diminished significantly (patients 31.73±4.22; controls 36.85±3.20; $p<0.01$; fig. 3.4, E) and the acceptor capacity of serum of adult CKD patients was increased (patients 49.68±4.34; controls 45.86±5.95; $p<0.05$; fig. 3.4, C) compared to healthy controls.

Similarily, the significant increase in the cholesterol efflux capacity of HMDM of dialysis patients to HDL ($p<0.01$; fig. 3.9, D) is in contrast to the results of cholesterol acceptor capacity of serum or HDL measured using THP-1 macrophages (see fig. 3.4, F), where no difference could be shown.

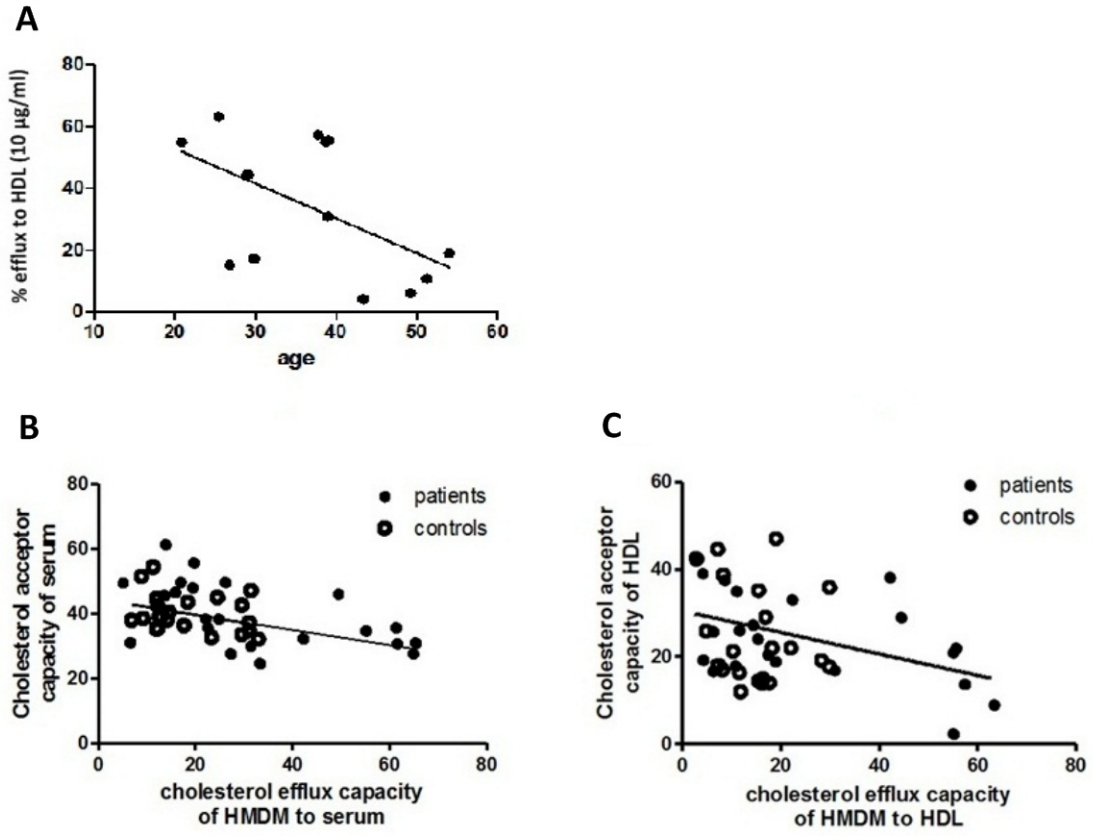
Fig. 3.10: Influence of uremia on the cholesterol efflux capacity of HMDM-overview



*Monocytes were isolated from blood samples and differentiated towards macrophages under RPMI w/o FCS with 10% autologous serum for 7 days. 24h prior to efflux experiments the cells were trace-labeled with [³H]-Cholesterol (1µCi). cholesterol efflux was measured on day 8 to 1% serum or 10 µg/ml HDL obtained from healthy volonteers. The results are expressed in % efflux. Statistical significance was analyzed using an unpaired t-test ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).*
The cholesterol efflux capacity of HMDM to control serum was increased significantly in dialysis patients compared to adult patients ($p<0.05$). Also, cholesterol efflux from HMDM to control HDL was increased significantly compared to adult patients ($p<0.05$).

Furthermore, the cholesterol efflux capacity of HMDM obtained from dialysis patients to control serum and HDL was increased significantly compared to adult CKD patients (serum: adult patients 20.1±12.4, dialysis patients 40.0±19.7, $p<0.05$, fig.3.10, A; HDL: adult patients 13.7±12.2, dialysis patients 33.5±22.2, $p<0.05$, fig. 3.10, B).

Fig. 3.11: cholesterol efflux from HMDM



cholesterol efflux from HMDM of dialysis patients to control HDL has a tendency to decrease with age (A; n.s.). In adult patients a negative correlation of cholesterol efflux capacity of HMDM and cholesterol acceptor capacity of control serum (B; R^2=0.281; p=0.0112) and HDL (C; R^2=0.372; p=0.0026) could be shown.

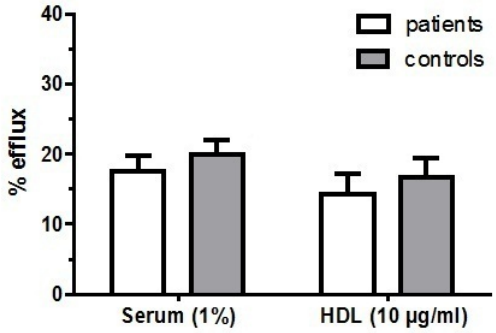
In HMDM no significant correlation between cholesterol acceptor capacity of HDL and age could be found although there might be a tendency of cholesterol efflux capacity to decrease with increasing age in dialysis patients (R^2=0.281; p=0.0624; fig.3.11, A). This could not be observed in pediatric or adult patients or efflux to serum respectively.

In adult patients, a negative correlation between the cholesterol acceptor capacity and the cholesterol efflux capacity from HMDM (R^2=0.281; p<0.05; fig. 3.11, B) and HDL (R^2=0.372; p<0.01; fig. 3.11, C) could be shown, which leads to the speculation that the cells try to compensate a diminished cholesterol acceptor capacity with a higher rate of cholesterol efflux.

3.2.5 Capacity of pediatric, uremic serum and HDL to act as an acceptor for cellular cholesterol exported by healthy HMDM

Attempting to validate the data obtained from the experiments in THP-1 macrophages (described in section 3.1.4, fig. 3.4 A) for HMDM, monocytes were isolated and differentiated towards macrophages under RPMI w/o FCS containing 10% autologous serum for 7 days. Efflux was performed on day 8 to 1% pooled serum and 10 µg/ml pooled HDL.

Fig. 3.12: Capacity of pediatric, uremic serum and HDL to act as an acceptor for cellular Cholesterolefluxed from healthy HMDM



Monocytes were isolated from blood samples obtained from a healthy volonteer (female) and differentiated towards macrophages under RPMI w/o FCS with 10% autologous serum for 7 days. 24h prior to efflux experiments the cells were trace-labeled with [³H]-Cholesterol (1µCi). cholesterol efflux was measured on day 8 to 1% pooled serum or 10 µg/ml pooled HDL obtained from pediatric patients/controls. Each bar represents the mean ± SD, n=5. The results are expressed in % efflux.

The cholesterol efflux capacity of THP-1 macrophages to serum or to HDL isolated from pediatric patients was not altered significantly.

The results for cholesterol efflux from HMDM to either 1% pooled serum or 10 µg/ml pooled HDL did not differ significantly (serum: patients 17.5±2.2; controls 19.9±2.0; HDL: patients 14.3±2.8; controls 16.6±2.9), although the efflux capacity of serum as well as of HDL seems to be slightly diminished in patients. HDL serum levels (mg/dl) were decreased in patients ($p<0.01$), while apoA-I serum levels (mg/dl) were not altered significantly compared to controls (see tab. 8).

4 Discussion

Atherosclerosis, a chronic, progressive disease, is highly prevalent in chronic kidney disease (Chade et al., 2005). The risk for CKD patients to die from a CV cause is excessively elevated, especially in children and young adults (Oh et al., 2002; Schiffrin et al., 2007).

The development of atherosclerosis in CKD is at least in part due to a disturbed cholesterol balance in macrophages of the arterial wall.

In the context of CKD cellular cholesterol influx into macrophages has been studied in more detail than cholesterol efflux. Cholesterol uptake from modified LDL is mediated by the scavenger receptors SR-A1/2 and CD36 and their expression is elevated in CKD patients (Chmielewski et al., 2005). It is known that CD36 is hardly expressed in monocytes but that its protein expression increases with the grade of differentiation (Huh et al., 1996). However, not only the state of differentiation, but also the presence of cytokines and growth factors contributes to the CD36 expression (Han et al., 1997). The expression of all three, SR-A1/2 and CD36, has been shown to be increased in blood monocytes of CKD patients, which possibly contributes to the risk of developing severe atherosclerosis. CD36 expression is even higher in dialysis than in CKD patients, yet, it is not entirely clear if the enhanced expression is due to the longer duration of CKD or to the impact of hemodialysis treatment (Chmielewski et al., 2005; Konishi et al., 1997).

Cellular cholesterol efflux is part of a process called reverse cholesterol transport in which excess cholesterol is removed from peripheral cells and transported back to the liver for excretion (Lusis, 2000). The initial step of RCT is the cellular cholesterol efflux to apoA-I, the main apolipoprotein of HDL. Alterations in HDL composition (Kwan et al., 2007; Marsche et al., 2008), as well as low HDL-cholesterol (Gonen et al., 1985) may contribute to an impairment of RCT during CKD, which leads to an increased risk for developing atherosclerotic disease (Cho, 2009). Thus, both the quantity and also the quality of HDL are critical for adequate cholesterol efflux (Chirinos et al., 2005; Cho, 2009), however, its exact role is still incompletely understood (Weichhart et al., 2012).

In addition to its role in reverse cholesterol transport Weichhart et al. (2012) showed that control HDL is able to inhihbit inflammatory cytokine production, whereas HDL obtained from ESRD patients had lost this property and, beyond that, even promoted the production of inflammatory cytokines (Weichhart et al., 2012). This pathophysiological mechanism is thought to enhance atherosclerosis development and may eventually lead to fatal CV events (Oh et al., 2002; Schiffrin et al., 2007).

This study aimed to investigate the cholesterol acceptor capacity of serum and HDL obtained from adult and pediatric CKD patients, dialysis patients and healthy matched controls, as well as the cholesterol efflux capacity of HMDM isolated from the study participants and healthy, matched controls.

The first aim, to examine the cholesterol acceptor capacity of serum and HDL isolated from the study participants, was investigated in experiments conducted in THP-1 macrophages as well as in HMDM.

To define the experimental conditions, first the cholesterol acceptor capacity of different concentrations of apoA-I (10, 25, 50 µg/ml) was measured in THP-1 macrophages. No difference was detected between the three apoA-I concentrations and HDL respectively (fig. 3.2).

The results from THP-1 macrophages could be verified in HMDM where, again, cholesterol efflux did not differ when measured to HDL and apoA-I at two different concentrations (10, 25 µg/ml; fig. 3.7). Taking into account the fact that apoA-I is the major apolipoprotein of HDL (Cho, 2009) the similar results in cholesterol acceptor capacity of HDL and apoA-I observed in these experiments are explicable.

To examine the cholesterol acceptor capacity of uremic serum and HDL, efflux was measured from THP-1 macrophages to patient's and control's individual serum (1%) and HDL (10 µg/ml) respectively. The cholesterol acceptor capacity of serum from adult patients was increased significantly ($p<0.05$; fig 3.4, C), whereas in dialysis patients the contrary, a significant decrease ($p<0.01$), could be shown (fig. 3.4, E). This might, at least to some extent, be explained by the significantly reduced concentration of serum apoA-I in the dialysis patients of this cohort. ApoA-I is known to promote cellular cholesterol efflux via the ABCA1 pathway (Ohashi et al., 2005). Indeed, the cholesterol acceptor capacity of serum isolated from adult CKD stage 3-5 and dialysis patients positively correlated with the apoA-I level (mg/dl; $p<0.05$; fig. 3.6, B), which was even more distinct in the group of dialysis patients ($p<0.01$; fig. 3.6, C). Also, pediatric CKD patients did not show a diminished cholesterol accceptor capacity of serum compared to controls, which might be due to the unaltered serum apoA-I concentration despite low HDL.

Investigating the cholesterol efflux to uremic and control HDL, a difference could exclusively be observed in the group of pediatric subjects, where the patient's cellular choles-

terol efflux was increased significantly ($p<0.05$; fig. 3.4, B). Notably, the cholesterol acceptor capacity of serum was not altered in this group. This again, might be related to the serum apoA-I concentration which, in contrast to the serum HDL-C concentration, was not altered in pediatric CKD patients compared to controls. In an attempt to confirm these data using HMDM, cholesterol efflux was measured from HMDM obtained from a healthy adult volunteer to either 1% pooled serum or 10 µg/ml pooled HDL of pediatric patients. In this experiment the cholesterol acceptor capacity of serum and HDL was subtly higher in control subjects, but did not differ significantly (fig. 3.11). This is in contrast to the findings in THP-1 macrophages where efflux to the patients individual HDL preparations was found to be increased significantly ($p<0.05$; fig. 3.4, B).

HDL synthesis and maturation are impaired during CKD and ESRD and as a consequence HDL levels are diminished. It has been speculated that uremia renders HDL dysfunctional by altering its composition and functionality. In 2000, Morena et al. showed impaired atheroprotective effects of HDL, functional and quantitative lipid abnormalities, and increased susceptibility to LDL oxidation during hemodialysis, and furthermore, an association between qualitative abnormalities of LDL during HD as well as a decrease in HDL antioxitative properties (Morena et al., 2000). Moreover, an enhanced susceptibility of HDL to lipid peroxidation, as well as a decrease of its antioxidant activity with increasing age, has been shown (Berrougui et al., 2007).

In 2011 Holzer et al. investigated the proteomic profile as well as the cholesterol acceptor capacity of uremic HDL isolated from patients on maintenance hemodialysis and reported that the altered composition of uremic HDL is linked to a diminished cholesterol efflux due to apoA-I, apoA-II and phospholipid depletion (Holzer et al., 2011a). This is in contrast to our finding of an unaltered cholesterol acceptor capacity of HDL in a similar patient group. Exclusion of diabetic patients in our study and the use of human THP-1 cells, rather than the mouse RAW267 macrophage line employed by Holzer et al, as well as differences in the techniques for HDL isolation may explain these differences. Moreover, it needs to be taken into account that the patients in the study of Holzer et al. were clearly older than the controls.

In our study the cholesterol acceptor capacity of HDL from all adult subjects significantly decrased with age ($p<0.05$; fig. 3.6, A). In dialysis patients there was a trend toward a negative correlation of cholesterol efflux capacity of HMDM and age, although this result did not reach statistical significance ($p=0.0624$; $R^2=0.2810$; fig. 3.11, A). The exact mechanism of this process remains to be elucidated. Similarly, Berrougui et al.

(2007) studied cholesterol efflux from THP-1 and J774 macrophages to HDL obtained from healthy, young and healthy, elderly subjects respectively, and showed a significantly diminished capacity of HDL from elderly subjects to promote cholesterol efflux (Berrougui et al., 2007).

Holzer et al. reported in 2011 that HDL carbamylated by myeloperoxidase (MPO) can be found in the vessel wall. In SR-B1 overexpressing THP-1 cells they were able to demonstrate that carbamylation of HDL *in vitro* results in net cholesterol uptake by destabilizing the HDL/SR-B1-dependent balance of cholesterol influx and efflux, thereby promoting the development of atherosclerosis in ESRD, where the urea-levels are extremely high. This effect, however, could not be shown in non-transfected control cells (Holzer et al., 2011b).

A very recent study by Weichhart et al. (2012) demonstrated an altered HDL protein composition in ESRD patients. Surfactant protein-B (SP-B), ApoC-II, serum-amyloid A (SAA), and alpha-1-microglobulin/bikunin precursor (AMBP) were found to be enriched in ESRD patients compared to healthy controls. Moreover, Weichhart et al. found SB-P in patients HDL exclusively. As they were able to detect SB-P and SAA in stage 4 CKD patients as well, this indicates that the changes in HDL composition are not only due to hemodialysis, as has been speculated before, but to uremia itself (Weichhart et al., 2012). In healthy controls SB-P was not detectable at all, hence its plasma concentration may be a novel biomarker for CKD progression as well as kidney failure, cardiovascular morbidity and mortality (Weichhart et al., 2012) and chronic heart failure (Pasquale et al., 2004).

After investigating the cholesterol acceptor capacity of THP-1 macrophages and HMDM, we examined the effect of LDL modifications occuring in CKD patients, namely carbamylation and oxidation, on the cholesterol efflux capacity of THP-1 macrophages.

In CKD subjects modified LDL are capable of stimulating CE accumulation in the macrophages of the vessel wall (Gonen et al., 1985), which is a key event in atherosclerosis development (Lusis, 2000). Especially carbamylated LDL has been shown to be remarkably elevated in serum of ESRD patients (281.5$\pm$46.9 mg/l compared to 86.1$\pm$29.7 mg/l in controls; $p<0.001$) (Apostolov et al., 2005). It has been suggested that cLDL holds a major part in the atherosclerotic process, especially in these patients (Basnakian et al., 2010). Furthermore, Ok et al. (2005) showed that cLDL also damages human coronary artery endothelial cells (HCAECs) in a dose- and time-dependent manner and that it is remarkably elevated in hemodialysis patients (Ok et al., 2005).

OxLDL is commonly divided in two forms: minimally modified LDL and extensively oxi-

dized LDL (oxLDL). While mmLDL is still recognized by the LDLR, oxLDL is recognized by scavenger receptors, but not vice versa (Levitan et al., 2010).

To investigate the influence of *in vitro* modified LDL (nLDL, cLDL, mmLDL) on the cholesterol efflux capacity of THP-1 macrophages cholesterol efflux to control serum and HDL was measured. In these experiments the cholesterol efflux capacity was found to be significantly increased in macrophages pretreated with mmLDL only, but not with cLDL ($p<0.01$ vs. nLDL; fig. 3.1).

In human monocyte-derived macrophages the influence of cLDL on the cholesterol efflux capacity of HMDM obtained from a healthy volunteer to control serum and HDL was investigated and compared to the influence of nLDL. The results did not show a significant difference (fig. 3.8), which is consistent with the findings in THP-1 macrophages, where an increase in efflux was observed in macrophages pretreated with mmLDL exclusively (fig. 3.1).

These data are in agreement with the results of Svensson et al. (2005), who found SR-B1, which promotes cellular cholesterol efflux in peripheral tissues (Pagler et al., 2007), to be upregulated under the influence of mmLDL ($p<0.05$) in HMDM of atherosclerosis patients and healthy controls (Svensson et al., 2005).

Han et al. (2001) reported SR-B1 expression to be time-dependent in a murine macrophage cell line (J774), when treated with oxLDL. They observed an increase in SR-B1 expression when oxLDL was added after 3 days in culture, but a decrease when added to fully differentiated macrophages after 10 days in culture, which is in contrast to the results of Hirano et al. (1999), who found SR-B1 to be markedly upregulated as the differentiation of human monocytes to macrophages progresses (Han et al., 2001; Hirano et al., 1999). In this study modified LDL was added to fully differentiated macrophages.

Cardinal et al. (2007) reported a down-regulation of ABCA1 and ABCG1 in human coronary arterial endothelial cells (HCAEC), that had been treated with 20% uremic plasma for 48 hours, which could lead to the speculation that efflux to apoA-I should be decreased in THP-1 macrophages incubated with uremic serum. This could not be observed in our study (see fig. 3.3). Still, efflux to HDL was diminished significantly in THP-1 macrophages pretreated with uremic serum ($p<0.05$; fig.3.3).

According to Maggi et al. (1994), who reported an increased level of oxLDL present in CKD and Cardinal et al. (2007) who, as mentioned above, reported decreased levels of ABCA1 and ABCG1 in HCAECs, the diminished cholesterol efflux capacity to HDL of THP-1 macrophages treated with uremic serum, could be explained. This is in full contrast to the results of Hirano et al. (1999), according to which cholesterol efflux would be upregulated under the influence of oxidativly modified LDL and thus during CKD where higher amounts of modified LDL are present.

Further research is needed to elucidate the underlying pathophysiological mechanisms.

Finally we studied the effect of CKD on the cholesterol efflux capacity of HMDM isolated from adult CKD patients, dialysis patients and healthy, matched controls to control serum and HDL.

In adult patients with CKD stage 3-5 as compared to controls no difference in cellular cholesterol efflux to serum or HDL could be observed. In dialysis patients, however, a significant increase in efflux to serum and HDL respectively could be found (serum $p<0.001$; HDL $p<0.01$; fig. 3.9, B, D). In addition, efflux to serum as well as to HDL was significantly augmented in dialysis patients compared to adult CKD patients without dialysis (both $p<0.05$; fig. 3.10). Notably, a negative correlation between the cholesterol acceptor capacity of serum and HDL, and the cholesterol efflux capacity of HMDM could be found (fig. 3.11, B, C). It is tempting to speculate that, when the cholesterol acceptor capacities of serum and HDL decrease, the cells try to compensate by enhancing the rate of cholesterol efflux.

In conclusion the data of this study suggests that the diminished capacity of serum to act as an acceptor for cellular cholesterol in HD patients, may be due to the lower quantity of HDL and apoA-I. Together with alterations in HDL composition reported to occur in CKD this may enhance the progression of atherosclerosis. Our results also point to the possibility that human monocyte-derived macrophages may adapt to the diminished cholesterol acceptor capacity of uremic serum by enhancing the rate of cholesterol efflux. Finally, our findings suggest that treatment modalities, which aim to raise the reduced cholesterol acceptor capacity of serum in hemodialysis patients by increasing plasma apoA-I levels or by applying apoA-I mimetic peptides may be promising approaches to slow down the rapid progression of atherosclerosis in this group of patients.

5 Abstract

Atherosclerosis, a chronic, progressive disease is highly prevalent in CKD. One of the primary events of atherosclerosis is the accumulation of modified LDL in the macrophages of the subendothelial matrix which leads to foam cell formation, subsequent formation of fatty streaks and eventually enhanced CV morbidity and mortality. The converse event, cellular cholesterol efflux, is part of a process referred to as reverse cholesterol transport, in which excess cholesterol is removed from peripheral cells and transported back to the liver for excretion. To prevent foam cell formation and atherosclerosis development efficient cholesterol efflux is crucial, but this process may be impaired in CKD due to alterations in HDL composition and function.

This study aimed to investigate the cholesterol acceptor capacity of serum and HDL obtained from adult and pediatric CKD patients, dialysis patients and healthy, matched controls, as well as the cholesterol efflux capacity of HMDM isolated from the study participants.

It could be shown that, while in adult CKD patients stage 3-5 the cholesterol acceptor capacity of serum was increased significantly, in dialysis patients the opposite, a significant decrease occured. In HMDM obtained from adult patients with CKD stage 3-5 cholesterol efflux capacity to serum and HDL was unaltered, while in dialysis patients both was increased significantly compared to controls.

The cholesterol acceptor capacity of serum from adult and dialysis patients correlated positively with serum apoA-I levels, which was even more pronounced in the group of dialysis patients. Moreover, the cholesterol accceptor capacity of serum measured in pediatric CKD patients was, despite low HDL levels, not diminished compared to controls, which might be due to their unaltered serum apoA-I concentration. These data indicate that the serum apoA-I concentration is an important predictor of serum cholesterol efflux capacity.

The cholesterol acceptor capacity of HDL isolated from all adult subjects was shown to significantly decrease with age. Interestingly, the cholesterol acceptor capacity of serum as well as of HDL both correlate negatively with the cholesterol efflux capacity of HMDM.

In conclusion, our data suggest that the diminished cholesterol acceptor capacity of serum on HD patients may be due to reduced HDL and apoA-I conentrations. Together with the alterations in HDL composition in CKD this may enhance the progression of atherosclerosis. Furthermore, human monocyte-derived macrophages may adapt to the diminished cholesterol acceptor capacity of uremic serum by enhancing the rate of cholesterol efflux.

6 Zusammenfassung

Atherosklerose ist eine chronische, progressive Krankheit, die insbesondere bei Patienten mit chronischem Nierenversagen häufig auftritt. Am Beginn dieser Erkrankung steht die Anreicherung von modifizertem LDL in Makrophagen der subendothelialen Matrix was in weiterer Folge zur Bildung von Schaumzellen, 'fatty streaks' und dann fortgeschrittenen atherosklerotischen Läsionen und schließlich zu erhöhter kardiovaskulärer Morbidität und Mortalität führt. Der entgegengesetzte Prozess, der zelluläre Cholesterolefflux, ist Teil eines als Reverser Cholesteroltransport bezeichneten Stoffwechselweges. Dabei wird überschüssiges Cholesterol von Zellen der Peripherie zur Leber transportiert und über diese ausgeschieden. Um die Bildung von Schaumzellen zu verhindern ist ein effizienter Cholesterolefflux essentiell. Dieser Prozess könnte jedoch wegen struktureller und funktioneller Veränderungen des HDL bei chronischer Nierenkrankheit beeinträchtigt sein.

Ziel dieser Studie war es, die Kapazität von urämischem Serum und HDL zur Aufnahme von zellulärem Cholesterol bei pediatrischen und erwachsenen Patienten mit chronischem Nierenversagen und bei chronischen Hämodialyse-Patienten zu untersuchen. Darüber hinaus wurde auch die Cholesteroleffluxkapazität von aus dem Blut der Studienteilnehmer isolierten HMDM studiert und mit gematchten Kontrollen verglichen.

Es konnte gezeigt werden, dass bei erwachsenen Patienten mit Nierenversagen Stadium 3-5 die Cholesterol-Akzeptor-Kapazität von Serum signifikant erhöht war. Bei Dialysepatienten zeigte sich hingegen eine signifikante Erniedrigung. Die Cholesteroleffluxkapazität von HMDM zu Serum und HDL war bei erwachsenen Patienten mit Nierenversagen Stadium 3-5 unverändert, während bei Dialysepatienten beide im Vergleich zu Kontrollen signifikant erhöht waren.

Darüber hinaus zeigte sich eine positive Korrelation zwischen der Cholesterolakzeptorkapazität von Serum und der Serum ApoA-I Konzentration sowohl bei erwachsenen Patienten mit Nierenversagen Stadium 3-5, als auch bei Dialysepatienten, wobei diese Korrelation bei Dialysepatienten stärker ausgeprägt war. Bei pädiatrischen Patienten mit CKD war die Cholesterolakzeptorkapazität von Serum trotz niedrigem HDL nicht vermindert, was möglicherweise durch eine unveränderte Konzentration von ApoA-I im Serum zu erklären ist. Diese Daten zeigen, dass die Konzentration von ApoA-I einen wichtigen Prädiktor der Cholesterolakzeptorkapazität des Serums darstellt.

Bei den erwachsenen Studienteilnehmern nahm die Cholesterolakzeptorkapazität von HDL mit zunehmendem Alter signifikant ab. Interessanterweise korrelierten sowohl die Cholesterolakzeptorkapazität von Serum als auch von HDL negativ mit der Cholesteroleffluxkapazität von HMDM.

Zusammenfassend zeigen unsere Ergebnisse eine verminderte Cholesterolakzeptorkapaz-

ität von Serum bei Hämodialysepatienten, welche auf eine erniedrigte Konzentration von ApoA-I und HDL zurückzuführen ist. Zusammen mit Veränderungen in der Struktur von HDL könnte dies das Fortschreiten der Atherosklerose bei Dialysepatienten verstärken. Darüber hinaus weisen unsere Daten darauf hin, dass HMDM sich an die verminderte Cholesterolakzeptorkapazität von urämischem Serum anpassen, indem sie die zelluläre Cholesteroleffluxrate steigern.

Addendum

	pediatric		adult		dialysis	
	controls	patients	controls	patients	controls	patients
N	12	12	17	17	14	14
Serum lipids						
Cholesterol (mg/dl)	157.0±24.0	174.0±45.6	171.8±29.3	186.7±45.7	188.0±38.0	169.7±51.7
Triglycerides (mg/dl)	64.2±26.9	186.2±100.8***	83.3±49.4	112.4±46.7	92.4±55.5	126.2±69.1
HDL (mg/dl)	48.5±9.9	32.2±13.2**	49.5±15.9	42.0±12.9	53.2±14.1	36.8±9.3**
LDL (mg/dl)	95.7±21.9	104.6±43.4	105.6±31.9	122.3±41.2	116.3±32.8	107.7±47.6
Free Cholesterol (mg/dl)	43.8±7.8	57.8±14.2**	50.2±8.2	55.7±13.3	54.4±11.2	53.4±18.1
Phospholipids (mg/dl)	179.7±21.2	207.1±44.3	216.1±26.9	210.9±40.1	299.6±35.9	213.1±65.2
Free fatty acids (mmol/l)	0.09±0.4	0.8±0.4	0.5±0.2	0.5±0.3	0.4±0.1	1.1±0.8**
Serum apoproteins						
Apo-A1 (mg/dl)	126.6±13.5	121.9±32.6	155.4±27.9	145.1±32.0	166.6±29.8	132.4±27.0**
Apo-A2 (mg/dl)	29.5±2.6	30.8±13.2	35.5±4.9	34.2±7.8	41.4±7.3	31.0±5.8***
Apo-B (mg/dl)	70.3±12.0	95.8±30.5*	71.3±22.5	88.0±26.4	76.6±27.1	68.1±18.4
Apo-C2 (mg/dl)	3.1±1.5	5.8±3.1*	2.9±1.5	3.8±1.6	3.2±1.5	4.5±2.9
Apo-C3 (mg/dl)	5.7±1.6	8.2±3.6*	7.6±2.5	11.2±7.1	11.1±4.4	17.7±11.0
Apo-C2/Apo-C3	0.5±0.1	0.9±0.8	0.4±0.2	0.4±0.2	0.3±0.1	0.3±0.1
Apo-E (mg/dl)	8.3±1.9	11.1±3.5*	7.9±1.6	8.9±1.9	8.9±2.8	14.1±14.1
Lp(a) (mg/dl)	17.7±18.4	13.8±16.8	10.2±9.5	29.4±31.3*	13.8±12.5	19.5±22.3
HDL composition (%)						
Cholesterol ester	14.99±5.14	11.77±3.85	14.31±3.61	13.42±3.62	15.70±4.20	13.51±2.78
Triglycerides	2.83±1.31	4.73±2.00*	4.01±1.48	4.34±1.61	3.58±1.44	3.52±1.74
Free Cholesterol	4.77±2.15	3.74±2.00	5.00±1.47	3.99±2.03	4.45±0.81	4.27±1.19
Phospholipids	28.06±5.15	26.55±5.18	34.19±8.28	30.70±5.57	31.80±3.58	31.69±4.79
Protein	49.34±9.23	53.22±7.85	42.48±12.88	47.56±11.02	44.45±5.57	47.01±8.43

Tab. 7: Clinical characteristics

Information für Kontrollpersonen und Einwilligungserklärung zur Teilnahme an der klinischen Studie

Auswirkung der Urämie auf den zellulären Cholesterol-Efflux und das Plasmalipidprofil bei chronischem Nierenversagen

Sehr geehrter Herr……..! Sehr geehrte Frau …….!

Wir laden Sie ein an der oben genannten klinischen Studie teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen ärztlichen Gespräch.

Die Teilnahme an einer klinischen Studie ist freiwillig und kann jederzeit ohne Angabe von Gründen durch Sie beendet werden.

Klinische Studien sind notwendig, um verlässliche neue medizinische Forschungsergebnisse zu gewinnen. Unverzichtbare Voraussetzung für die Durchführung einer klinischen Studie ist jedoch, dass Sie Ihr Einverständnis zur Teilnahme an dieser klinischen Studie schriftlich erklären. Bitte lesen Sie den folgenden Text als Ergänzung zum Informationsgespräch mit Ihrem Arzt sorgfältig durch und zögern Sie nicht Fragen zu stellen.

Bitte unterschreiben Sie die Einwilligungserklärung nur

- wenn Sie Art und Ablauf der klinischen Studie vollständig verstanden haben,
- wenn Sie bereit sind, der Teilnahme zuzustimmen und
- wenn Sie sich über Ihre Rechte als Teilnehmer an dieser klinischen Studie im Klaren sind.

Zu dieser klinischen Studie, sowie zum diesem Informationsblatt mit Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

1. Was ist der Zweck der klinischen Studie?

Es gibt Hinweise darauf, dass bei Patienten mit chronischen Nierenerkrankungen häufig die Blutfette erhöht sind. Das kann dazu führen, dass die Blutgefäße geschädigt werden.

Der Zweck dieser klinischen Studie ist es, Veränderungen im Fettstoffwechsel, die bei chronischem Nierenversagen zu einer frühzeitigen Veränderung der Gefäße (Atherosklerose) führen können zu studieren und mit jenen von gesunden Kontrollpersonen zu vergleichen. Dadurch sollen die Fettstoffwechselstörungen bei chronischen Nierenerkrankungenn Zukunft besser und gezielter behandelt werden können.

2. Wie läuft die klinische Studie ab?

Diese klinische Studie wird an der Universitätsklinik für Kinder- und Jugendheilkunde mit Unterstützung durch die III. Medizinische Klinik der Medizinischen Universität Wien durchgeführt, und es werden insgesamt ungefähr 105 Personen daran teilnehmen.

Die Teilnahme an dieser klinischen Studie wird voraussichtlich ein halbe Stunde dauern.

Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt:

Während dieser klinischen Studie werden einmal ca. 30ml Blut (3 Röhrchen) abgenommen.

3. Worin liegt der Nutzen einer Teilnahme an der Klinischen Studie?

Durch Ihre Teilnahme an dieser klinischen Studie können auch bei Ihnen Risikofaktoren für Veränderungen der Gefäßwände, insbesondere erhöhte Blutfette, frühzeitig erkannt werden. Dadurch wird es möglich diese Risikofaktoren zu behandeln und eventuell die Veränderungen der Gefäßwände günstig zu beeinflussen.

4. Gibt es Risiken, Beschwerden und Begleiterscheinungen?

Im Rahmen dieser klinischen Studie durchgeführten Maßnahmen kann es im Rahmen der Blutabnahme zu Schmerzen kommen.

5. Hat die Teilnahme an der klinischen Studie sonstige Auswirkungen auf die Lebensführung und welche Verpflichtungen ergeben sich daraus?

Die einzige die Lebensführung beeinträchtigenden Verpflichtung besteht darin, dass die Teilnehmer vor der Blutabnahme 8 Stunden nüchtern (keine Nahrungsaufnahme) sein müssen.

6. Wann wird die klinische Studie vorzeitig beendet?

Sie können jederzeit auch ohne Angabe von Gründen, Ihre Teilnahmebereitschaft widerrufen und aus der klinischen Studie ausscheiden ohne dass Ihnen dadurch irgendwelche Nachteile für Ihre weitere medizinische Betreuung entstehen.

Ihr Arzt/ Ihre Ärztin wird Sie über alle neuen Erkenntnisse, die in Bezug auf diese klinische Studie bekannt werden, und für Sie wesentlich werden könnten, umgehend informieren. Auf dieser Basis können Sie dann Ihre Entscheidung zur weiteren Teilnahme an dieser klinischen Studie neu überdenken.

Es ist aber auch möglich, dass Ihr Arzt/ Ihre Ärztin entscheidet, Ihre Teilnahme an der klinischen Studie vorzeitig zu beenden, ohne vorher Ihr Einverständnis einzuholen. Die Gründe hierfür können sein:

Fig. 6.1: Information for control subjects

a) Sie können den Erfordernissen der klinischen Studie nicht entsprechen;

b) Ihr behandelnder Arzt/ Ihre behandelnde Ärztin hat den Eindruck, dass eine weitere Teilnahme an der klinischen Studie nicht in Ihrem Interesse Ihres ist.

7. In welcher Weise werden die im Rahmen dieser klinischen Studie gesammelten Daten verwendet?

Sofern gesetzlich nicht etwas anderes vorgesehen ist, haben nur die Prüfer und deren Mitarbeiter Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt werden. Diese Personen unterliegen der Schweigepflicht.

Die Weitergabe der Daten erfolgt ausschließlich zu statistischen Zwecken und Sie werden ausnahmslos darin nicht namentlich genannt. Auch in etwaigen Veröffentlichungen der Daten dieser klinischen Studie werden Sie nicht namentlich genannt.

8. Entstehen für die Teilnehmer Kosten? Gibt es einen Kostenersatz oder eine Vergütung?

Durch Ihre Teilnahme an dieser klinischen Studie entstehen für Sie keine zusätzlichen Kosten.

9. Möglichkeit zur Diskussion weiterer Fragen

Für weitere Fragen im Zusammenhang mit dieser klinischen Studie stehen Ihnen Ihr Prüfarzt und seine Mitarbeiter gern zur Verfügung. Auch Fragen, die die Rechte Ihres Kindes als Patient und Teilnehmer an dieser klinischen Studie betreffen, werden Ihnen gerne beantwortet.

Name der Kontaktperson: **ao. Univ. Prof. Dr. Martin Haas**

Ständig erreichbar unter: Tel: **01 40400 4217**

E-Mail: **martin.haas@meduniwien.ac.at**

10. Einwilligungserklärung

Name in Druckbuchstaben: .....

Geb. Datum: ..... Code: .....

Ich erkläre mich bereit, an der klinischen Studie „Auswirkungen der Urämie auf den zellulären Cholesterol-Efflux und das Plasmalipidprofil bei Kindern und jungen Erwachsenen mit chronischem Nierenversagen“ teilzunehmen.

Ich bin von Herrn/Frau Dr.med. ..... ausführlich und verständlich über mögliche Belastungen und Risiken, sowie über Wesen, Bedeutung und Tragweite der klinischen Studie und die sich für mich daraus ergebenden Anforderungen aufgeklärt worden. Ich habe darüber hinaus den Text dieser Patientenaufklärung und Einwilligungserklärung, die insgesamt 4 Seiten umfasst gelesen. Aufgetretene Fragen wurden mir vom Prüfarzt verständlich und genügend beantwortet. Ich hatte ausreichend Zeit, mich zu entscheiden. Ich habe zurzeit keine weiteren Fragen mehr.
Ich werde den ärztlichen Anordnungen, die für die Durchführung der klinischen Studie erforderlich sind, Folge leisten, ich behalte mir jedoch das Recht vor, meine freiwillige Mitwirkung zu beenden.

Ich bin zugleich damit einverstanden, dass die im Rahmen dieser klinischen Studie von mir ermittelten Daten aufgezeichnet werden. Um die Richtigkeit der Datenaufzeichnung zu überprüfen, dürfen Beauftragte des Auftraggebers und der zuständigen Behörden beim Prüfarzt Einblick in meine personenbezogenen Krankheitsdaten nehmen.

Beim Umgang mit den Daten werden die Bestimmungen des Datenschutzgesetzes beachtet.

Eine Kopie dieses Informationsblatts und der Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Prüfarzt.

.....
(Datum und Unterschrift des Patienten)

.....
(Datum, Name und Unterschrift des verantwortlichen Arztes)

(Die Kontrollperson erhält eine unterschriebene Kopie Iinformationsblattes und Einwilligungserklärung, das Original verbleibt im Studienordner des Prüfarztes.)

Fig. 6.2: Information and informed consent form for control subjects

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