Innovative Lipid Biomarkers and Long-Term Outcomes

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DOI:
10.33612/diss.192996734

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Studies in patients treated with renal replacement therapy and in the general population

Josephine L.C. Anderson

Financial support for the printing of this thesis from the University of Groningen, the Dutch Heart Foundation, the Graduate School of Medical Sciences of the University Medical Center Groningen and ChipSoft is gratefully acknowledged. Additional financial support was kindly provided by the Dutch Heart Foundation (hartstichting).

Cover	Ilse Modder | www.ilsemodder.nl
Lay-Out	Ilse Modder | www.ilsemodder.nl
Printed by	Gildeprint, Enschede | www.gildeprint.nl

university of groningen

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Studies in patients treated with renal replacement therapy and in the general population

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Wednesday 8 December 2021 at 12.45 hours

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INTRODUCTION

1

INTRODUCTION

Kidney transplantation is the gold standard treatment for end-stage renal disease (ESRD) and kidney failure. Despite offering numerous advantages above hemodialysis therapy, this solution is far from perfect, as the transplantation procedure introduces a new array of problems into the patient's life.

Renal transplant recipients (RTR) face a vastly increased risk of cardiovascular disease (CVD), with an incidence that is 4-6 times higher compared to the general population. This increased risk of CVD is however insufficiently explained by traditional risk factors, which results in a lack of adequate risk prediction and suitable treatment options. Instead, factors that are specific to this patient population seem to be of greater importance.[1,2] Specifically, the use of immunosuppressive medication, kidney function and a history of hemodialysis are important contributing factors.[1,2] Immunosuppressive regimes have a large impact on RTRs health. Although vital for the survival of the donor graft they have an array of detrimental side effects. Standard immunosuppressive regimes consist of a calcineurin inhibitor, anti-proliferation agent and prednisolone.[3]

In addition to the threat of CVD, RTR constantly face the risk of graft loss, resulting in re-transplantation or return to hemodialysis. In recent decades tremendous progress has been made with immunosuppressive medication, resulting in a reduced incidence of acute rejection. Long term graft survival, however, remains disappointing, with little improvement in the last decades. The average survival of a kidney graft has stagnated at 10.7 years.[4] In fact, graft failure is such a dominant problem that, in patients younger than 50 years of age, it is now the most important reason for placing patients on the wait list for transplantation, surpassing all other forms of ESRD.[5]

However, of potentially even more concern is the exceptional emotional burden that RTR face. After spending months to years on transplantation waiting lists prior to transplantation, the potential of graft failure and subsequent re-transplantation or return to dialysis is deeply concerning to patients. Multiple studies have shown that patients would in fact prefer to die, rather than face graft failure.[6,7] Moreover, renal transplantation often depends on a sacrifice by a close friend or family member, in the form of a living donation. Obviously, optimal protection of donated kidneys from development of graft failure in the recipients is a major clinical, as well as ethical, necessity.

It is therefore imperative that long term outcomes are improved for kidney transplant recipients. Not only will this directly improve the quality of life of RTR, but an improved graft survival will also result in the need for fewer re-transplantations, which will take away pressure from waiting lists. A higher availability of donor organs means that patients will spend less

time on hemodialysis as well as that optimal organs can be chosen for transplantation, both of which will again positively influence patient survival and graft survival. Lipid parameters are thus far an under-utilized modifiable modality when attempting to improve the morbidity and mortality of RTR.

The main hypothesis of this thesis is that classical lipid biomarkers as well as dynamic novel measures of functionality of lipoproteins play an important role in determining both development of CVD and development of graft failure in RTR. The relation between levels of lipoproteins and development of CVD is a well-known phenomenon in the general populations (REFS), but less obviously established in the population of RTR (REFS). Interestingly, it has also been suggested that in RTR, the arterial vasculature in the transplanted kidney, in contrast to the arterial vasculature in native kidneys of individuals in the general population, is susceptible to the process of atherosclerosis [8] , which also underlies development of certain forms of cardiovascular disease.

LIPOPROTEINS: DEFINITION AND PHYSIOLOGY

Lipoproteins are complex particles that carry a vast variety of protein and lipid cargo. They consist of a hydrophilic phospholipid and free cholesterol outer shell and carry hydrophobic cholesterol esters and triglycerides in their core to facilitate movement in the aqueous blood (figure 1). Lipoproteins can therefore be considered cholesterol transporters; however, their many associated proteins have a variety of other functions with regards to inflammation and oxidation.[9,10] The proteins that are embedded in the lipoprotein shell are termed apolipoproteins. A vast number of different apolipoproteins subclasses have been identified, with a wide range of different physiological activity.[11] Lipoproteins are classified based on the respective density of these particles, ranging from high density lipoproteins (HDL), lipoproteins with a high protein content, to very low-density lipoproteins (VLDL) with a low protein content and a higher respective content of triglycerides and cholesterol esters (figure 1). Measurement of the amount of cholesterol that is associated with the different types of lipoproteins is a frequently carried out laboratory test in the general practitioner's practice, as well as in secondary and tertiary care. Of note, these standard laboratory techniques show the amount of cholesterol carried by the respective lipoproteins, termed for example LDL cholesterol (LDL-C), and not the number or concentration of lipoprotein particles.[12] It is therefore also a misconception that LDL is 'bad cholesterol', it is the cholesterol that is associated with the LDL particle that is problematic.

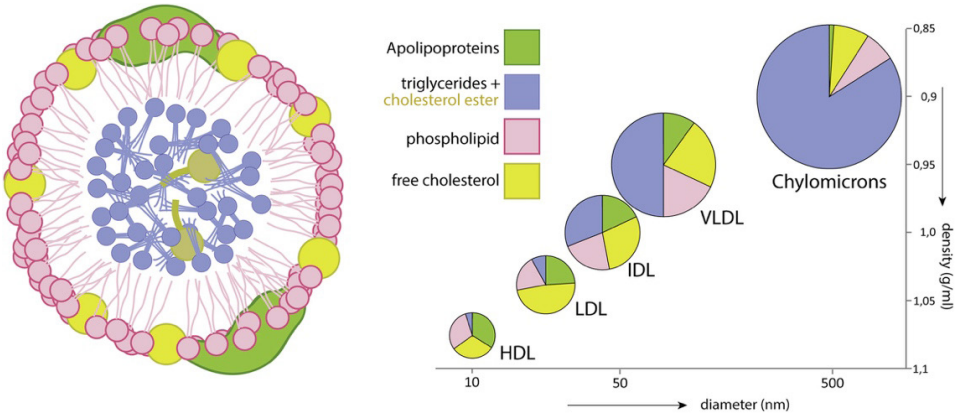

Figure 1. Lipoprotein composition[13]

LIPOPROTEIN FUNCTIONALITY IN THE GENERAL POPULATION

Cardiovascular disease

Cardiovascular disease (CVD), a grouping of diseases involving the heart and vessels, is the leading cause of death in the western world. CVD includes four major areas: i. coronary heart disease (CHD), manifest by myocardial infarction (MI), angina pectoris or subsequent heart failure, ii. cerebrovascular disease, manifested by stroke and transient ischemic attacks, iii. Peripheral artery disease, manifested by intermitted claudication and critical limb ischemia and iv. arrhythmias. Atherosclerosis is central to the pathophysiology of CHD, cerebrovascular disease and peripheral artery disease, and these diseases are therefore termed atherosclerotic CVD.

Atherosclerosis is a complex inflammatory process. As Steinberg put it, hypercholesterolemia and inflammation are partners in crime,[14] as chronic inflammation of artery walls with resulting endothelial dysfunction precedes atherosclerotic plaque formation. LDL particles that enter the intima of the vessel wall are retained and oxidized, followed by phagocytosis of macrophages. The accumulation of these lipid laden macrophages, termed foam cells, leads to the emergence of a fatty streak.[15] Different type of immune cells, including T-cells and monocytes, are then recruiting which results in a chronic inflammatory state.[16][16] Deposition of extracellular matrix components in turn increase the retention of atherogenic lipoproteins. As the plaque grows remodeling takes places with subsequent necrosis in its core, which promotes further recruitment of inflammatory cells.[17] Buildup of plaques eventually leads, rupture and consequent occlusion of a vessel, which can manifest itself as e.g. a myocardial infarction or ischaemic stroke, depending on the vessel in which the occlusion occurs. It can also lead to rupture of a vessel, e.g. a ruptured acute aortic abdominal aneurysma or a hemorrhagic stroke.

In addition to a pivotal role of inflammation, auto-immune components of atherosclerosis are increasingly being described. Modification of the HDL particle can lead to increase atherogenic potential[18] and auto-antibodies can directly effect atherosclerotic plaque vulnerability.[19]

The role of lipoproteins in atherosclerotic CVD is well established. LDL-C is uniformly accepted as causative factor of atherosclerosis. Furthermore, large population based studies, such as the Framingham or PROCAM established that plasma levels of HDL-C are strongly inversely correlated with the future risk of CVD events.[20,21] However, genetic as well as pharmacological intervention studies called this concept into question. The drug niacin successfully raised HDL-C levels, however this did not translate into a decreased CVD risk.[22,23] Similarly, studies involving CETP-inhibitors raised HDL-C levels, again without a significantly decreased risk of all-cause or CVD specific mortality[24] or even an increased risk of mortality.[25] Furthermore, Mendelian disorders of HDL metabolism that lead to lifelong high levels of HDL-C did not uniformly contribute to a lower incident of CVD.[26] In addition, Mendelian randomization studies showed that genetic mechanism that raise plasma HDL-C don't lower risk of MI.[27] This apparent discrepancy led to the hypothesis that it is not measures of HDL-C levels which have a protective effect for CVD, but rather functional properties of the HDL particles. This is biologically plausible, since HDL are highly complex lipoproteins; more than 100 proteins have been identified on HDL, of which the majority have anti-inflammatory and proteolytic functions, strengthening the hypothesis that HDL functionality rather than levels are important for lowering CVD risk.[9]

Key anti-atherosclerotic functional properties of HDL are: (i) increasing cholesterol efflux, as first step of reverse cholesterol transport.[28] Cholesterol efflux describes the movement of cholesterol from atherosclerotic plaques, for either uptake into hepatocytes and secretion into bile with subsequent elimination in feces or to a lesser extent through transintestinal cholesterol excretion pathway[28,29] (ii) Decreasing the inflammatory activation of endothelial cells; thereby decreasing the recruitment of inflammatory cells into the vascular wall (iii) protecting native low density lipoprotein (LDL) against oxidative modification, and (iv) promoting vascular nitric oxide production.[28]

Chronic kidney disease

Chronic kidney disease (CKD) describes a heterogenous group of disorders, defined by the presence of kidney damage or decreased kidney function for three or more months, irrespective of the cause.[30] CKD is very prevalent, with 10% of the adult population in developed countries being affected.[31] Severity of CKD varies enormously and is therefore subdivided into a number of categories, ranging from asymptomatic proteinuria and eGFR decline to end-stage renal disease and the need for renal replacement therapy. Although the exact pathophysiological mechanism behind CKD is complex, multifactorial and

incompletely understood, there is growing evidence for a pivotal role of dyslipidemia. An association between cholesterol level and progression of CKD has been shown,[32] with lipid deposits being found in biopsies in glomerulosclerosis, nephrotic syndrome, diabetic nephropathy, chronic glomerulonephritis and heroin nephropathy.[33]

LIPOPROTEIN FUNCTIONALITY IN HIGH RISK POPULATIONS

Cardiovascular disease in renal transplant recipients

The vastly increased risk of CVD in RTR is not sufficiently explained by traditional risk factors, such as levels of LDL-C or HDL-C.[8,34,35] Thus, the identification of predictive biomarkers represents an unmet clinical need in this patient population. Recent studies indicated that assays capturing the functional properties of HDL lipoproteins might provide clinical information beyond cholesterol levels within these, exemplified by HDL cholesterol efflux capacity being able to predict CVD in the general population [36–38] , independent of HDL-C levels. However, throughout the atherogenic process especially LDL particles play a central and pivotal role[39]. Specifically, binding of LDL particles to proteoglycans in the vessel wall is an early key event in the initiation of atherosclerotic lesions, as summarized in the now widely accepted response-to-retention hypothesis of atherogenesis [40]. However, thus far the concept that measures of LDL functionality can be used as predictive biomarkers has not been widely explored.

Graft failure in renal transplant recipients

In addition to an increased cardiovascular risk, largely driven by atherosclerosis, RTR also face a danger of graft failure. Late graft failure and atherosclerotic CVD are potentially different expressions of the same pathological basis. Transplant vasculopathy (TV) is acknowledged as a major contributing factor to late graft failure.[41] Interestingly, TV lesions closely resemble classic atherosclerosis, and factors influencing the development of atherosclerosis also have been implicated in TV.[8] It is therefore plausible to believe that the same risk factors that lead to atherosclerosis, also play a role in transplant vasculopathy and therefore late graft loss.

Hemodialysis patients

ESRD describes those CKD patients with such a limited renal function that renal replacement therapy is indicated. CVD represents the single largest cause of morbidity and mortality in patients with reduced kidney function or uremia, reflected by a 30-fold increase in age-adjusted CVD mortality in end-stage renal disease (ESRD) patients.[42,43] Although a number of classical as well as non-classical risk factors have been reported to contribute to this excessive increase in CVD mortality, the underlying pathophysiological basis for these findings is still insufficiently understood.[44] ESRD is associated with a chronic pro-inflammatory state with a vast amount of oxidative stress, defined as a disbalance between oxidative products, such as reactive oxygen species and nitrogen species, and antioxidants.

Oxidative stress triggers the oxidation and damage of molecules such as proteins lipids, nucleic acids and carbohydrates.[45]

It is plausible that extensive oxidation damages the HDL particle and therefore decreases its functionality and atheroprotective effects in ESRD. This could conceivably contribute to the vastly increased CVD risk in this patient group.

AIMS OF THE THESIS

PART 1: THE ROLE OF LIPOPROTEIN FUNCTIONALITY IN PATIENTS TREATED WITH RENAL REPLACEMENT THERAPY

In part 1 of this thesis the role of different lipid parameters in patients with ESRD and in kidney transplant recipients was examined.

In **chapter 2** the ability of HDL from ESRD patients to perform two major steps in reverse cholesterol transport was assessed. Furthermore, it investigated whether this lack of function can be ascribed to oxidative modifications caused by hemodialysis therapy.

Apolipoprotein A-1 (apoA-1) is the major protein component of the HDL particle. Recently the role of autoantibodies against apoA-1 (anti-apoA-1 IgG) for prediction of CVD risk is emerging in the general population, as well as in some high-risk patients. In **chapter 3** the association with anti-apoA-1 IgG with CVD specific mortality, overall mortality and late graft failure was assessed. Furthermore, an attempt was made to delineate the relationship of anti-apoA-1 IgG levels and HDL functionality.

Raised TG levels are known to contribute to atherosclerosis, and therefore form a major risk factor for CVD. RTR have marked dyslipidemia, including raised TG levels and decreased HDL, and it is conceivable that these abnormalities contribute to the formation of TV lesions and subsequent late graft failure. However, in contrast to HDL-C TG levels vary considerably based on feeding status and is therefore a poor biomarker despite large clinical implications. In **chapter 4a** it was examine whether the combination of TG and HDL-C in form of a ratio, which represents a considerably more stable measure of dyslipidemia, is associated with late graft failure in RTR. In **chapter 4b** the potential impact of the TG/HDL-C ratio on premature mortality was assessed.

As late rejection is commonly accompanied by de novo atherogenesis, it was hypothesised that the same risk factors apply to CVD as well as to late graft failure. **Chapter 5** therefore assessed whether the FRS, a commonly applied cardiovascular risk score, could also serve to stratify RTR at risk of developing chronic graft failure.

Statins achieve potent LDL lowering in the general population, with translates into a decreased incidence of CVD events and mortality. Treatment guidelines for RTR include statins, however conclusive evidence of improved CVD outcomes has not been uniformly provided. Furthermore, concerns have been raised about the simultaneous administration of the immunosuppressive drug cyclosporine and statin, as they share a similar metabolic pathway. **Chapter 6** therefore examined whether the use of statins has an effect on CVD events and death, with subgroup analysis of cyclosporine-using RTR.

The relationship of lipid levels versus lipoprotein function in relation to outcomes in RTR is further examined in **chapter 7**. Binding of LDL to proteoglycans in the vessel wall is an early key event in atherosclerotic plaque formation. The susceptibility with which LDL particles bind to proteoglycans is highly variable, but potentially influenced by a variety of chemical modification of the LDL particle. It is plausible that such a process of oxidative modification has occurred in RTR, who usually have a period of hemodialysis prior to transplantation, in which they are in a uremic state with increased oxidative stress. Therefore, it was assessed whether lipoprotein-proteoglycan binding susceptibility of LDL is prospectively associated with two atherosclerotic driven end-points in RTR, namely CVD and late graft failure.

PART 2: THE ROLE OF LIPOPROTEIN FUNCTIONALITY IN THE GENERAL POPULATION

In part two of this thesis the role of lipids in the general population was investigated. **Chapter 8** aimed to determine whether the anti-inflammatory capacity of HDL is prospectively associated with incident CVD events in a general population cohort. In order to ascertain the relevance of this metric of HDL function irrespective of HDL cholesterol a case-control design was chosen.

Atherosclerotic disease can manifest in a multitude of ways. It is reasonable to believe that CVD and CKD are expressions of the same disease, through plaque buildup in vessels, leading to micro-infarctions in the kidney, ischemia and loss of function. The close correlation between CVD and CKD suggests that similar risk factors are of relevance. **Chapter 9** assessed whether the triglyceride/HDL-C ratio, an effective and stable measure of dyslipidemia, is associated with CKD in the general population.

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PART I

STUDIES IN PATIENTS WITH RENAL REPLACEMENT THERAPY

2

HIGH DENSITY LIPOPROTEIN (HDL) PARTICLES FROM END-STAGE RENAL DISEASE PATIENTS ARE DEFECTIVE IN PROMOTING REVERSE CHOLESTEROL TRANSPORT

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Scientific Reports, 2017

ABSTRACT

Atherosclerotic cardiovascular disease (CVD) represents the largest cause of mortality in end-stage renal disease (ESRD). CVD in ESRD is not explained by classical CVD risk factors such as HDL cholesterol mass levels making functional alterations of lipoproteins conceivable. HDL functions in atheroprotection by promoting reverse cholesterol transport (RCT), comprising cholesterol efflux from macrophage foam cells, uptake into hepatocytes and final excretion into the feces. ESRD-HDL (n=15) were compared to healthy control HDL (n=15) for their capacity to promote *in vitro* (i) cholesterol efflux from THP-1 macrophage foam cells and (ii) SR-BI-mediated selective uptake into ldla[SR-BI] cells as well as (iii) *in vivo* RCT. Compared with HDL from controls, ESRD-HDL displayed a significant reduction in mediating cholesterol efflux ($p<0.001$) and SR-BI-mediated selective uptake ($p<0.05$), two key steps in RCT. Consistently, also the in vivo capacity of ESRD-HDL to promote RCT when infused into wild-type mice was significantly impaired ($p<0.01$). *In vitro* oxidation of HDL from healthy controls with hypochloric acid was able to fully mimic the impaired biological activities of ESRD-HDL. In conclusion, we demonstrate that HDL from ESRD patients is dysfunctional in key steps as well as overall RCT, likely due to oxidative modification.

INTRODUCTION

Plasma levels of high density lipoprotein (HDL) cholesterol are strongly inversely correlated with the risk of atherosclerotic cardiovascular disease (CVD) in populations with normal kidney function.[1,2]I The beneficial effects of HDL are largely ascribed to the role of HDL in reverse cholesterol transport (RCT), i.e. the transport of excess cholesterol from the periphery back to the liver for excretion into bile.[2–5] For efficient RCT two steps are of critical importance, (i) cholesterol efflux from macrophage foam cells mainly mediated by ABCA1 and ABCG1[2,5] and (ii) SR-BI-dependent cholesterol uptake into hepatocytes.[2,6]

CVD represents the single largest cause of morbidity and mortality in patients with reduced kidney function or uremia, reflected by a 30-fold increase in age-adjusted CVD mortality in end-stage renal disease (ESRD) patients.[7,8] Although a number of classical as well as non-classical risk factors have been reported to contribute to this excessive increase in CVD mortality, the underlying pathophysiological basis for these observations is still insufficiently understood.[9] Chronic kidney disease itself might not result in an substantial impairment of the cholesterol efflux function of HDL,[10] while HDL from patients on hemodialysis exhibits an apparent reduction in the capacity to accept cholesterol from macrophages.[11–14] However, the ability of ESRD-HDL to function in the whole RCT pathway has not been investigated thus far.

Therefore, the present study not only tested the functional properties of HDL from ESRD patients for the two major steps of RCT *in vitro*, namely cholesterol efflux from macrophages and SR-BI-mediated cholesterol delivery but also the ability of ESRD-HDL to promote RCT from ^{3}H-cholesterol-loaded macrophages *in vivo* in mice. Our results indicate that ESRD-HDL is less efficient than control HDL in mediating RCT, conceivably due to oxidative modifications of HDL apolipoproteins.

RESULTS

HDL FROM ESRD PATIENTS DISPLAYS DEFECTIVE CHOLESTEROL UPTAKE AS WELL AS CHOLESTEROL DELIVERY PROPERTIES *IN VITRO*

Two important functional properties enable HDL to serve as an efficient mediator of RCT, namely (i) to elicit cholesterol efflux from macrophage foam cells and (ii) to deliver cholesterol to cells via the SR-BI-mediated selective uptake pathway. To test these properties for HDL from ESRD patients we first performed cholesterol efflux experiments. Compared with HDL from control subjects, ESRD-HDL displayed a significant reduction in mediating cholesterol efflux (5.87 ± 0.26 vs. 4.05 ± 0.41 %, $p<0.001$, figure 1A). Next, we tested the capacity of ESRD-HDL to deliver cholesterol into ldla cells stably transfected with SR-BI. Also cellular cholesterol uptake from ESRD-HDL via SR-BI was significantly impaired for HDL from ESRD

patients compared with controls (20.2 ± 1.2 vs. 13.4 ± 1.6 %, p<0.05, figure 1B). These data demonstrate that ESRD-HDL is defective in both properties crucial for functional RCT.

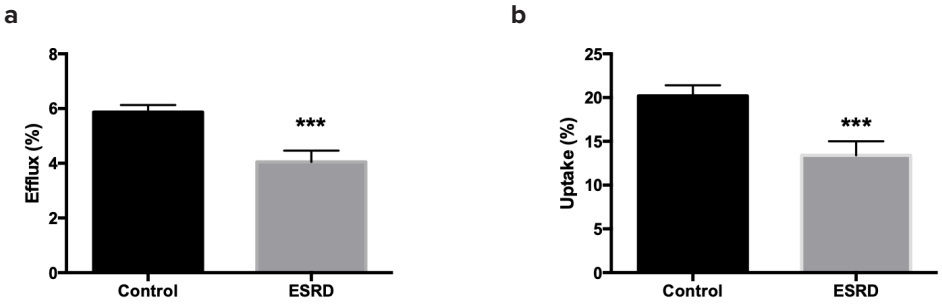

Figure 1. ESRD-HDL displays defective cholesterol uptake as well as cholesterol delivery properties. (a) Cholesterol efflux from primary mouse peritoneal macrophages towards HDL from ESRD-patients (n=15) compared with healthy control subjects (n=15). (b) Cellular SR-BI mediated selective cholesterol uptake from ESRD-HDL or control HDL (each n=15) into ldla cells stably transfected with SR-BI. Data are presented as means ± SEM. ***p<0.001

HDL FROM ESRD PATIENTS DISPLAYS AN ALTERED LIPID AND PROTEIN COMPOSITION

Compared with controls, HDL particles from ESRD patients were significantly enriched in triglycerides (Supplemental table S2, p=0.001). While the cholesteryl ester content of HDL was decreased in ESRD patients (p<0.05), free cholesterol content was increased (p<0.05). Phospholipid and protein contents did not differ between the two experimental groups. Regarding HDL proteins associated with impaired functionality of the particle we found ESRD HDL significantly enriched in both serum amyloid A (SAA, 27.4 ± 5.5 vs. 3.1 ± 0.5 µg/dl, p<0.001) and apoC-III (10.3 ± 0.7 vs. 8.1 ± 0.8 mg/dl, p<0.05).

In ESRD patients, HDL cholesterol was positively associated with both the efflux (r=0.58, p<0.05) and selective uptake function (r=0.53, p<0.05), while no correlations were detected with the HDL triglyceride content. In addition, SAA within HDL correlated inversely to efflux (r= -0.71, p<0.01) but not significantly to selective uptake (r= -0.43, p=0.11). No correlations were found with the apoC-III content of HDL and either efflux (r= -0.26, n.s.) or selective uptake (r= -0.12, n.s.).

HDL FROM ESRD PATIENTS IS DEFECTIVE IN MEDIATING RCT *IN VIVO*

Since HDL from ESRD patients showed impaired cholesterol uptake and delivery properties *in vitro*, we next tested the functional behaviour of ESRD-HDL in an integrated *in vivo* physiological setting of RCT. Mice that had received macrophage foam cells loaded with radioactively labeled cholesterol were infused with either PBS, control HDL or ESRD-HDL, and appearance of the tracer in different compartments was followed over time.

First, we assessed mass changes in cholesterol in the plasma compartment in response to the different treatments. As shown in Figure 2A, only the group receiving control HDL exhibited a increase in plasma total cholesterol at the early 4h time point, essentially due to significantly higher plasma free cholesterol levels ($p<0.01$, Figure 2B). The group receiving the ESRD-HDL was not different in these parameters from PBS controls. In agreement with the mass data also appearance of macrophage-derived ^{3}H-cholesterol in plasma was only higher at the 4h time point in the control-HDL group ($p<0.05$, Figure 2C). These data are consistent with ESRD-HDL having a reduced capacity to elicit cholesterol efflux *in vivo* analogous to our *in vitro* findings.

At the 48h time point livers from PBS injected mice contained significantly less macrophage-derived ^{3}H-cholesterol (2.97 ± 0.10 %) than livers from mice infused with either control HDL (4.10 ± 0.23 %, $p<0.05$) or ESRD-HDL (3.78 ± 0.14 %, $p<0.05$), while there was no difference between control and ESRD-HDL receiving groups.

Cholesterol can either be excreted from the body within the fecal neutral sterol fraction or after metabolic conversion to bile acids. The mass fecal excretion of neutral sterols (Figure 2D) and bile acids (Figure 2E) did not change upon the different treatments. On the other hand, control HDL resulted in a significant increase in tracer excretion within neutral sterols (Figure 2F) as well as within bile acids ($p<0.05$ for ESRD, $p<0.01$ for PBS, Figure 2G) causing an overall substantial increase in RCT. However, ESRD-HDL failed to have any significant effect on the fecal exretion of the macrophage-derived ^{3}H-cholesterol, indicating that the *in vivo* capacity to mediate effective RCT is significantly impaired in these particles in comparison to HDL from healthy controls.

OXIDATION OF CONTROL HDL IN VITRO RESULTS IN IMPAIRED CHOLESTEROL UPTAKE AS WELL AS DELIVERY PROPERTIES

Since ESRD patients show a substantial increase in inflammatory load and oxidative stress,[15–17] we speculated that a possible mechanism underlying the decreased RCT functionality of ESRD-HDL might be of oxidation of apolipoproteins contained within the HDL particle, which are of crucial importance to its function. Therefore, we next determined TBARS levels within HDL as a measure of oxidative modification. While in control HDL TBARS were detectable at a considerably low level, all ESRD-HDL tested contained substantial amounts of TBARS consistent with our hypothesis (0.7 ± 0.1 vs. 4.2 ± 0.6 nmol/mg, $p<0.001$). In addition, TBARS content of HDL correlated negatively with the two functional parameters determined in our study, namely cholesterol efflux ($r= -0.58$, $p=0.02$) and selective uptake ($r= -0.52$, $p<0.05$). Further, we aimed to test the pathophysiological consequences of HDL oxidation on the two functional properties important for RCT, cholesterol uptake and delivery. HDL oxidatively modified by incubation with HOCl displayed a significantly reduced capacity to serve as acceptors for macrophage-mediated cholesterol efflux compared with control HDL (7.02

± 0.36 vs. 4.96 ± 0.28 %, p<0.01, figure 3A). In addition, also the SR-BI-mediated uptake of cholesterol from oxidized HDL was significantly decreased (21.3 ± 0.7 vs. 15.5 ± 0.8 %, p<0.01, figure 3B). These data show that *in vitro* oxidatively modified HDL are defective in both properties, mediating cholesterol efflux and delivering cholesterol to cells via SR-BI, comparable with the functional deficits observed for ESRD-HDL.

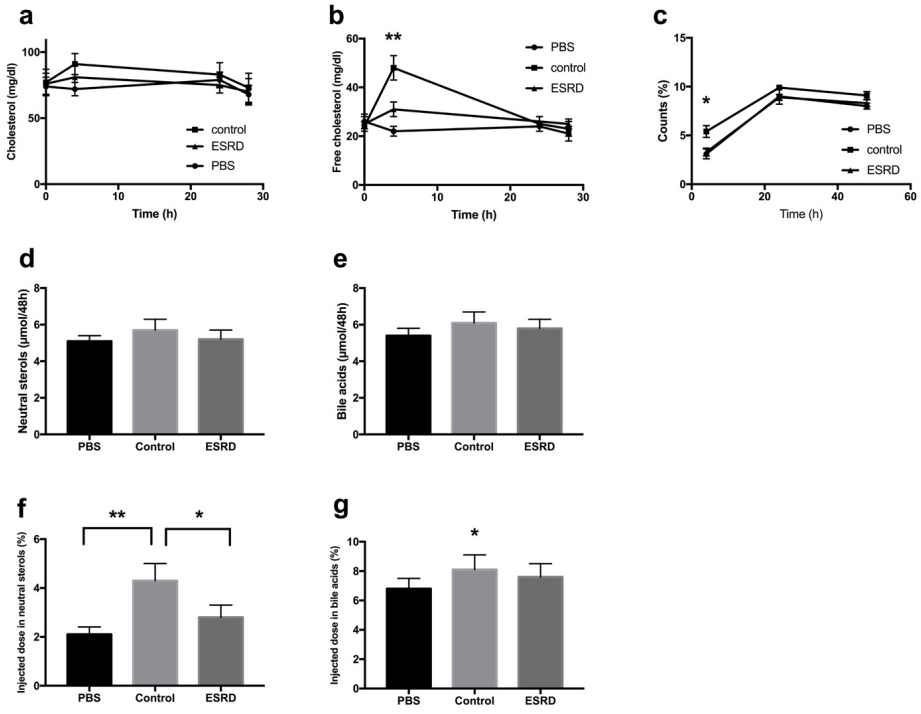

Figure 2. HDL from ESRD patients are defective in medicating RCT in vivo. Mice injected with macrophage foam cells loaded with radioactively labelled ^{3}H-cholesterol were infused with either PBS, control HDL or ESRD-HDL as detailed in methods. (a) Mass changes in plasma total cholesterol, (b) mass changes in plasma free cholesterol, (c) appearance of the ^{3}H-cholesterol tracer in plasma, (d) mass fecal neutral sterol excretion over 48h, (e) mass fecal bile acid excretion over 48h, (f) fecal ^{3}H-cholesterol tracer excretion within neutral sterols, (G) fecal ^{3}H-cholesterol tracer excretion within bile acids. Data are presented as means ± SEM, n=6-8 mice/group. * p<0.05, ** p<0.01.

HDL OXIDIZED IN VITRO IS DEFECTIVE IN MEDIATING IN VIVO RCT

Next, we tested the *in vivo* functionality of oxidized HDL in RCT. HOCl-modified HDL had a significantly decreased capacity to mobilize macrophage-derived ^{3}H-cholesterol to the plasma compartment compared with native HDL at the 48h time point (p<0.05, Figure 4A). In addition, counts recovered in the fecal neutral sterol fraction (p<0.01, Figure 4B) as well as in

the fecal bile acid fraction ($p<0.05$, Figure 4C) were significantly lower with HOCl-modified HDL, indicating that oxidative modification of healthy control HDL substantially decreases its *in vivo* capacity to function in RCT.

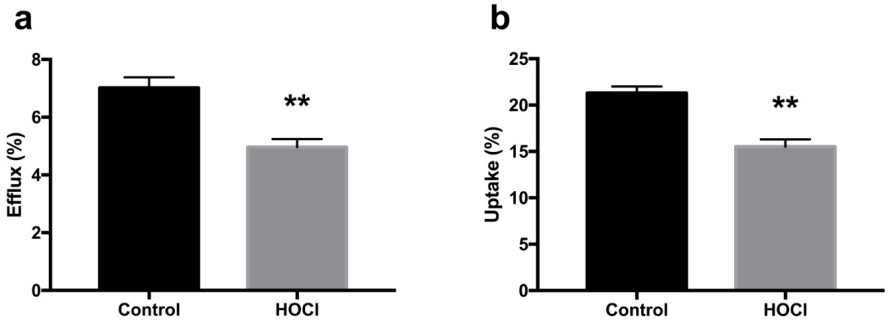

Figure 3. Oxidation of HDL *in vitro* results in impaired cholesterol uptake as well as delivery properties. (a) Cholesterol efflux from primary mouse peritoneal macrophages towards native, control HDL compared with control HDL following HOCl incubation (n=10). (b) Cellular SR-BI mediated selective cholesterol uptake form control HDL or HOCl-modified HDL (n=10) into ldla cells stably transfected with SR-BI. Data are presented as means ± SEM. **p<0.01

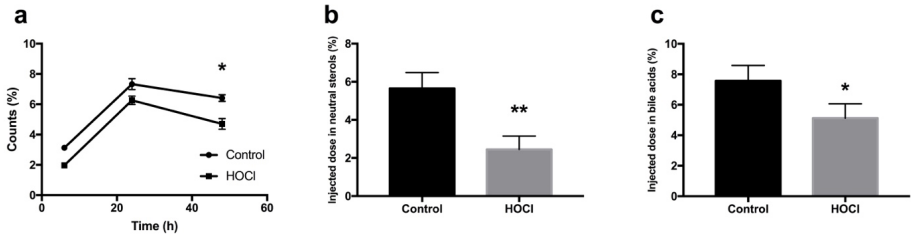

Figure 4. HDL oxidized in vitro is defective in mediating in vivo RCT. Mice injected with macrophage foam cells loaded with ^{3}H-cholesterol were infused with either control HDL or HOCl-modified HDL as detailed in methods. (A) HOCl-modified HDL had a significantly decreased capacity to mobilize macrophage-derived ^{3}H-cholesterol to the plasma compartment compared with native HDL at the 4h time point ($p<0.05$). (B) Fecal ^{3}H-cholesterol tracer recovery in the neutral sterol fraction, (C) Fecal ^{3}H-cholesterol tracer recovery in the bile acid fraction. Data are presented as means ± SEM, n=8 mice/group. * $p<0.05$, ** $p<0.01$.

DISCUSSION

Combined, the results of this study demonstrate that HDL from patients with ESRD is dysfunctional in mediating RCT, a key atheroprotective property,[2,3] conceivably due to extensive oxidative modifications of HDL associated proteins. Reduced RCT is thus expected to contribute to the excessive increase in CVD risk observed in ESRD patients.

A progressive reduction in kidney function is known to associate with a significant increase in CVD risk.[7,8,18] This relationship culminates in an approximately 30-fold increase in age-adjusted CVD risk in ESRD patients.[7,8] Plasma HDL cholesterol levels in ESRD patients are decreased,[19,20] however, to our knowledge the ability of HDL particles from ESRD patients to function in overall RCT has not been investigated thus far. In the present study we used in addition to *in vitro* studies an *in vivo* approach to directly measure RCT from macrophages to feces.[3,5,6,21] Our data thereby add a reduction in the RCT functionality of HDL to pathophysiological concepts of increased CVD in patients with reduced kidney function and uremia.

An interesting question is the underlying pathophysiology and therefore the mechanistic basis for the oxidative modifications observed in this study within HDL apolipoproteins in ESRD. It is established that ESRD patients display a proinflammatory state and suffer from an increased oxidative stress burden.[22–24] Myeloperoxidase (MPO) is an enzyme expressed by macrophages and neutrophils that is released in response to proinflammatory stimuli.[25] Interestingly, plasma levels of MPO have been shown to be significantly increased in patients with ESRD and also to be a predictor of mortality in hemodialysis patients.[26,27] MPO has been previously demonstrated to cause oxidative modifications of HDL apolipoproteins that might impact their functionality.[25,28] Hypochloric acid (HOCl) can mimic MPO-induced oxidation *in vitro*. By modifiying HDL from healthy controls with HOCl we were able to replicate the functional deficits displayed by ESRD-HDL, namely a decreased efficacy in promoting cholesterol efflux as well as a reduced ability to deliver cholesterol to cells via SR-BI. These data provide an additional line of evidence that MPO might play a key role in causing the decreased functionality of HDL in ESRD and that MPO might therefore represent an interesting target for pharmacological inhibition in ESRD patients.

Testing HDL functionality in addition to measuring mass HDL cholesterol and apoA-I levels is an emerging concept in the field of HDL research.[29–32] However, although multiple potentially beneficial effects have been described for HDL,[33] there are limitations for the clinical setting, since reliable and reproducible assays to test these functions are lacking.[30] Thus far, several dysfunctionalities were ascribed to HDL from ESRD patients. It was reported that ESRD is less effective in protecting LDL against copper mediated oxidation *in vivo*.[34] These data could be interpreted as a further indication that HDL from ESRD patients is already oxidized to a substantial extent and therefore cannot properly fulfil anti-oxidative functions. Furthermore, a decreased functionality of HDL-mediated cholesterol efflux has been demonstrated in ESRD patients.[13,35] Regarding testing properties related to RCT *in vitro* our data suggest that also assays addressing selective uptake through SR-BI from given HDL preparations might be valuable, since this also represents a key step for effective RCT that might be differentially affected compared with cholesterol efflux.

For a balanced interpretation of our results the following points should be taken into account. (i) We demonstrate that ESRD-HDL is dysfunctional in mediating RCT in wild-type mice. RCT in mice largely depends on HDL and the HDL selective uptake receptor SR-BI. In contrast, in humans RCT is mainly based on the LDL receptor mediating hepatic cholesterol uptake following transfer of cholesteryl esters out of HDL into apoB-containing lipoproteins by the cholesteryl ester transfer protein (CETP), which is not expressed in rodents.[36] Although ESRD-HDL apparently has a decreased interaction with several relevant components of the HDL metabolism system, it remains to be formally established that also in humans ESRD-HDL is defective in mediating *in vivo* RCT. (ii) In addition, we focussed in our *in vitro* studies on the classical RCT pathway, investigating macrophage efflux and hepatic uptake. We did not assess a contribution of transintestinal cholesterol excretion (TICE) to RCT, which might also have relevance here.[37] In previous work, we estimated the contribution of TICE to RCT to be around 30% under baseline conditions in wild-type mice,[4] while others found higher values.[38] However, results from lipoprotein kinetic studies indicate that HDL does not represent the lipoprotein subclass that donates cholesterol to the TICE pathway,[4,39] at least not directly. In a system expressing CETP such as humans, this contribution might be higher, but this questions remains to be experimentally answered. (iii) Furthermore, the patient and the control group differed in several aspects other than the presence/absence of ESRD such as diabetes, smoking or use of lipid lowering medication and we cannot formally exclude that these differences impact the results. However, there were no differences within the ESRD group between either smokers/non-smokers, diabetic/non-diabetic patients and patients with or without lipid lowering medication (data not shown). Furthermore, also the results for cholesterol efflux as well as selective uptake were not different between ESRD patients displaying all additional risk factors combined (smoking, diabetes, lipid lowering medication) and those without, while each ESRD group differed significantly from controls (supplemental figure S1). These data indicate that the presence of ESRD might have a substantially stronger effect on the impairment of HDL function than any of these potential confounders. Also, none of the conclusions changed when the smokers were excluded from analysis (data not shown). (iv) The design of our study does not enable us to draw a firm conclusion, if the observed differences in HDL function are due to the hemodialysis treatment or the presence of ESRD. Future studies including patients with ESRD naïve to hemodialysis are warranted to address this issue.

In summary, our data demonstrate that HDL from ESRD patients is extensively oxidatively modified and displays reduced efficacy in key protective functions related to CVD, namely promoting (i) cellular cholesterol efflux, (ii) SR-BI-mediated cholesterol delivery and (iii) overall functional RCT *in vivo*. These results might have major implications to explain the excessive increase in CVD risk in uremic patients.

MATERIALS AND METHODS

PATIENTS AND CONTROL SUBJECTS

EDTA plasma was collected under fasting conditions from patients with ESRD and age- and sex-matched controls (n=15 each, see Supplemental table S1 for clinical and biochemical characteristics). Blood samples were placed on ice immediately after collections and stored at -80ºC until analysis. Patients and controls were in a stable clinical condition and free from infectious complications for at least 3 months. None of the ERSD patients had residual renal function. Blood samples of the ESRD group were taken before regular hemodialysis sessions. Informed consent was obtained from all subjects. Blood collection was approved by the responsible medical ethics committee of the Charité Berlin and methods were carried out in accordance with the approved guidelines.

CHOLESTEROL EFFLUX EXPERIMENTS

Thioglycollate-elicited mouse peritoneal macrophages[40] were loaded with ^{3}H-cholesterol (1 µCi/ml, NEN Life Sciences Products), and 50 µg acetylated LDL for 22h as described[11] followed by equilibration in RPMI with 0.2% BSA for 18h. Following another wash with PBS, acceptors were added (individual HDL samples isolated by ultracentrifugation as detailed below, 50 µg of protein, experiments were performed in triplicates). After 5 h the supernatant was taken off and radioactivity within the medium was determined by liquid scintillation counting (Beckman LS6500, Beckman Instruments, Palo Alto, CA). Next, 0.1 M NaOH was added to cells, plates were incubated for 30 min at room temperature and the radioactivity remaining within the cells was then also assessed by liquid scintillation counting. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells). Values for unspecific efflux determined as release of ^{3}H-cholesterol from macrophages in the absence of HDL were subtracted from the individual values.

HDL UPTAKE EXPERIMENTS

LdlA cells lacking LDL receptor expression as well as ldlA cells stably transfected with a murine SR-BI cDNA (ldlA[mSR-BI]) were kindly provided by Dr. Monty Krieger (MIT, Boston, USA) and cultured as described.[41] For HDL cholesterol uptake experiments, 5% lipoprotein-depleted serum was used. HDL was isolated from individual plasma samples as described below and labeled with cholesteryl hexadecyl ether (cholesteryl-1,2-^{3}H, NEN Life Sciences Products), a non-hydrolyzable analogue of cholesteryl ester with identical selective uptake properties as ^{3}H-cholesteryl ester, essentially as described.[42,43] Ten µg/ml of ^{3}H-CE HDL was added to the cells, experiments were performed in duplicates. After a 5-h incubation, the cells were washed three times with PBS (pH 7.4) and lysed with 0.5 ml of 0.1 M NaOH. Tracer uptake was calculated as counts recovered from the cells as percentage of the total dose (counts from cells added to the counts from media).

HDL COMPOSITION ANALYSIS

For the analysis of HDL composition, HDL was isolated from 200 µl of plasma by tabletop sequential ultracentrifugation ($1.063 < d < 1.21$) as described,[44] and total and free cholesterol, phospholipids and triglycerides were determined enzymatically using commercially available reagents (Wako Pure Chemical Industries, Neuss, Germany). Protein concentrations were measured with the BCA assay (Pierce, Rockford, IL, USA). Commercially available ELISA kits were used according to the manufacturer's instructions to determine human SAA (Biosupply, Bradford, UK) and apoC-III (Abcam, Cambridge, UK) in HDL.

IN VIVO *RCT*

C57BL/6J mice, 8 weeks of age, were purchased from Charles River (Sulzfeld, Germany). The animals were kept in animal rooms with alternating 12-hour periods of light (from 7:00 a.m. to 7:00 p.m.) and dark (from 7:00 p.m. to 7:00 a.m.), with ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in accordance with the national laws. All protocols were approved by the responsible ethics committees of the Landesamt für Gesundheit, Ernährung und technische Sicherheit Berlin (LAGETSI) and the University of Groningen. Thioglycollate-elicited macrophages were isolated, cultured and loaded with AcLDL and ^{3}H-cholesterol exactly as detailed above. At the end of the equilibration, macrophages were carefully harvested from the plate and injected intraperitoneally into mice (2x10E6 cells/mouse). Directly following the i.p. injection of the macrophages, individually housed mice were administered i.v. either PBS (200 µl) or pooled HDL (200 µl) either ~~isolated~~ from the ESRD patient~~s~~ group or from the group of control subjects at a dose of 2 mg HDL cholesterol/mouse. The choice to base injections on HDL cholesterol was made to be able to detect potential functional differences of HDL independent of the established clinical biomarker HDL cholesterol, whose validity has recently been called into question[36]. Injections were repeated at 24h. Blood samples were drawn at time points 0h, 4h, 24h and 48h and tracer within plasma was determined by liquid scintillation counting. After 48h, mice were sacrificed, the liver was harvested and total feces produced over the 48h period of the experiment were collected.

Radioactivity in plasma was counted directly, counts for ^{3}H-cholesterol taken up by the liver were determined by incubating a piece of liver with Solvable (Packard, Meriden, CT, USA) according to the manufacturer's instructions to dissolve the tissue as previously published.[45] Counts recovered from a respective piece of liver were backcalculated to total liver mass and expressed as percent of injected dose per whole organ. Feces were thoroughly dried, ground and aliquots were separated into the bile acid and the neutral sterol fractions.[46] Counts recovered from the respective aliquots were related to the total amount of feces produced within 24 h and expressed as percentage of the injected radiotracer dose.

IN VITRO *OXIDATIVE MODIFICATION OF HDL*

Oxidized HDL was generated following a previously published procedure.[47] Briefly, 1 mg/ml of total HDL protein isolated from healthy controls (1.063 < *d* < 1.21) was incubated with NaOCl solution at a molar ratio of 200 for 60 min at 37 °C, adjusted to a final pH of 7.4. Preparations of modified HDL were dialyzed against PBS and used within 24h. For uptake experiments, oxidized HDL were labeled with cholesteryl hexadecyl ether as described above.

STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical package for social sciences (SPSS; SPSS Inc., Chicago, IL). Data are presented as means ± SEM. Statistical differences between two groups were assessed using the Mann-Whitney U-test. To compare more than two groups ANOVA followed by a Bonferroni post-hoc test was used. Statistical significance for all comparisons was assigned at $P<0.05$.

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AUTOANTIBODIES TO APOLIPOPROTEIN A-1 AS INDEPENDENT PREDICTORS OF CARDIOVASCULAR MORTALITY IN RENAL TRANSPLANT RECIPIENTS

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Journal of Clinical Medicine, 2019

ABSTRACT

Renal transplant recipients (RTRs) are known to have a high cardio-vascular disease (CVD) burden only partly explained by traditional CVD risk factors. The aim of this paper was therefore to determine: i) the prognostic value of autoantibodies against apoA-1 (anti-apoA-1 IgG) for incidence of CVD mortality, all-cause mortality and graft failure in RTR. Four hundred and sixty two (462) prospectively included RTRs were followed for 7.0 years. Baseline anti-apoA-1 IgG were determined and associations with incidence of CVD mortality (*n* = 48), all-cause mortality (*n* = 92) and graft failure (*n* = 39) were tested. Kaplan–Meier analyses demonstrated significant associations between tertiles of anti-apoA-1 IgG and CVD mortality (log rank test: P = 0.048). Adjusted Cox regression analysis showed a 54% increase in risk for CVD mortality for each anti-apoA-1 IgG levels standard deviation increase (hazard ratio [HR]: 1.54, 95% Confidence Interval [95%CI]:1.14–2.05, *p* = 0.005), and a 33% increase for all-cause mortality (HR: 1.33; 95%CI:1.06-1.67, *p* = 0.01), independent of CVD risk factors, renal function and HDL function. The association with all-cause mortality disappeared after excluding cases of CVD specific mortality. The sensitivity, specificity, positive predictive value, and negative predictive value of anti-apoA-1 positivity for CVD mortality were 18.0%, 89.3%, 17.0%, and 90.0%, respectively. HDL functionality was not associated with anti-apoA-1 IgG levels. This prospective study demonstrates that in RTR, anti-apoA-1 IgG are independent predictors of CVD mortality and are not associated with HDL functionality.

INTRODUCTION

Impaired kidney function is a major risk factor for cardiovascular diseases (CVD) through all stages of renal dysfunction, amounting to a substantial 40-fold increased risk of CVD mortality in end-stage renal disease (ESRD) patients.[1,2] Even after renal transplantation the CVD risk remains four to six times higher in age-adjusted analyses, with half of all deaths of renal transplant recipients (RTRs) being attributable to a CVD origin [3]. Traditional CVD risk factors, such as the ones aggregated in the Framingham risk score (FRS), are known to be of little use in CVD risk prediction in RTRs.[4,5] Thus, accurate CVD risk stratification in RTRs represents an unmet clinical need in this constantly increasing patient population.

Autoantibodies against apoA-1 (anti-apoA-1 IgG) represent a recently identified biomarker with a high potential to predict increased CVD risk. Increased levels of these antibodies are associated with a pro-atherogenic lipid profile, a systemic pro-inflammatory state,[6-8] as well as high-density lipoprotein (HDL) dysfunction,[9,10] and were shown to be associated with increased CVD risk and poorer prognosis in high-risk patients, as well as in the general population.[7,11-14] When administered to mouse models of atherosclerosis, anti-apoA-1 IgG enhanced atherogenesis, myocardial necrosis and premature death indicating that anti-apoA-1 IgG have the potential to serve as a causative biomarker for CVD.[15-17] A previous study showed that ESRD patients have a high prevalence of elevated anti-apoA-1 IgG levels, which was associated with dialysis vintage, and was a major determinant of cardiovascular outcomes in these patients.[18] The reasons underpinning such association in ESRD are still elusive, but may suggest that a prolonged exposure to the uremic milieu, characterized by increased oxidative stress and inflammation, could increase apoA-1 immunogenicity leading to an anti-apoA-1 IgG response. Accordingly, as RTR are exposed to a uremic milieu prior to transplantation, they could also constitute a particularly risk-prone group to such a humoral autoimmunity phenomenon, despite receiving immunosuppressive treatment.

The aim of our present study was to determine: i) the prognostic value of anti-apoA-1 IgG for incidence of CVD specific mortality, overall mortality and graft failure in RTR and ii) to delineate the relationship of anti-apoA-1 IgG with apoA-1 levels and HDL functionality.

EXPERIMENTAL SECTION

STUDY DESIGN AND STUDY POPULATION

This study included all adult RTR who visited the University Medical Centre Groningen (UMCG) outpatient clinic between August 2001 and July 2003 with a functioning renal graft for at least 1 year. Of 847 eligible patients, 606 consented to participate in the overall study. Exclusion criteria consisted of congestive heart failure, malignant disease other than cured

skin cancer, as well as endocrine abnormalities other than diabetes, or suspected acute infection upon inclusion, indicated by a CRP value above 15 mg/L. This way 477 patients were initially included in the present study; serum was available from 462 RTRs, in which subsequently anti-apoA-1 IgG levels were measured. All relevant patient characteristics were obtained from the "Groningen Renal Transplant Database". Patients were followed for a period of 7 years, and no patients were lost during follow-up. More detailed definitions of the characteristics of the database patients' baseline characteristics, as well as the routine laboratory methods used have been previously described.[19,20] The study was approved by the local Medical Ethics Committee (METc2001/039), and is in accordance with the Declaration of Helsinki and Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'.

OUTCOME MEASURES

The main outcome measure in this study is the level of anti-apoA-1 IgG. The primary endpoints consisted in CVD mortality, all-cause mortality and graft failure. As previously reported.[21,22] graft failure was defined as return to dialysis or re-transplantation. Cause of death was obtained by linking the number on the death certificate to the primary cause of death, as coded by a physician from the Central Bureau of Statistics. CVD mortality was defined as deaths in which the principal cause of death was cardiovascular in nature, using ICD-9 codes 410–447. The secondary endpoint was a possible association between anti-apoA-1 IgG levels and apoA-1 levels, as well as a key HDL functionality, namely macrophage cholesterol efflux capacity (CEC).

SENSITIVITY ANALYSES

Sensitivity analyses were performed, in which the association of anti-apoA-1 IgG with non CVD-mortality was assessed, in order to assess the specificity of anti-apoA-1 with CVD.

DETERMINATION OF ANTI-apoA-1 IgG

Anti-apoA-1 IgG were measured using RTR serum aliquots stored at −80°C, as previously described.[6-8, 11-13] The experiments demonstrating the specificity of our assay against the native and unmodified form of apoA-1 are available in the Supplementary Material.

DETERMINATION OF HDL FUNCTION

To determine HDL-mediated CEC, a previously published method was used.[21,22] For further details see the Supplementary Material.

STATISTICAL ANALYSIS

In order to eliminate bias due to gender specific differences in levels of anti-apoA-1 IgG renal transplant recipients were divided into gender-stratified tertiles based on levels of anti-apoA-1 IgG. This was done by first dividing the group into males and females, then

computing tertiles based on the levels of anti-apoA-1 IgG, and subsequently merging the groups back together. Differences between baseline characteristics were tested. For continuous variables with a skewed distribution differences were tested by Kruskal–Wallis test. Differences for normally distributed continuous variables were tested by one-way analysis of variance followed by Bonferroni post-hoc test. Differences in categorical data were tested by chi-squared test.

Thereafter, multivariable linear regression analysis was performed to evaluate which variables predict levels of anti-apoA-1. Baseline characteristics with a P-value of ≤0.2 between tertiles of anti-apoA-1 IgG were first fitted into a univariate linear regression. Variables that had a significant association with anti-apoA-1 IgG in a univariate analysis were then entered into a multivariate linear regression.

The association of anti-apoA-1 IgG levels with the primary endpoints was assessed by the log-rank test and by Cox proportional hazards regression. Kaplan-Meier curve analyses were performed across anti-apoA-1 IgG tertiles and according to anti-apoA-1 IgG seropositivity, based upon a predefined and validated anti-apoA-1 IgG cut-off value (an OD value >0.64 and a percentage of the positive control above 37%).[7,8,11–14,18] Differences were assessed using a log-rank test. Cox regression analyses were used to calculate hazard ratios (HR) and reported with their 95% confidence intervals (95%CI). Univariate and multivariate Cox regression analyses were performed per standard deviation (SD, 0.316) increase of anti-apoA-1 IgG levels, and according to anti-apoA-1 IgG seropositivity. Multivariate analyses were performed using different models, taking into account traditional CV risk factors, renal function, HDL functionality, and all the variables that had significant association with anti-apoA-1 levels in linear regression. Schoenfeld residuals test was used to test the proportional hazard assumption for the outcomes of CVD mortality, all-cause mortality and graft failure for analysis per standard deviation increase (P=0.18, P=0.20 and P=0.69 respectively) and for analysis with seropositivity (P=0.38, P=0.09 and P=0.77 respectively), and was found not to be violated. Sensitivity (SN), specificity (SP), positive predictive and negative predictive values (PPV and NPV, respectively) for anti-apoA-1 IgG seropositivity were computed. P-values <0.05 were considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 24 (IBM SPSS, IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

BASELINE DEMOGRAPHIC CHARACTERISTICS

In this longitudinal follow-up study the levels of anti-apoA-1 IgG were measured in 462 RTR. Of these patients, 92 (20%) died within the follow-up of 7 years, 48 of these from a

confirmed CVD cause, as determined by ICD-9 codes 410–447 (10% of included patients, 52% of all recorded deaths). A total of 39 (8%) patients experienced graft failure. Overall, the prevalence of high levels of anti-apoA-1 IgG (anti-apoA-1 IgG seropositivity according to a previously defined cut-off value based on data from the general population) was 11.5 % (53/462).[7,11–13,18] In order to better explore the architecture of anti-apoA-1 IgG in RTR, patients were divided into gender-stratified tertiles of anti-apoA-1 IgG, with median values of 0.15 (range 0–0.24), 0.31 (range 0.24–0.49), and 0.64 (range 0.50–2.09) for the first, second and third tertile, respectively (Table 1). Analyses between tertiles showed a significant difference for the history of myocardial infarction (MI), which was most common in patients with the highest levels of anti-apoA-1 IgG (P=0.047), as well as for diabetic nephropathy (P= 0.04) as the primary renal disease. A trend was also observed for tubulo-interstitial disease (P=0.05), which is characterised by acute or chronic inflammation of the renal tubules and interstitium, and primary glomerular disease (P=0.08), which covers a group of conditions in which there is primary injury in the glomerulus.[23]

Table 1. Baseline characteristics according to gender stratified tertiles of anti-apoA-1 IgG

Characteristic	Gender stratified Tertiles of Anti-apoA-1 levels			P value
	First (n=154)	Second (n=154)	Third (n=154)	
Recipient demographics				
Age, years	50.5 [41.6-59.4]	53.4 [44.7-61.1]	52.1 [44.0-60.8]	0.12
Male gender, *n* (%)	84 (55)	84 (55)	84 (55)	1.00
Current smoking, *n* (%)	28 (18)	33 (21)	22 (14)	0.26
Previous smoking, *n* (%)	70 (46)	67 (44)	72 (47)	0.85
Metabolic syndrome, *n* (%)	83 (57)	94 (65)	84 (60)	0.34
Body composition				
BMI, kg/m^2	26.1 ±4.3	26.1±4.2	25.9±4.2	0.86
Lipid Profile				
Total cholesterol, mmol/l	5.6±1.0	5.7 ±0.9	5.7 ±1.3	0.49
LDL cholesterol, mmol/l	3.5 ±1.0	3.6±0.8	3.6 ±1.2	0.82
HDL cholesterol, mmol/l	1.1 ±0.3	1.3 ± 0.3	1.1±0.3	0.55
Apolipoprotein A-I, g/l	1.6 ± 0.3	1.6 ±0.3	1.6 ±0.3	0.75
Triglycerides, mmol/l	2.1 [1.3-2.7]	2.1 [1.4-2.5]	2.2 [1.4-2.7]	0.22
Cholesterol efflux percentage	7.3 [6.2-8.4]	7.5 [6.3-8.3]	7.6 [6.5-8.9]	0.11
Use of statins, *n* (%)	79 (51)	88 (57)	74 (48)	0.27
Cardiovascular disease history				
History of MI, *n* (%)	12 (7)	8 (5)	20 (13)	0.047
TIA/CVA, *n* (%)	5 (3)	8 (5)	9 (6)	0.54
Blood pressure				
Systolic blood pressure, mmHg	152.2 ±23.9	151.0±21.4	154.1 ±22.0	0.47
Diastolic blood pressure, mmHg	89.8 (±9.8)	89.0 (±9.5)	90.1 (±10.0)	0.59
Use of ACE inhibitors, *n* (%)	55 (36)	49 (32)	58 (38)	0.55
Use of *β*-blockers, *n* (%)	90 (58)	93 (60)	95 (61)	0.84
Use of diuretics, *n* (%)	59 (38)	75 (49)	63 (41)	0.16
Number of antihypertensive drugs, *n (%)*	2 [1-3]	2 [1-3]	2 [1-3]	0.16

Table 1 continued.

Characteristic	Gender stratified Tertiles of Anti-apoA-1 levels			P value
	First (n=154)	Second (n=154)	Third (n=154)	
Glucose homeostasis				
Glucose, mmol/l	4.9 [4.1-5.0]	4.8 [4.1-5.0]	4.8 [4.1-5.1]	0.69
Insulin, μmol/l	11.3 [8.7-16.5]	10.6 [7.8-14.8]	11.4 [7.6-15.4]	0.16
HbA1c, %	6.3 [5.8-6.9]	6.3 [5.8-7.0]	6.4 [5.7-7.1]	0.47
HOMA-IR	3.1 [1.7-3.6]	2.7 [1.5-3.4]	2.8 [1.5-3.4]	0.21
Post-Tx diabetes mellitus, *n* (%)	24 (15)	29 (19)	29 (19)	0.69
Use of anti-diabetic drugs, *n* (%)	17 (11)	25 (16)	21 (14)	0.41
Use of insulin, *n* (%)	7 (5)	9 (6)	13 (8)	0.36
Inflammation				
hsCRP, mg/l	3.4 [0.7-4.4]	3.3 [0.9-4.1]	3.3 [1.0-4.2]	0.43
Anti-apoA-1 IgG, AU (OD405 nm)	0.15 [0.11-0.19]	0.31 [0.26-0.36]	0.64 [0.52-0.82]	<0.001
Framingham risk score	17.2 [7.6-27.3]	20.8 [9.6-32.9]	20.7 [8.6-31.3]	0.28
Donor demographics				
Age, years	37.6 [23.0-50.0]	37.2 [23.8-50.0]	37.1 [23.0-51.3]	0.76
Male gender, *n* (%)	76 (49)	90 (59)	85 (56)	0.24
Living kidney donor, *n* (%)	28 (18)	17 (11)	15 (10)	0.06
(Pre)transplant history				
Dialysis time, months	34.8 [12.0-48.3]	37.0 [14.8-51.0]	33.6 [12.8-45.0]	0.44
Primary renal disease				
Primary glomerular disease, *n* (%)	35 (23)	40 (26)	52 (34)	0.08
Glomerulonephritis, *n* (%)	11 (7)	6 (4)	12 (8)	0.32
Tubulo-interstitial disease, *n* (%)	33 (21)	24 (16)	17 (11)	0.05
Polycystic renal disease, *n* (%)	26 (17)	31 (20)	24 (16)	0.56
Dysplasia and hypoplasia, *n* (%)	7 (5)	8 (5)	2 (1)	0.15
Renovascular disease, *n* (%)	11 (7)	12 (8)	6 (4)	0.32
Diabetic nephropathy, *n* (%)	3 (2)	2 (1)	9 (6)	0.04
Other or unknown cause, *n* (%)	28 (18)	31 (20)	32 (21)	0.84
Immunosuppressive medication				
Daily prednisolone dose, mg	9.2 [7.5-10.0]	9.1 [7.5-10.0]	9.1 [7.5-10.0]	0.33
Calcineurin inhibitors, *n* (%)	120 (78)	126 (82)	124 (81)	0.68
Proliferation inhibitors, *n* (%)	124 (81)	109 (71)	108 (70)	0.07
Renal allograft function				
Creatinine clearance, ml/min	47.3 ± 14.6	48.2 ±15.9	46.5 ±16.1	0.62
Urinary protein excretion, g/24 h	0.3 [0.0-0.3]	0.2 [0.1-0.2]	0.4[0.1-0.4]	0.07

Normally distributed continuous variables are presented as mean±SD. Continuous variables with a skewed distribution are presented as median [25th to 75th percentile]. Categorical data are summarized by n (%), and differences were tested by chi-squared test. MI, myocardial infarction; TIA, transient ischemic attack; CVA, cerebrovascular event; ACE, angiotensin-converting enzyme; Tx, transplantation.

When participating RTR were stratified according to anti-apoA-1 IgG seropositivity, there was again a significant association with a higher prevalence of previous MI (p = 0.02), glomerular disease (p = 0.03) and tubulo-interstitial disease (p = 0.03) as the primary renal disease (supplementary Table 1). Furthermore, anti-apoA-1 IgG seropositive patients tended to have received grafts from older donors (p = 0.05) and showed a higher urinary protein excretion (p = 0.04, supplementary Table 1). Importantly, cholesterol efflux capacity as central HDL function metric did not differ between patients seropositive for anti-apoA-1 IgG and those seronegative for these antibodies (supplementary Table 1).

Subsequently, univariate and thereafter multivariate linear regression was performed to deduce which variables are independently associated with levels of anti-apoA-1 IgG (Table 2). A positive, independent association was seen between anti-apoA-1 IgG and a history of myocardial infarction (β = 0.103, *p* = 0.026) and primary glomerular disease (β = 0.116, *p* = 0.016). A negative association was seen with tubule-interstitial disease (β = −0.106, *p* = 0.028). No significant relationship between anti-apoA-1 IgG and the metric of HDL function, CEC, was discernible, nor with concentrations of HDL-C or with apoA-1. There was also no association with immunosuppressive drugs, either individually or combined.

Table 2. Multivariate linear regression for baseline characteristics that are significantly associated with anti-apoA-1 in a univariate linear regression

	Unstandardized coefficient	95% CI	Standardized coefficient	P value
Primary glomerular disease	0.086	0.016-0.156	0.116	0.016
History of MI	0.121	0.015-0.227	0.103	0.026
Tubulo-interstitial disease	-0.0.96	-0.182- -0.010	-0.106	0.028

Variables are listed in decreasing order of strength of association. R^2=.0.043 (Cox & Snell). Model x^2: 49.8 p<0.001

ASSOCIATION WITH INCIDENCE OF CVD MORTALITY, ALL-CAUSE MORTALITY, AND GRAFT FAILURE

As shown in Figure 1, Kaplan Meier curves showed a significant association of tertiles of anti-apoA-1 IgG with CVD mortality (*p* = 0.048), but not with all-cause mortality (*p* = 0.22) or graft failure (*p* = 0.13).

When Kaplan Meier curves were generated comparing anti-apoA-1 IgG seropositive versus anti-apoA-1 IgG seronegative RTR (Supplementary figure 1), the same associations were retrieved, namely significance for CVD mortality (*p* = 0.035), but not for all-cause mortality or for incident graft failure.

At the pre-specified cut-off for anti-apoA-1 IgG positivity, sensitivity was 18.0% (95% CI: 9–32), specificity 89.3% (95% CI: 86–92), positive predictive value 17.0% (95% CI: 9–30) and negative predictive value 90.0% (95% CI: 87–92) for CVD-related deaths.

Finally, as shown in Table 3 Cox regression analyses showed that anti-apoA-1 IgG levels were significantly associated with CVD mortality in a model adjusted for age and gender (model 1, HR: 1.56, *p* = 0.002). This association remained significant, independent of adjustment either for the Framingham risk score (FRS, model 2, HR: 1.56, *p* = 0.002), eGFR (model 3, HR: 1.54, *p* = 0.004) or both parameters combined (model 4, HR: 1.54, *p* = 0.004), HDL CEC (model 5, HR: 1.54, *p* = 0.003), history of MI (model 6, HR: 1.45, *p* = 0.0013), primary renal disease (model 7, HR: 1.53, *p* = 0.005) and time between transplantation and baseline (model 8, HR: 1.56, *p* =0.002). Importantly, FRS itself was not associated with CVD mortality

in our RTR cohort (unadjusted HR: 1.00 [0.99-1.02], p = 0.51; age and gender adjusted HR: 1.00 [0.98–1.03], p =0.95). For all-cause mortality, the same associations were retrieved (models 3 and 4; Table 3). On the other hand, no significant associations were detected between anti-apoA-1 IgG levels and incident graft failure (Table 3). In our sensitivity analyses there was no association between anti-apoA-1 IgG levels and non-CVD mortality, further supporting the specificity of the relationship between anti-apoA-1 IgG and CVD mortality (Table 3).

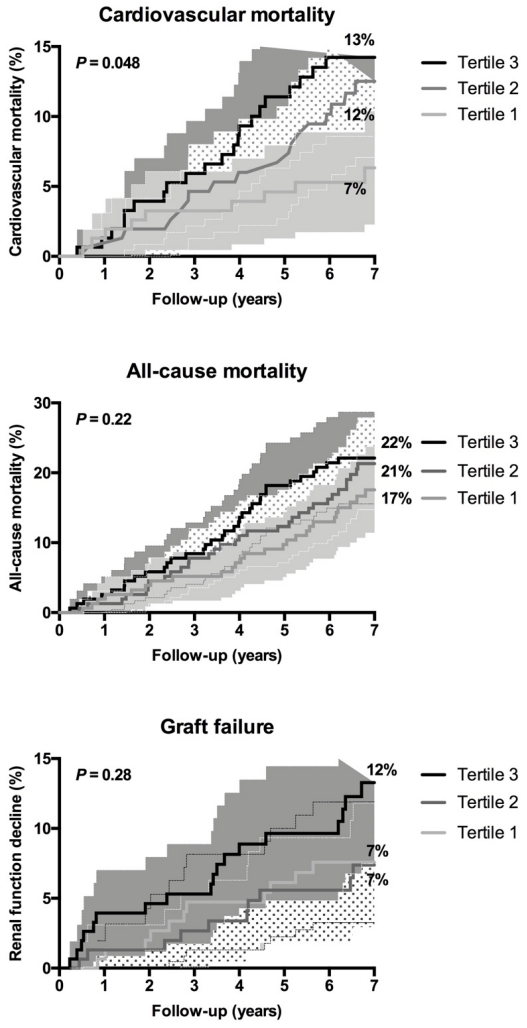

Figure 1. Higher levels of anti-apoA-1 IgG are associated with an increased incidence of cardiovascular mortality and all-cause mortality in renal transplant recipients. Kaplan-Meier curves depicting (A) cardiovascular mortality, (B) all-cause mortality, and (C) graft failure according to tertiles of anti-apoA-1 IgG. The corresponding P value was obtained from log-rank tests.

Table 3. Hazard ratios for cardiovascular mortality, all-cause mortality, and graft failure per one standard deviation increase of anti-apoA-1 IgG.

	CVD mortality		All-cause mortality		Graft failure		All-cause mortality Sensitivity analysis	
	HR [95%CI] per 1-SD increase	P value	HR [95%CI] per 1-SD increase	P value	HR [95%CI] per 1-SD increase	P value	HR [95%CI] per 1-SD increase	P value
Model 1	1.56 [1.17-2.07]	0.002	1.36 [1.09-1.70]	0.007	1.17 [0.93-1.48]	0.18	1.41 [0.95-2.09]	0.09
Model 2	1.56 [1.17-2.08]	0.002	1.36 [1.09-1.70]	0.007	1.18 [0.94-1.49]	0.16	1.41 [0.94-2.11]	0.09
Model 3	1.54 [1.15-2.06]	0.004	1.32 [1.05-1.67]	0.017	1.14 [0.91-1.42]	0.26	1.39 [0.92-2.08]	0.11
Model 4	1.54 [1.15-2.07]	0.004	1.32 [1.04-1.66]	0.020	1.15 [0.92-1.44]	0.22	1.39 [0.92-2.10]	0.12
Model 5	1.54 [1.16-2.05]	0.003	1.36 [1.09-1.71]	0.007	1.19 [0.94-1-50]	0.15	1.44 [0.98-2.11]	0.06
Model 6	1.45 [1.08-1.94]	0.013	1.32 [1.05-1.66]	0.016	1.14 [0.90-1.45]	0.27	1.39 [0.90-2.14]	0.14
Model 7	1.53 [1.14-2.05]	0.005	1.33 [1.06-1.67]	0.016	1.17 [0.93-1.48]	0.18	1.45 [0.96-2.20]	0.08
Model 8	1.56 [1.18-2.09]	0.002	1.37 [1.09-1.17]	0.07	1.17 [0.93-1.48]	0.18	1.47 [0.98-2.23]	0.07

Model 1: adjustment for recipient age and gender; model 2: model 1 + adjustment for FRS; model 3: model 1 + adjustment for eGFR; model 4: model 1 + adjustment for FRS and eGFR; model 5: model 1 + adjustment for cholesterol efflux capacity; model 6: model 1 + adjustment for history of MI; model 7: model 1 + adjustment for primary renal disease; model 8: model 1 + adjustment for time between transplantation and baseline. In sensitivity analysis non-CVD deaths were used as endpoint. One standard deviation is equivalent to 0.316. HR: Hazard ratios.

When Cox regression analyses were performed according to anti-apoA-1 IgG seropositivity, the aforementioned associations remained unchanged, at the exception of all-cause mortality which remained significant after adjusting for renal function, and for which the association became close to significance after adjusting for previous MI on top of age and gender (Supplementary Table 2). Again, no association between anti-apoA-1 IgG levels and graft failure could be observed (Supplementary Table 2).

DISCUSSION

The novel finding of this prospective study is that anti-apoA-1 IgGs are an independent predictor of CVD mortality in a RTR cohort with a follow-up of 7 years. Our observations indicate that traditional CVD risk factors were presently not associated with CVD mortality. Furthermore, no association was observed between anti-apoA-1 IgG and non-CVD mortality in the preformed sensitivity analysis. This reinforces both the possible clinical relevance and the CVD specificity of the present findings. Indeed, to the best of our knowledge and at the exception of renal function markers,[3–5,21,22] no specific biomarkers of CVD outcome independent of renal function have been identified so far in RTR. Considering the absence of currently validated tools for CVD risk prediction in RTR, a rule-out test with a 90% NPV could conceivably be of clinical interest as a first step in the field of CV risk stratification in these patients. In this context, we hypothesize that a simple standard follow-up could be particularly well adapted to RTR patients with low anti-apoA-1 IgG values. Further validation studies are now required to challenge this hypothesis before any clinical recommendations can be made. The second notable finding of this study is that anti-apoA-1 IgG were not associated with

graft failure, nor with HDL CEC. Although further reinforcing the specificity between anti-apoA-1 IgG and CV outcomes, these results were somehow unexpected, as anti-apoA-1 IgG have been previously shown to be associated with impaired HDL CEC,[9,10] lately reported as being an independent predictor of incident graft failure.[21] The reasons for such differences are still elusive, most likely numerous, and possibly related to pathophysiological differences between CVD and graft atherosclerosis. Indeed, rupture of vulnerable atherosclerotic plaques is known to underlie most cases of acute CVD events, while this is not thought to play an important role in chronic transplant vasculopathy-induced graft failure, where progressive arteriolar luminal narrowing due to the intimal accumulation of degenerating smooth muscle-like cells and adventitial fibrosis represent the major pathogenic processes.[23] Furthermore, another explanation could lie in the fact that RTR represent a unique patient population in terms of oxidative stress exposure and persistent loss of HDL function, when compared to e.g. systemic lupus erythematosus patients[10] or dyslipidaemic subjects with preserved renal function.[9] Finally due to the heterogeneity of methodological protocols underlying the numerous unstandardized HDL functional assays, we cannot exclude that an analytical difference between our HDL assay and those from other groups could undermine the present observation.[24] Therefore, further studies are warranted to determine if this absence of correlation between anti-apoA-1 IgG and HDL functionality in RTR is intrinsically disease-specific.

Thirdly, this study strengthens previous observations and provides the first insights of the anti-apoA-1 IG architecture in RTR. Indeed, the association between these antibodies with previous MI has been consistently reported across different populations with preserved renal function.[7,8,11,13] Reproducing this association reinforces the notion that a previous acute coronary event is an important acquired factor, that could, together with niacin therapy[9] and genetic determinants,[25] contribute to better understand the reasons underlying the existence of anti-apoA-1 IgG in individuals without overt signs of clinical autoimmunity. In this context, we report for the first time specific associations with primary glomerular disease and tubulo-interstitial disease as primary renal diseases possibly associated to the existence of anti-apoA-1 IgG.

Lastly, the somewhat lower than expected prevalence of anti-apoA-1 IgG seropositivity retrieved presently (11.5%) when compared to maintenance hemodialysis patients and the general population (20%),[8,18] is worth a comment, as we would have expected an increased prevalence as previously reported in all other clinical situations with a high CV risk.[7,8,10,12–14,18] A conceivable explanation for this observation might be that RTRs are under chronic immunosuppressive medication, known to improve features of autoimmunity and thus decrease autoantibody levels. The trend toward a decrease in the prevalence of proliferation inhibitors along the increasing anti-apoA-1 IgG tertiles may lend weight to this hypothesis and warrants further investigations.

Although the results of the present study lend further weight to the growing body of evidence indicating that humoral autoimmunity contributes to CVD, we could not explore the mechanisms by which anti-apoA-1 IgG levels may associate with CVD in RTR. So far, previous animal and in-vitro studies showed that anti-apoA-1 IgG could be active mediators of atherogenesis, inducing myocardial necrosis and death in mice through toll-like receptors (TLR)[2,4] and CD14 heterodimer signaling.[6,7,11,15–17,26] Since these deleterious effects could potentially be amended by immunomodulation therapies, either using a specific apoA-1 mimetic peptide or intravenous immunoglobulins, anti-apoA-1 IgGs have been proposed as emergent therapeutic targets.[11,27] In accordance with these in vitro and animal experiments, a functional CD14 polymorphism was recently shown to be a strong modulator of anti-apoA-1 IgG-related CVD risk prediction in the general population.[12] As CD14 expressing monocytes display higher TLR2 and 4 expression in RTR,[28] knowing whether CD14 and/or TLR2/4 polymorphisms together with the presence of anti-apoA-1 IgG could further improve prognosis assessment in RTR remains to be investigated. Given the important pathophysiological differences between atherogenesis and transplant vasculopathy,[23] knowing whether the aforementioned molecular mechanisms could also explain the increased CV risk ascribed to these antibodies in RTR remains unknown and constitutes an important limitation of the present study. Further, despite the relatively large number of included RTR in this adequately powered study, the number of events was still somewhat low, leading to restricted possibilities with regards to statistical analysis. Since this investigation was also carried out in a single center, further validation of our findings in a larger multicentre cohort appears desirable. In addition, it would be interesting to analyse whether RTR with high anti-apoA-1 IgG titers show a differential response to an intervention with cardiovascular treatment strategies. A further limitation resides in the fact that we did not measure other autoantibodies of possible CV relevance, such as auto-antibodies to β2 glycoprotein I domain I and IV, cardiolipin, heat-shock protein 60, and to phosphorylcholine. Because anti-apoA-1 IgG were shown to display the strongest and independent prognostic accuracy for major adverse cardiovascular events in non-autoimmune settings when compared to the aforementioned auto-antibodies,[29] we focused our work specifically on this class of antibodies. Knowing whether the present association could be reproduced with other auto-antibodies remains to be shown. Also, before utilizing anti-apoA-1 IgGs as clinical biomarker, it would be interesting to screen kidney graft donors to learn, whether intra-individual variability in titers has a potential impact on outcomes after transplantation.

In conclusion, we report anti-apoA-1 IgG as a novel prognostic biomarker for CVD mortality in RTR, independent of traditional CVD risk factors and HDL functionality. These data indicate that anti-apoA-1 IgG holds potential as a clinical biomarker for CVD risk stratification in RTR patients, a high CVD risk population with altered functionality of the immune system. Further investigations to define the potential usefulness of anti-apoA-1 IgG assessments in clinical decision making are required, as well as studies to delineate the intrinsic pathophysiological

pathways that these antibodies activate to subsequently result in an increased CVD risk in RTR.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found at www.mdpi.com/xxx/s1.

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THE TRIGLYCERIDE TO HDL-CHOLESTEROL RATIO AND CHRONIC GRAFT FAILURE IN RENAL TRANSPLANTATION

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Journal of Clinical Lipidology, 2021

ABSTRACT

BACKGROUND

Transplant vasculopathy (TV) is a major contributing factor to chronic graft failure in renal transplant recipients (RTR). TV lesions resemble atherosclerosis in several ways, and it is plausible to believe that some risk factors influence both atherosclerotic plaque formation and formation of TV.

OBJECTIVE

The objective of this prospective longitudinal study was to determine if dyslipidemia reflected by the triglyceride (TG)/high-density lipoprotein cholesterol (HDL-C) ratio is prospectively associated with death censored chronic graft failure in RTR.

METHOD

454 prospectively included RTR with a functioning graft for at least one year, were followed for a median of 7 years. RTR were matched based on propensity scores to avoid potential confounding and subsequently the association of the TG/HDL-C ratio with the endpoint chronic graft failure, defined as return to dialysis or re-transplantation, was investigated.

RESULTS

Linear regression analysis showed that concentration of insulin, male gender, BMI and number of antihypertensives predict the TG/HDL-C ratio. Cox regression showed that the TG/HDL-C ratio is associated with chronic graft failure (HR= 1.43, 95%CI = 1.12-1.84, p = 0.005) in competing risk analysis for mortality. Interaction testing indicated that the relationship of the TG/HDL-C ratio with graft failure is stronger in subjects with a higher insulin concentration.

CONCLUSION

Our results demonstrate that the TG/HDL-C ratio has the potential to act as a predictive clinical biomarker. Furthermore, there is a need for closer attention to lipid management in RTR in clinical practice with a focus on triglyceride metabolism.

KEYWORDS

triglyceride/HDL-C ratio, transplantation, dyslipidemia, chronic graft failure, HDL, triglycerides, kidney

INTRODUCTION

Renal transplantation is the gold standard treatment for end-stage renal disease (ESRD). In many countries the number of renal transplant recipients (RTR) is by now even surpassing that of haemodialysis patients.[1] Availability of donor kidneys is sparse, and patients often spend years on waiting lists, or depend on a sacrifice by a family member or friend in the form of a living donation. Therefore, protection of donor kidneys and improving long-term graft survival is a major clinical, as well as ethical, necessity. However, although short-term graft survival is steadily improving, chronic graft failure still represents an important clinical challenge, eventually resulting in return to haemodialysis or re-transplantation.[2]

In addition to the risk of graft failure, renal transplant recipients also face an increased risk of atherosclerotic and other cardiovascular disease (CVD). Kidney disease is associated with an increased risk of CVD throughout the spectrum of decreased kidney function, with early stages of CKD translating to a 4 fold increased risk of CVD events, whereas in end-stage renal disease this rises to a 30 fold increased risk.[3,4] Even after transplantation the risk remains 4-6 times higher compared to the general population.[5]

Transplant vasculopathy (TV) is acknowledged as a major contributing factor to chronic graft failure. Interestingly, TV lesions resemble atherosclerosis in several ways, and factors influencing the development of atherosclerosis also have been implicated in TV.[6] Through various factors relating to the transplantation RTR frequently display dyslipidemia. The prevalence of dyslipidemia in RTR is estimated to be around 80%,[7,8] with mean reported triglyceride levels values ranging from 160 to 200 mg/dL (1.8 to 2.26 mmol/L).[11–139] It has been shown that raised triglyceride (TG) levels, but not hypercholesterolemia, are associated with chronic graft failure.[10,11] TG levels however fluctuate substantially based on feeding status, limiting its utility as a predictive biomarker.[12,13] Combining TG levels with high-density lipoprotein-cholesterol (HDL-C) levels leads to a far more consistent measure of dyslipidemia and could therefore overcome this problem. And indeed, recent studies established that the combination of TG and HDL-C in form of a ratio has a greater predictive value for CVD events.[14,15] Conceivably, a high TG/HDL-C ratio could also reflect accelerated TV lesion formation. However, the possible impact of the TG/HDL-C ratio on chronic graft failure in RTR has not been investigated to date. In the present work we therefore aim to determine the association of the TG/HDL-C ratio with incident chronic graft failure in a well-characterised prospective cohort of RTR.

MATERIALS AND METHODS

STUDY POPULATION

In this study all RTR with a functioning graft for at least 1 year, who visited the University Medical Center Groningen (UMCG) between 2001 and 2003, were invited to join. Patients were excluded from the study if they had congestive heart failure or cancer, other than cured skin cancer, as well as endocrine abnormalities other than diabetes mellitus (DM). Patients were followed over a median of 7 years (interquartile range [IQR] 6.1 - 7.5 years), and there was no loss during follow-up. Of the 847 eligible patients, 624 patients gave written informed consent (figure 1). Of these patients, 170 were excluded due to a suspected infection, indicated by a high sensitivity C-reactive protein (hsCRP) value of above 10 mg/l at the time of blood sampling. The included 454 patients did not differ from the entire cohort with regards to baseline characteristics and are therefore a valid representation of the whole. None of the included patients received triglyceride lowering treatment. A more complete description of the study design and the obtained measurements has been published previously.[16] The study has been approved by the local Medical Ethics Committee (METc2001/039) and is in accordance with the Declaration of Helsinki. The TxL-IRI Biobank and Cohort Study is registered at ClinicalTrials.gov with identifier NCT03272854.

MEASUREMENTS AND DEFINITIONS

Metabolic syndrome was defined based on the criteria of the National Cholesterol Education Program Expert Panel.[17] In 2008 the American Diabetes Association (ADA) suggested to use a lowered cut-off value for impaired fasting glucose at 5.6 mmol/l. This adaptation was used in our definition. Diabetes was defined as a fasting plasma glucose of 7.0 mmol/l or use of antidiabetic medication, in accordance with the ADA guidelines.[18] Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in meters squared.

Blood samples were drawn after a 8-12h fasting period and routine laboratory measurements were conducted, as previously described.[16] Total cholesterol was determined using the cholesterol oxidase-phenol aminophenazone method (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). LDL-cholesterol was calculated using the Friedewald equation.[19] HDL-cholesterol was measured with the cholesterol oxidase-phenol aminophenazone method on a Technikon RA-1000 (Bayer Diagnostics, Mijdrecht, The Netherlands). Plasma triglycerides were determined with the glycerol-3-phosphate oxidase-phenol aminophenazone method (Roche Diagnostics). ApoB levels were determined by nephelometry using commercially available reagents from Dade Behring (BN II; Dade Behring, Marburg, Germany). Plasma hsCRP was assessed by ELISA.[16] The glucose-oxidase method was used to determine plasma glucose levels. Plasma insulin was measured using an AxSym autoanalyzer. HbA1c was assessed by high-performance liquid chromatography. Insulin resistance was calculated using the homeostasis model assessment-estimated insulin resistance (HOMA-

IR) as follows: HOMA-IR=glucose (mmol/l) x insulin (µU/ml)/22.5.

END POINTS AND OUTCOME MEASURES

The main predictor in this study was the TG/HDL-C ratio, which was computed by dividing the triglyceride concentration by the HDL-C concentration (both in mmol/l). The primary end point is graft failure, which is defined as return to dialysis therapy or re-transplantation.

STUDY DESIGN

In order to reduce potential confounding we used propensity score matching to compare the incidence of death censored graft failure between subjects of high and low TG/HDL-C ratio values. Since there is no validated cut-off for the TG/HDL-C ratio, RTRs were dichotomized into high versus low TG/HDL-C ratio by dividing the group at the median (1.27). A logistic regression was fitted for high versus low TG/HDL-C ratio, including variables that, based on literature, are related to the outcome. This included patient demographics (age and sex), lifestyle factors (BMI), renal disease history (primary renal disease, dialysis time), transplantation demographics (type of transplantation, number of human leukocyte antigen [HLA] mismatches, acute rejection), medication use (use of calcineurin inhibitors, use of proliferation inhibitors, prednisolone dose, number of anti-hypertensives), lipid factors (use of statins, total cholesterol), renal function (estimated glomerular filtration rate [eGFR], urinary protein excretion) and co-morbidities (diabetes, HbA1c, insulin concentration).[20] Propensity scores were obtained from the outcome of the logistic regression.

Subjects with high versus low TG/HDL-C ratios were matched by one to one nearest-neighbor matching with replacement based on propensity scores, meaning that a control subject could be used in multiple case-control pairs, allowing for more optimal matching.[21] Quality of matching was graphically evaluated (supplemental figure) and the reduction of bias assessed using a t-test for equality of means, the standardized percentage bias and the variance ratio (supplemental table 1).

STATISTICAL ANALYSIS

Differences in baseline characteristics were tested between groups of high versus low TG/HDL-C ratio in the propensity matched cohort. Due to matching with replacement and categorizing the low TG/HDL-C group as control group, there were fewer subjects in the low TG/HDL-C ratio category in the propensity matched cohort. Categorical values are given as absolute numbers (percentages) and differences were tested by the chi-squared test. Normally distributed continuous variables are given as mean ± standard deviation and differences were tested by one-way analysis of variance (ANOVA). Skewed continuous variables are presented as median [25th to 75th percentile] and differences between groups were determined by Kruskal-Wallis test.

In order to identify variables independently associated with the TG/HDL-C ratio all characteristics with a P<0.10 between high versus low TG/HDL-C ratio in the entire, unmatched cohort at baseline were entered into a step-wise multivariable linear regression model with backward elimination (P<0.05). This included urinary protein excretion, eGFR, daily prednisone dose, hsCRP, use of antidiabetics, HbA1c, insulin concentration, glucose concentration, use of diuretics, use of beta blockers, use of ace inhibitors, number of antihypertensives, use of statins, total cholesterol, concentration of apolipoprotein B (apoB), BMI and sex.

Cumulative incidence curves with competing risk for mortality were computed in order to assess the association of the TG/HDL-C ratio with graft survival. The association of the TG/HDL-C ratio levels with graft failure was evaluated using Cox proportional hazards regression. Competing-risk regression for mortality using the Fine and Gray model was performed.[22] Cox proportional hazards regression was performed in the propensity matched cohort, using weighted estimations based on the frequency with which a single observation was used as a match. Cumulative hazards were computed for the endpoint. Analyses were performed both crude, as well as with further adjustment for covariates for which balance was not achieved with matching, as indicated by significant differences between groups, namely presence of the metabolic syndrome.

Furthermore, subgroup analysis using interaction tests were performed in which HR were determined across categories of baseline characteristics. For continuous variables the median value was used as cut-off. To assess the functional relationship of the TG/HDL-C ratio with graft failure we used a functional polynomial Cox regression model. The proportional hazards assumption was tested using log-log graphs, and was found not to be violated.

Since acute inflammation impacts lipid metabolism,[23] we performed a sensitivity analysis where we excluded all patients with a hsCRP above 5mg/l. Furthermore, we also assessed the association of the TG/HDL-C ratio with graft failure with traditional survival analysis, not taking into account propensity score matching.

A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using STATA® Statistical Software, Release 15.1 (StataCorp, College Station, TX).

RESULTS

BASELINE DEMOGRAPHIC CHARACTERISTICS

A total of 624 subjects from the "TransplantLines Insulin Resistance and Inflammation Biobank and Cohort Study" were assessed for eligibility (figure 1). After exclusion due to suspected acute infection, as determined by a hsCRP >10 mg/l, 454 subjects were eligible for inclusion in the cohort. The matching procedure matched 57 subjects with a low TG/HDL-C ratio to 153 subjects with a high TG/HDL-C ratio. Due to matching with replacement, this means that 153 case-control pairs were matched. Standardized percentage bias and the variance ratio are shown in supplemental table 1.

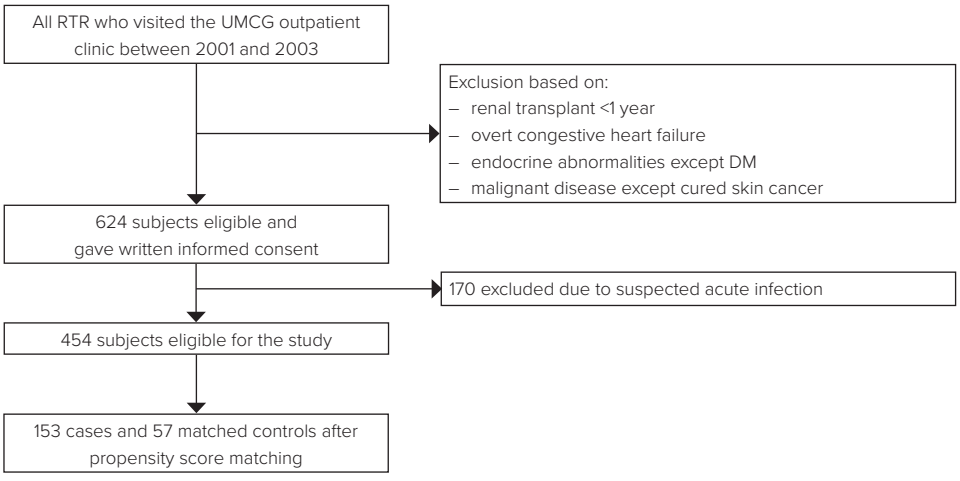

Figure 1. Inclusion of renal transplant recipients
RTR, renal transplant recipients; UMCG, University Medical Center Groningen; DM, diabetes mellitus

Baseline characteristics for subjects with low TG/HDL-C ratio versus high TG/HDL-C ratio are summarized in table 1. Good balance was achieved with matching, leading to few differences between groups. In sensitivity analysis the analysis was repeated in subjects with a hsCRP of below 5mg/l. Results were largely comparable to using the 10 mg/l cut-off, however, significantly more subjects used diuretics in the high TG/HDL-C group (32% versus 50%, p=0.029, supplementary table 2).

Backward multiple linear regression analysis was used to assess which variables are determinants of the TG/HDL-C ratio in renal transplant recipients. Concentration of insulin, male sex, BMI and number of antihypertensives were positively associated with the TG/HDL-C ratio, whereas concentration of apoB was inversely associated (table 2). Model R2 was 0.16.

Table 1. Baseline characteristics according to low and high levels of the triglyceride/HDL-cholesterol ratio in the propensity matched cohort

Characteristics	Low TG/HDL-C ratio (n= 57)	High TG/HDL-C ratio (n=153)	P value
Triglyceride/ HDL-C ratio	1.3 [0.9, 1.5]	2.6 [2.1, 3.3]	<0.001
Recipient demographics			
Age, years	52.4 (12.4)	52.4 (11.3)	0.98
Male gender, *n* (%)	23 (40.4%)	73 (47.7%)	0.34
Current smoking, *n* (%)	9 (15.8%)	27 (17.6%)	0.75
Previous smoking, *n* (%)	27 (47.4%)	59 (38.6%)	0.25
Waist circumference, cm	97 (10.3)	99 (13.0)	0.26
Body composition			
BMI, kg/m2	25.9 (3.5)	26.7 (4.3)	0.19
Lipids			
Total cholesterol, mmol/l	5.4 [4.9, 6.0]	5.6 [4.9, 6.2]	0.53
LDL-cholesterol, mmol/l	3.6 [3.1, 4.0]	3.5 [2.9, 4.1]	0.28
HDL-cholesterol, mmol/l	1.2 [1.1, 1.4]	0.9 [0.8, 1.1]	<0.001
Triglycerides, mmol/l	1.5 [1.1, 1.9]	2.4 [2.0, 3.0]	<0.001
Apolipoprotein B, g/l	1.0 (0.2)	1.2 (0.2)	0.06
Use of statins, *n* (%)	34 (59.6%)	87 (56.9%)	0.72
Cardiovascular disease history			
History of MI, *n* (%)	2 (3.5%)	14 (9.2%)	0.17
History of TIA/CVA, *n* (%)	5 (8.8%)	10 (6.6%)	0.58
Blood pressure			
Systolic blood pressure, mmHg	153.8 (23.0)	153.5 (23.0)	0.98
Diastolic blood pressure, mmHg	90.0 (9.5)	90.3 (10.1)	0.59
Use of ACE inhibitors, *n* (%)	2 (3.5%)	14 (9.2%)	0.17
Use of β-blockers, *n* (%)	5 (8.8%)	10 (6.6%)	0.58
Use of diuretics, *n* (%)	2 (3.5%)	14 (9.2%)	0.17
Number of antihypertensive drugs, *n*	5 (8.8%)	10 (6.6%)	0.58
Glucose homeostasis			
Glucose, mmol/l	4.7 [4.3, 5.2]	4.6 [4.2, 5.1]	0.38
Insulin, μmol/l	11.9 [8.9, 16.5]	11.7 [9.0, 15.3]	0.94
HbA1c, %	6.4 [5.9, 6.8]	6.3 [5.8, 7.0]	0.67
HOMA-IR	2.7 [1.8, 3.4]	2.4 [1.8, 3.6]	0.64
Use of anti-diabetic drugs, *n* (%)	7 (12.3%)	19 (12.4%)	0.98
Inflammation			
hsCRP, mg/l	1.5 [0.7, 3.4]	1.8 [0.9, 3.7]	0.23
Donor demographics			
Age, years	35.0 [24.0, 51.0)	40.0 [23.0, 52.0)	0.64
Male gender, *n* (%)	34 (59.6%)	88 (58.3%)	0.86
Living kidney donor, *n* (%)	7 (12.3%)	16 (10.5%)	0.71
(Pre)transplant history			
Dialysis time, months	35.0 [16.0, 48.0]	29.0 [13.0, 48.0]	0.41
HLA mismatch	1.0 [0.0, 2.0]	1.0 [0.0, 2.0]	0.91
Acute rejection, *n* (%)	33 (%)	90 (%)	0.90
Graft age, years	4.7 [2.7, 10.4]	5.7 [3.2, 10.7]	0.52
Primary renal disease			
Primary glomerular disease			
Glomerulonephritis	6 (10.5%)	9 (5.9%)	0.25
Tubulo-interstitial disease	11 (19.3%)	19 (12.4%)	0.21
Polycystic renal disease	10 (17.5%)	32 (20.9%)	0.59
Dysplasia and hypoplasia	0 (0.0%)	7 (4.6%)	0.10
Renovascular disease	3 (5.3%)	10 (6.5%)	0.73
Diabetic nephropathy	1 (1.8%)	1 (0.7%)	0.47
Other or unknown cause	9 (15.8%)	36 (23.5%)	0.22

Table 1 continued.

Characteristics	Low TG/HDL-C ratio (n= 57)	High TG/HDL-C ratio (n=153)	P value
Immunosuppressive medication			
Daily prednisolone dose, mg/dl	10 [9, 10]	10 [9, 10]	0.89
Calcineurin inhibitors, *n* (%)	43 (75.4%)	127 (83.0%)	0.21
Proliferation inhibitors, *n* (%)	44 (77.2%)	112 (73.2%)	0.56
Renal allograft function			
eGFR, ml/min	50.2 (16.3)	46.4 (16.8)	0.15
Proteinuria ≥ 0.5 g/24 h, *n* (%)	16 (28.1%)	37 (24.2%)	0.56

Normally distributed continuous variables are presented as mean (SD), and differences were tested with one-way analysis of variance (ANOVA). Continuous variables with a skewed distribution are presented as median [25th, 75th percentile], and differences were tested by Kruskal–Wallis. Categorical data are summarized as n (%), and differences were tested by chi-squared test. To calculate cholesterol in mg/dL, multiply by 38.7. To calculate triglyceride in mg/dL, multiply by 88.6. Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; MI, myocardial infarct; TIA, transient ischemic attack; CVA, cerebrovascular event; HOMA-IR, homeostasis model assessment-estimated insulin resistance; hsCRP, high sensitivity C-reactive protein; HLA, human leukocyte antigens; eGFR, estimated glomerular filtration rate.

Table 2. Predictors of triglyceride/HDL-C ratio

	β	95% CI	Standardized β	*p*
Concentration of apoB	1.52	0.80, 2.23	0.27	<0.001
Patient gender	0.75	0.39, 1.12	0.18	<0.001
Number of anti-hypertensives	0.29	0.14, 0.45	0.17	<0.001
BMI	0.07	0.02, 0.11	0.13	0.007
Concentration of insulin	0.06	0.03, 0.08	0.21	<0.001

All variables with p>0.1 between high versus low triglyceride/HDL-C ratio at baseline in the whole cohort before propensity score matching were entered into a stepwise linear regression with backward elimination. Abbreviations: apoB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; CI, confidence interval; BMI, body mass index.

TIME TO EVENT ANALYSIS

The endpoint was reached in 21 of the included RTR in the propensity matched cohort, of which 5 were in the low TG/HDL-C ratio group and 16 in the high TG/HDL-C group. Graft failure occurred due to chronic allograft nephropathy in 11 subjects (52%), chronic allograft dysfunction in 8 subjects (38%) and return of primary disease in 2 subjects (10%). A cumulative incidence curve demonstrated that a lower TG/HDL-C ratio is associated with improved graft survival. In a crude Cox regression analysis with competing risk for mortality the TG/HDL-C ratio levels were significantly associated with graft failure (HR= 1.43, 95%CI = 1.12-1.84, p = 0.005). In order to avoid residual confounding, we adjusted for variables that remained significantly different after matching, namely metabolic syndrome, which did not considerably impact the association (HR= 1.51, 95%CI =1.17 – 1.94, p =0.002, competing risk model). In sensitivity analysis we repeated the Cox regression for subjects with a hsCRP of below 5mg/l. Results did not differ considerably from those reached with handling the 10 mg/l cut-off (crude HR=1.58, 95%CI = 1.21 – 2.07, p = 0.001, competing risk model). Furthermore, optimal balance was not achieved with regards to use of diuretics, as indicated by a significant difference between groups (supplementary table 2). Therefore,

additional adjustment was performed for use of diuretics, which did not considerably alter the association (HR=1.46, 95%CI = 1.15 – 1.85, p = 0.002, competing risk model). Fractional polynomial regression showed that a TG/HDL-C ratio of under 2.2 (in mmol/L, or 5.0 in mg/dL) is inversely associated with graft failure, where after any rise in the TG/HDL-C ratio is associated with an increased risk of graft failure (figure 3).

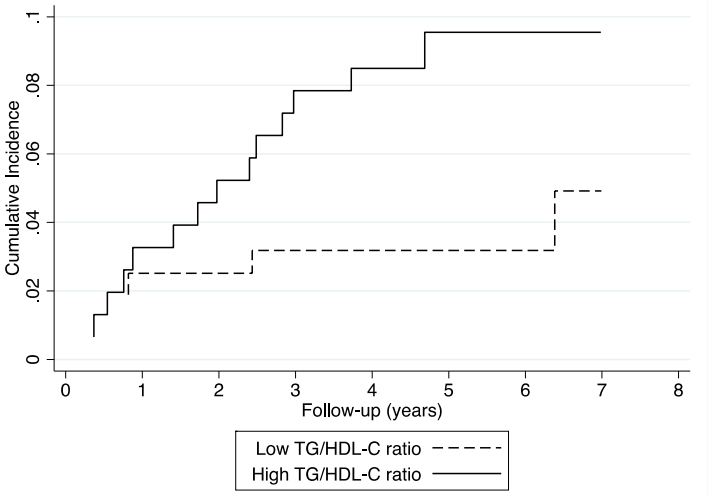

Figure 2. A lower triglyceride/HDL-C ratio is associated with an increased graft survival.
Cumulative incidence curves of the association of a high versus low TG/HDL-C ratio with chronic graft failure. Abbreviations: TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

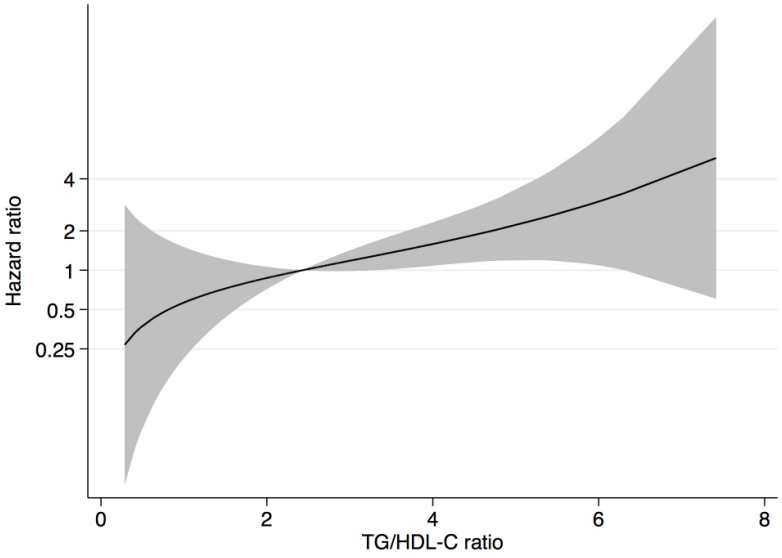

Figure 3. A higher triglyceride/HDL-C ratio is associated with an increased incidence of graft failure.
Hazard ratios (95% confidence interval) obtained by Cox regression of fractional polynomials.

In sensitivity analyses the association of the TG/HDL-C ratio with graft failure was assessed using Cox proportional hazard regression in the overall cohort not matched based on propensity scores. This confirmed that the TG/HDL-C ratio is associated with graft failure in a continuous scale in a crude model (HR per unit change=1.10, 95%CI=1.03-1.17, p=0.003). This association was not significantly impacted by subsequent adjustment for potential confounders (supplementary table 3).

The association of the TG/HDL-C with graft failure was different for subjects with a low versus low insulin concentration (p for interaction = 0.019, figure 4), showing that the relationship of the TG/HDL-C with graft failure is stronger in subjects with a higher insulin concentration. A number of other participant characteristics did not have a significant impact (Figure 4).

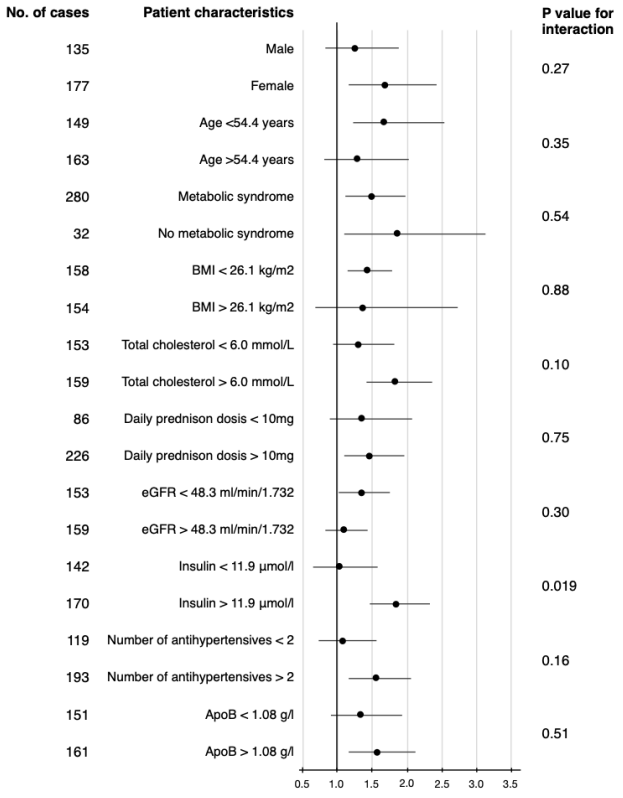

Figure 4. Hazard ratios of TG/HDL-C ratio for incident graft failure, by several participant level characteristics

Data are hazard ratios (95% confidence interval) for incident graft failure obtained with Cox regression with competing risk for mortality. Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; ApoB, apolipoprotein B.

DISCUSSION

The results of this study demonstrate that a higher TG/HDL-C ratio is associated with a significant increase in the incidence of chronic graft failure, independent of a large number of other risk factors. These data stress the importance of good lipid control in clinical practice, particularly of triglycerides.

RTR are a complex patient group, frequently presenting with an altered lipid profile. Several factors contribute to this dyslipidaemia. Immunosuppressive medication, in particular calcineurin inhibitors and mTOR inhibitors, as well as steroids cause both hypercholesterolemia and raised triglycerides.[24–26] Also, a large number of RTR have underlying diabetes, a frequent cause of ESRD leading to transplantation. Furthermore, RTR face a high incidence of new onset diabetes after transplantation. Insulin resistance and diabetes are well characterized pathophysiological states associated with high serum TG and low HDL-C.[27] Finally, RTR generally have a reduced kidney function, and consequently suffer from an uremic proinflammatory state, conceivably also contributing to raised TG levels.[28]

The disappointing lack of long-term improvement of graft survival stresses the clinical need to further assess possible mechanisms and predictors. TV, the formation of atherosclerosis-like lesions in the kidney graft,[29] is an important limitation in long-term graft survival. Indeed, within 5 years 50% of RTR will have significant TV lesions, and within 10 years 90% of patients are affected.[6] Numerous studies have shown a causal association between triglyceride levels and cardiovascular events.[30,31] Very low density and remnant lipoproteins rich in TG penetrate the arterial intima and can be bound and retained by the connective tissue matrix, thus contributing to the development and progression of atherosclerotic plaques.[32] Furthermore, postprandial TG have been linked to impaired vasodilation, upregulated pro-inflammatory cytokine production, increased inflammatory response and monocyte activation.[33–36]

Considering that the pathology of TV resembles that of atherosclerosis it is plausible to believe that TG also effect chronic graft failure. However, the clinical utility of TG measures is limited by the fact that plasma levels are highly dependent on feeding status. TG levels are inversely correlated to HDL-C and it has been suggested that HDL-C acts as a stable marker of average TG levels and can therefore be used to monitor long term TG changes.[37] Combining TG and HDL-C in a ratio therefore more accurately reflects dyslipidemia and allows for more stable, fasting independent measures. Based on our data and studies in the general population, it is plausible that the dyslipidemia reflected by the TG/HDL-C ratio contributes to the pathogenesis of TV. It is unknown whether TV is reversible, which is supposedly the case in atherosclerotic lesions.[29,38] It is therefore conceivable that good

lipid management at least limits the progression, but might also be able to reverse existing TV lesions, thereby decreasing the incidence of chronic graft failure.

Apart from de novo atherosclerosis in the kidney graft in the form of TV dyslipidemia reflected by the TG/HDL-C ratio might also impact chronic graft failure through immunomodulatory processes. TG rich lipoproteins, as well as their remnants, are associated with inflammation. A 1 mmol/l increase in non-fasting remnant cholesterol has been shown to translate into a 37% higher CRP level.[39] HDL-C on the other hand has well-documented anti-inflammatory capacities.[40,41] A higher TG and a lower HDL-C level, as reflected by an increased TG/HDL-C ratio, therefore potentially contributes to inflammation, an established risk factor for graft loss.[42–44]

We suggest that lipid levels are routinely monitored in RTR, including TG and HDL-C levels. In case of a high TG/HDL-C ratio, lifestyle changes are first warranted. The focus should be placed on elimination of sucrose- or fructose-sweetened beverages, avoidance of excessive and sometimes even moderate alcohol, limitation of refined carbohydrates,[45] weight loss[46,47] and aerobic exercise.[48] Whereas fibrates have been shown to be safe and effective in lowering risk of coronary events in the general population,[49] insufficient information is available about the safety in RTR. Studies with a low level of evidence indicated that initiation of fibrate treatment led to higher serum urea in RTR.[50,51] Omega 3 fatty acids however have a potent TG lowering effect and have been shown to be safe and effective in RTR.[52,53] Triglyceride lowering in the general population is an active field of study, reflected by numerous ongoing phase 3 trials of new emerging therapies.[45] Apolipoprotein C-III is an interesting potential target, with promising results in phase 2 clinical trials using antisense oligonucleotides showing an 80% reduction in TG levels.[54] However, similar to other antisense oligonucleotide therapies some adverse effects were documented in the treatment group, namely decreased platelet counts and injection-site reactions.[55] Pharmacological lowering of angiopoietin-like protein 3 is another emerging treatment modality, with TG reductions of 75% in a single-group, open-label study of homozygous familial hypercholesterolemia patients.[56] We would like to stress that statin therapy, although effective to lower LDL-C, has insufficient effects on either TG or HDL-C levels and is thus not a valid treatment option for hypertriglyceridemia.[57] More research is warranted to evaluate the optimal TG lowering treatment in RTR, as well as establishing a definite clinical cut-off value.

Some limitations warrant consideration. The study was conducted in a single center and all included RTR shared the same ethnicity. Despite the reasonable number of included RTR in this adequately powered study, the number of events was somewhat limited, leading to restricted possibilities with regards to statistical analysis. In particular, we cannot separate effects associated with TG/HDL-C ratio from those of closely related variables such as

insulin resistance. Furthermore, single measures of lipids were taken, therefore we can not comment on the biological variability of lipid values over time.

In conclusion, our study shows that the TG/HDL-C ratio has potential to be utilized as a simple and valuable tool to predict chronic graft failure in RTR, a field in which limited improvement has been made in the last decade. The results of the present work demonstrate a need for closer attention to lipid management in clinical practice. Due to the low costs and broad availability of the measurements, using the TG/HDL-C ratio in daily clinical practice is realistic and potentially very valuable. Further research is required, with subsequent re-evaluation of existing guidelines in order to improve care for the vulnerable patient group of RTR.

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4b

TRIGLYCERIDE/HDL-CHOLESTEROL RATIO AND PREMATURE ALL-CAUSE MORTALITY IN RENAL TRANSPLANT RECIPIENTS

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Nephrology Dialysis Transplantation, 2020

Numbers of renal transplant recipients (RTR) are constantly increasing, already surpassing in several countries those of patients receiving hemodialysis treatment.[1] However, RTR still suffer an exceptionally high, but poorly understood, mortality burden.[2] This is partly attributable to a declining allograft function, and partly to dyslipidemia, including decreased high density lipoprotein cholesterol (HDL-C), and increased triglycerides.[3] Frequently, HDL-C is low when triglycerides are high, a relationship particularly well reflected in the TG/HDL-C ratio.[4] As the only existing guideline which addresses lipid treatment in RTR, KDIGO solely advises lifestyle changes to combat raised triglyceride levels.[5] In the general population, the TG/HDL-C ratio constitutes a useful predictor of mortality, whereas interestingly in hemodialysis patients a protective effect is seen.[6,7] Comparable data from RTR cohorts are not available. Therefore, we aimed to determine, if the TG/HDL-C ratio associates prospectively with mortality risk in RTR.

The detailed design of this longitudinal cohort study has been published.[8] The study was approved by the local ethics committee (METc 2001/039). Briefly, all RTR visiting the UMCG outpatient clinic between August 2001 and July 2003 with a functioning allograft for at least one year were eligible. Exclusion criteria were congestive heart failure, malignant disease, endocrine abnormalities other than diabetes and evidence of acute inflammation at time of inclusion (hsCRP values >20 mg/l). None of the RTR received lipid modulating therapy other than statins. Of 847 invited patients, 606 gave informed written consent; a full dataset was available for the current analysis in 495 RTR (median follow-up 7 years, comparable in their baseline characteristics with the entire group of eligible patients). The main outcome measure was the TG/HDL-C ratio determined in the fasting state, the primary end was point all-cause mortality. Statistical analyses were performed using SPSS 24.0. A P-value of <0.05 was considered statistically significant.

The 495 included RTR (table) were followed for a median of 7 years. During follow-up 102 patients died. Patients were divided into gender-stratified tertiles based on the TG/HDL-C ratio (table). Patients with higher TG/HDL-C ratios had higher BMI, more dyslipidemia, used more statins and anti-hypertensive drugs, were more insulin resistant/diabetic, had a higher inflammatory load and worse graft function. Since immunosuppressive medications can have a considerable impact on dyslipidemia [9], we assessed the correlation between different immunosuppressants and the TG/HDL-C ratio. The use of cyclosporine was positively correlated with the TG/HDL-C ratio (r = 0.11, P=0.021), whereas use of tacrolimus showed a negative correlation with the TG/HDL-C ratio (r = -0.11, P=0.024). No correlation was seen with daily prednisolone doses (r = 0.08, P=0.088) or use of proliferation inhibitors (r = -0.02, P=0.60). Cox proportional hazard analyses demonstrated a strong significant association between the TG/HDL-C ratio and all-cause mortality in an age- and gender-adjusted model (HR=1.49 [95%CI=1.14-1.95], P=0.004). Therefore, patients in the third tertile, with a TG/HDL-C ratio between 2.7 and 4.4 mmol/l, have a 49% higher risk of mortality

than those in the first tertile. This association remained significant when further adjusted for HOMA-IR, insulin concentration and HbA1c (HR=1.48 [95%CI=1.11-1.97], P=0.008), use of statins (HR=1.48 [95%CI=1.12-1.95], P=0.006), calcineurin inhibitors (HR=1.49 [95%CI=1.14-1.96], P=0.004), total cholesterol (HR=1.49 [95%CI=1.14-1.95], P=0.004), eGFR and proteinuria (HR=1.32 [95%CI=1.03-1.69], P=0.03), hs-CRP (HR=1.48 [95%CI=1.13-1.94], P=0.005) and non-HDL-C (HR=1.49 [95%CI=1.12-1.97], P=0.005). ROC analysis identified 1.8 as best discriminating cut-off value for the TG/HDL ratio, which corresponds to the second tertile in this study.

Table. Baseline characteristics according to gender stratified tertiles of triglyceride/HDL cholesterol ratio

Characteristic	Gender stratified tertiles of triglyceride/HDL cholesterol ratio			P value
	First (n=166)	Second (n=164)	Third (n=165)	
Triglyceride/ HDL-C ratio	1.0 [0.7-1.1]	1.7 [1.5-2.1]	3.3 [2.7-4.4]	<0.001
Recipient demographics				
Age, years	51.9 [43.4-60.0]	54.2 [43.6-61.4]	51.3 [43.4-59.5]	0.88
Male gender, *n* (%)	90 (54)	90 (55)	89 (54)	1.00
Current smoking, *n* (%)	39 (24)	29 (18)	37 (22)	0.39
Metabolic syndrome, *n* (%)	83 (57)	94 (65)	84 (60)	0.34
Body composition				
BMI, kg/m2	24.7 ± 3.8	25.8 ± 4.3	27.3 ± 4.2	<0.001
Lipids				
Total cholesterol, mmol/l	5.5 ± 0.9	5.5 ± 1.0	5.9 ± 1.3	0.008
LDL cholesterol, mmol/l	3.6 ± 0.8	3.6 ± 0.9	3.5 ± 1.2	0.55
HDL cholesterol, mmol/l	1.4 ± 0.31	1.1 ± 0.24	0.9 ± 0.21	<0.001
Triglycerides, mmol/l	1.2 [0.9-1.5]	1.9 [1.6-2.2]	2.9 [2.5-3.5]	<0.001
Use of statins, *n* (%)	70 (42)	88 (54)	94 (57)	0.018
Cardiovascular disease history				
History of MI, *n* (%)	12 (7)	15 (9)	15 (9)	0.79
TIA/CVA, *n* (%)	6 (4)	10 (6)	9 (5)	0.58
Blood pressure				
Systolic blood pressure, mmHg	150.2 ± 22.7	153.1 ± 23.7	154.7 ± 22.4	0.20
Number of antihypertensive drugs, *n*	2 [1-2]	2 [1-3]	2 [2-3]	<0.001
Glucose homeostasis				
Glucose, mmol/l	4.4 [4.0-4.8]	4.6 [4.0-5.0]	4.7 [4.2-5.5]	<0.001
Insulin, μmol/l	9.5 [6.4-13.0]	10.5 [7.8-14.5]	12.7 [9.6-18.9]	<0.001
HbA1c, %	6.2 [5.7-6.7]	6.4 [5.9-7.1]	6.5 [5.9-7.4]	0.002
HOMA-IR	1.9 [1.2-2.8]	2.1 [1.5-3.1]	2.7 [1.9-4.3]	<0.001
Post-Tx diabetes mellitus, *n* (%)	22 (13)	26 (16)	41 (25)	0.016
Use of anti-diabetic drugs, *n* (%)	17 (10)	23 (14)	28 (17)	0.20
Use of insulin, *n* (%)	9 (5)	11 (7)	12 (7)	0.78
Inflammation				
hsCRP, mg/l	1.5 [0.7-3.4]	2.1 [0.7-4.9]	2.1 [1.1-5.2]	0.005
Donor demographics				
Age, years	34.0 [22.0-50.0]	39.5 [24.0-51.8]	40 [23.5-50.0]	0.23
Male gender, *n* (%)	91 (55)	97 (59)	68 (53)	0.49
Living kidney donor, *n* (%)	24 (15)	19 (12)	20 (12)	0.71
(Pre)transplant history				
Dialysis time, months	24.5 [12.0-46.0]	29.5 [13.0-50.0]	29.0 [14.0-49.0]	0.16
HLA mismatch	2.0 [0-3.0]	2.0 [1.0-2.8]	2.0 [1.0-3.0]	0.65

Table continued.

Characteristic	Gender stratified tertiles of triglyceride/HDL cholesterol ratio			P value
	First (n=166)	Second (n=164)	Third (n=165)	
Primary renal disease				
Primary glomerular disease	41 (25)	47 (29)	46 (28)	0.69
Glomerulonephritis	14 (8)	11 (7)	7 (4)	0.30
Tubulo-interstitial disease	34 (21)	17 (10)	29 (18)	0.04
Polycystic renal disease	22 (13)	30 (18)	35 (21)	0.16
Dysplasia and hypoplasia	6 (4)	4 (2)	8 (5)	0.51
Renovascular disease	11 (7)	10 (6)	10 (6)	0.97
Diabetic nephropathy	11 (7)	3 (2)	3 (2)	0.02
Other or unknown cause	27 (16)	42 (26)	27 (16)	0.05
Immunosuppressive medication				
Daily prednisolone dose, mg/dl	10.0 [7.5-10.0]	10.0 [8.8-10.0]	10.0 [8.1-10.0]	0.17
Calcineurin inhibitors, *n* (%)	127 (77)	130 (79)	134 (81)	0.57
Proliferation inhibitors, *n* (%)	123 (74)	124 (76)	120 (73)	0.84
Renal allograft function				
eGFR, ml/min	51.9 ± 14.0	45.7 ±15.6	44.3 ±16.8	<0.001
Urinary protein excretion, g/24 h	0.1 [0.0-0.2]	0.1 [0.0-0.3]	0.1 [0.1-0.4]	0.011

Normally distributed continuous variables are presented as mean±SD, and differences were tested with one-way analysis of variance followed by Bonferroni post hoc test. Continuous variables with a skewed distribution are presented as median [25th to 75th percentile], and differences were tested by Kruskal–Wallis test followed by Mann–Whitney U test. Categorical data are summarized as n (%), and differences were tested by chi-squared test. TIA, transient ischemic attack; CVA, cerebrovascular event; Tx, transplantation; MI, myocardial infarct; HOMA-IR, homeostasis model assessment-estimated insulin resistance; HLA, human leukocyte antigens.

Our results establish the TG/HDL-C ratio in RTR as a relatively simple, cost-efficient, routinely available lipid biomarker with a substantial clinical impact. Thereby, this study identifies an unmet clinical need, since TG modulating therapy is thus far not represented as a treatment goal by current guidelines. Even when adjusted for a number of strong potential confounders, such as kidney function, the TG/HDL-C ratio remained significantly associated with future mortality. Interestingly, if we would apply the NCEP cut-off values for 'borderline high TG' and 'low HDL-C',[10] the resulting recommended TG/HDL-C ratio would be 5, a value which is higher than the highest tertile in our study, therefore posing at least 2/3 of the RTR population at increased risk. Based on these data, we believe that special awareness is required to triglyceride levels in RTR. Furthermore, we think that in RTR prospective intervention trials with the treatment goal to lower triglycerides are warranted. A multicenter approach would be helpful to establish TG and TG/HDL-C ratio normal values specifically for RTR. We would also like to stimulate lipid guidelines for RTR to take account of the mortality risk associated with disturbances in triglyceride metabolism.

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5

THE FRAMINGHAM RISK SCORE IS ASSOCIATED WITH CHRONIC GRAFT FAILURE IN RENAL TRANSPLANT RECIPIENTS

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Journal of Clinical Medicine, 2021

ABSTRACT

BACKGROUND

Predicting chronic graft failure in renal transplant recipients (RTR) is an unmet clinical need. Chronic graft failure is often accompanied by transplant vasculopathy, the formation of de novo atherosclerosis in the transplanted kidney. We therefore determined whether the 10-year Framingham risk score (FRS), an established atherosclerotic cardiovascular disease prediction module, is associated with chronic graft failure in RTR.

METHOD

In this prospective longitudinal study, 600 well-characterized RTR with a functioning renal graft for at least one year, were followed for 10 years. The FRS was calculated and RTR were stratified into low (<10%), medium (10-19%), and high risk (≥20%), based on pre-defined cut-offs. The association with death-censored chronic graft failure (n=81, 13.5%) was computed.

RESULTS

An extended Cox model showed that each one percent increase of the FRS significantly increased the risk of chronic graft failure by 4% (HR: 1.04, P<0.001). This association remained significant after adjustment for potential confounders, including eGFR (HR: 1.03, P=0.014). Adding the FRS to eGFR resulted in a higher AUC in a receiver operating curve (AUC = 0.79, P<0.001) than eGFR alone (AUC = 0.75, P<0.001), and an improvement in the model likelihood ratio statistic (67.60 to 88.39) with a highly significant likelihood-ratio test (p<0.001). These results suggest that a combination of the FRS and eGFR improves risk prediction.

CONCLUSION

The easy to determine and widely available FRS has clinical potential to predict chronic graft failure in RTR. Therapeutic interventions targeted to reduce the FRS can be expected to extend graft survival.

INTRODUCTION

The number of patients with end-stage renal disease (ESRD) has been growing unwaveringly over the past decades,[1] resulting in a rising demand of donor kidneys. Renal transplant recipients (RTR) already outnumber dialysis patients in many countries.[2] Even after transplantation RTR still face major clinical problems, namely graft failure and a 4-6-fold increased incidence of cardiovascular disease (CVD),[3] the leading cause of death with a functioning graft.[4] Substantial clinical progress has been made over the past decades regarding acute rejection, but clinical advances concerning chronic graft failure stagnated, eventually leading to return to haemodialysis or re-transplantation in substantial numbers of patients.[5] An important contributing factor to chronic graft failure is transplant vasculopathy (TV). Histopathologically, TV represents the build-up of lesions within the graft that closely resemble classic *de novo* atherosclerotic plaque formation.[6,7] Therefore atherosclerosis negatively impacts RTR in two distinct ways, as classic atherosclerosis underlying CVD events and as de-novo atherosclerosis in the form of TV.

Due to the post-transplantation increase in cardiovascular risk, there is rather extensive literature concerning CVD prediction in RTR using traditional risk stratification.[8–10] In the general population, the Framingham Risk Score (FRS), that considers, gender, age, smoking, systolic blood pressure, total cholesterol, and HDL-C, is commonly used.[11] Unfortunately, the CVD risk stratification in renal transplant recipients has been disappointing, seemingly due to the complex aetiology of CVD in this specific patient population.[12,13] This could be because the years that RTR are on haemodialysis prior to transplantation are damaging to the cardiovascular system in specific ways, rendering risk predictors derived from a general population sample, that was essentially CVD free at time of inclusion, inadequate to predict CVD events and mortality in this specific population.

However, with respect to chronic graft failure, no consensus has been reached regarding prediction models, although there is a clear clinical need for such an approach given the poor 10-year graft survival. A successfully validated risk prediction module would also allow to tailor targeted intervention approaches, e.g. with pharmacotherapy, to prolong graft survival. Although the overall cardiovascular status of RTR is mostly poor, the allograft often has a normal vascular make-up at the time of transplantation. As chronic rejection is commonly accompanied by de novo atherogenesis, we hypothesise that the FRS as a commonly applied cardiovascular risk score could also serve to stratify RTR at risk of developing chronic graft failure.

MATERIALS AND METHODS

STUDY POPULATION

All RTR with a normally functioning renal graft for at least one year who visited the outpatient clinic in the University Medical Centre Groningen (UMCG) between August 2001 and July 2003 were invited to join the study. Exclusion criteria were overt congestive heart failure, endocrine abnormalities except diabetes mellitus, and cancer other than cured skin cancer. Of the 847 eligible patients, 606 opted to participate in the study (72%). The baseline characteristics showed no difference between participants and the original cohort, making it an accurate representation of the whole. Out of the 606 patients, five had to be excluded due to insufficient data to calculate the Framingham risk score, and one patient was lost to follow-up. Patients were followed for a period of 10 years. The study was approved by the local Medical Ethics Committee (METc2001/039), and is in accordance with the Declaration of Helsinki. All included subjects gave written informed consent. The TxL-IRI Biobank and Cohort Study is registered at ClinicalTrials.gov with identifier NCT03272854.

MEASUREMENTS AND DEFINITIONS

Diabetes was defined as a fasting plasma glucose ≥7.0 mmol/L or the use of antidiabetic medication, in accordance with ADA guidelines. [14]

After an 8-12 hour fasting period blood was drawn and routine laboratory measurements were conducted, as previously described.[15] Glucose was measured using the glucose-oxidase method (YSI 2300 Stat Plus; Yellow Springs, OH). Total cholesterol (TC) was determined using the cholesterol oxidase-phenol aminophenazone method. HDL cholesterol was measured with the cholesterol oxidase-phenol aminophenazone method on a Technikon RA-1000 (Bayer Diagnostics, Mijdrecht, The Netherlands). Plasma hs-CRP was determined using in-house enzyme linked immunosorbent assays (ELISAs). Creatinine concentrations were quantified in both urine and plasma using a modified version of the Jaffé method (MEGA AU 510; Merck Diagnostica). Creatinine clearance was computed from 24-hour urinary creatinine excretion and plasma estimated glomerular filtration rate (eGFR, calculated using the CKD-EPI formula). Body Mass Index (BMI) and waist circumference were measured, as previously described. [15]

END POINTS AND OUTCOME MEASURES

The main predictor of this study was the 10-year Framingham cardiovascular risk score, which was calculated using the 10-year cardiovascular risk algorithm of the Framingham Heart Study.[11,16] The primary end point was death-censored graft failure, defined as return to dialysis therapy or re-transplantation. Time to graft failure was measured from the date of inclusion until occurrence of graft failure or censoring due to death.

STATISTICAL ANALYSIS

Renal transplant recipients were divided into three pre-defined groups based on the FRS, namely "low risk" (<10%), "intermediate risk" (10-20%) and "high risk" (≥20%), as established in earlier studies. [17,18] Differences in baseline characteristics were tested between these groups. Categorical values are given as absolute numbers (percentages) and differences are tested with a chi-squared test. Normally distributed continuous variables are given as mean ± standard deviation (SD) and differences are tested by one-way analysis of variance (ANOVA). Skewed continuous variables are presented as median [25th to 75th percentile] and differences between groups are tested by the Kruskal-Wallis test.

A multivariable Cox regression analysis was performed to calculate the hazard ratio (HR), with 95% confidence intervals (CI), for chronic graft failure. However, the Schoenfeld residuals test was significant, indicating a violation of the proportional hazard assumption (P<0.05). In order to account for the varying effect of the FRS over time, an extended Cox model was used. A time varying covariate (TVC) was computed, therefore rendering the proportional hazards assumption obsolete.[19] The TVC of the FRS was included as an independent variable in all further analysis.

Adjustment was performed for potential confounders, defined as known risk factors of chronic graft failure in RTR, namely: Hba1c and concentration of insulin, primary kidney disease, hs-CRP as biomarker of inflammatory load, use of proliferation inhibitors, use of calcineurin inhibitors, the daily dose of prednisolon, time on haemodialysis pre-transplantation, proteinuria, time between renal transplantation and inclusion, type of renal transplant (living/dead), human leukocyte antigen – antibody (HLA-ab) mismatch, acute rejection and estimated glomerular filtration rate (eGFR). Factors that are included in the calculation of the FRS were not adjusted for.

The contribution of the FRS to risk prediction was assessed. ROC curves were computed for the TVC of the FRS, eGFR and a combined model of both the eGFR and FRS, in order to assess the prognostic value of these variables for chronic graft failure. Considering the time-varying aspect of the Framingham risk score, the ROC curves were made with the FRS as a function of time. Furthermore, due to the nested nature of the analysis, the addition of the FRS to the eGFR was assessed using likelihood ratio statistics.

A P-value of <0.05 is considered statistically significant. All statistical analyses were performed using Stata version 15.

RESULTS

In this prospective longitudinal study, the FRS was assessed as a potential clinical tool for predicting graft failure in 600 renal transplant recipients. A total of 81 patients (14%) experienced chronic graft failure during the 10-year follow-up.

Analyses were conducted by dividing patients into the predefined FRS categories of low (<10%, n=155), medium (10-20%, n=158), and high (≥20%, n=287) risk, and differences between baseline characteristics were assessed (Table 1). As expected, all parameters included in the FRS behaved consistent with higher risk in increasing risk groups. Additionally, the levels of glucose, HbA1c, and use of statins increased significantly with a higher FRS (P<0.001), likely also due to the inclusion of diabetes and total cholesterol as parameters in the FRS. A high body weight, BMI and higher levels of hs-CRP were also associated with a higher FRS (P<0.001). The number of patients using proliferation inhibitors was highest in the low-risk group (P=0.021). Graft function declined with increasing FRS, though this association was stronger for eGFR (P<0.001) than for creatinine clearance (p=0.02).

Table 1. Baseline characteristics according to low, medium and high Framingham risk score

Characteristics	Framingham risk score groups low (<10%), medium (10-20%), high (≥20%)			
	Low (n=148)	**Medium (n=151)**	**High (n=301)**	**P value**
Framingham risk score				
FRS	5.2 (3.3-7.5)	14.5 (12.3-16.9)	37.5 (26.8-49.5)	<0.001
Age, years	37.4 (31.5-44.1)	49.8 (42.8-55.5)	59.7 (53.0-64.8)	<0.001
Male gender, *n* (%)	58 (39.2)	80 (53.0)	194 (64.4)	<0.001
Smoking, *n* (%)	58 (39.2)	80 (53.0)	194 (64.5)	<0.001
Diabetes, *n* (%)	5 (3.4)	13 (8.6)	84 (27.9)	<0.001
Total cholesterol, mg/dL	204.2 (181.9-225.8)	209.2 (187.6-232.1)	225.1(199.5- 249.4)	<0.001
HDL cholesterol, mg/dL	44.3 (35.6-52.8)	40.2 (34.4-50.3)	39.1 (31.7-48.3)	<0.001
Mean systolic blood pressure, mmHg	134 (125-146)	145 (133-156)	162(149-176)	<0.001
Use of antihypertensive medication, n (%)	110 (74.3%)	129 (85.4%)	284 (94.4%)	<0.001
Recipient characteristics				
Weight, kg	70.0 (61.5-79.5)	76.5 (67.5-87.0)	79.0 (71.0-88.0)	<0.001
BMI, kg/m2	23.7 (21.6-27.0)	25.4 (23.0- 29.1)	26.4 (24.0-28.7)	<0.001
Use of statins, n (%)	52 (35.1)	82 (54.3)	160 (53.2)	<0.001
Cardiovascular disease history				
History of MI, *n* (%)	9 (6.2)	12 (8.0)	27 (9.0)	0.60
History of TIA/CVA, *n* (%)	7 (4.8)	7 (4.6)	19 (6.3)	0.69
Glucose homeostasis				
Glucose, mmol/l	4.3 (4.0-4.8)	4.5 (4.1-4.8)	4.7 (4.2-5.5)	<0.001
Insulin, μmol/l	10.6 (8.2-14.3)	11.9 (8.1-16.9)	11.2 (7.8-16.1)	0.33
HbA1c, %	5.8 (5.4-6.4)	6.3 (5.9-6.7)	6.7 (6.1-7.5)	<0.001
Use of anti-diabetic drugs, *n* (%)	3 (2.0)	9 (6.0)	63 (20.9)	<0.001
Inflammation				
hsCRP, mg/l	1.3 (0.6-3.8)	2.1 (0.7-4.6)	2.2 (1.1-5.9)	<0.001

Table 1 continued.

Characteristics	Framingham risk score groups low (<10%), medium (10-20%), high (≥20%)			
	Low (n=148)	**Medium (n=151)**	**High (n=301)**	**P value**
Donor demographics				
Age, years	35.0 (21.0-47.5)	37.0 (23.0-49.0)	40.0 (24.0-51.0)	0.14
Male gender, *n* (%)	81 (55.1)	85 (57.0)	158 (52.5)	0.64
Living kidney donor, *n* (%)	37 (25.0)	21 (13.9)	25 (8.3)	<0.001
(Pre)transplant history				
No haemodialysis	15 (10.1)	13 (8.6)	20 (6.6)	0.42
Dialysis time, months	26.5 (12.5-47.0)	26.0 (14.0-51.0)	28.0 (14.0-50.0)	0.65
Amount of transplantations	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	0.61
HLA mismatch	1.00 (0.00, 2.00)	1.00 (0.00, 2.00)	1.00 (0.00, 2.00)	0.58
Acute rejection, *n* (%)	72 (48.65%)	70 (46.36%)	127 (42.19%)	0.39
Primary renal disease, *n* (%)				
Primary glomerular disease	42 (28.4)	50 (33.1)	77 (25.6)	0.24
Glomerulonephritis	18 (12.2)	7 (4.6)	14 (4.7)	0.006
Tubulo-interstitial disease	33 (22.3)	26 (17.2)	33 (11.0)	0.006
Polycystic renal disease	12 (8.1)	30 (19.8)	63 (20.9)	0.002
Dysplasia and hypoplasia	11 (7.4)	4 (2.7)	6 (2.0)	0.01
Renovascular disease	5 (3.4)	8 (5.3)	20 (6.6)	0.36
Diabetic nephropathy	3 (2.0)	4 (2.7)	16 (5.3)	0.16
Other or unknown cause	24 (16.2)	22 (14.6)	72 (23.9)	0.03
Immunosuppressive medication				
Daily prednisolone dose, mg/dl	10.0 (7.5-10.0)	10.0 (7.5-10.0)	10.0 (7.5-10.0)	0.84
Calcineurin inhibitors, *n* (%)	115 (77.7)	112 (74.2)	244 (81.1)	0.23
Proliferation inhibitors, *n* (%)	118 (80.8)	116 (76.8)	207 (69.2)	0.021
Renal allograft function				
Creatinine clearance ml/min/1.73m^2	65.0 [22.3]	64.3 [23.7]	59.6 [21.8]	0.02
eGFR, ml/min	51.8 (40.2-61.9)	47.7 (36.8-58.6)	44.9 (34.0-55.8)	<0.001
Proteinuria ≥ 0.5 g/24 h, *n* (%)	39 (26.4)	39 (26.0)	90 (30.0)	0.58
Graft failure, n(%)	24 (16.2)	23 (15.2)	34 (11.3)	0.28

Normally distributed continuous variables are presented as mean±SD, and differences were tested with one-way analysis of variance [ANOVA]. Continuous variables with a skewed distribution are presented as median (25th to 75th percentile), and differences were tested by Kruskal–Wallis test. Categorical data are summarized as n (%), and differences were tested by chi-squared test. Abbreviations: FRS, Framingham risk score; HDL, high density lipoprotein; BMI, body mass index; MI, myocardial infarct; TIA, transient ischaemic attack; CVA, cerebrovascular accident; HbA1c, haemoglobin A1c; hs-CRP, high sensitivity C-reactive protein; HLA, human leukocyte antigens; eGFR, estimated glomerular filtration rate.

Since the Schoenfeld Residuals test was significant (P=0.003), a multivariable extended Cox model was carried out including a TVC of the FRS, which is shown in Table 2. A univariable extended Cox regression demonstrated a strong and highly significant association between FRS (in percentages) and graft failure (hazard ratio [HR]: 1.04, 95%CI:1.02-1.06, P<0.001, model 1), indicating a 4% increased risk in developing graft failure over the course of 10 years, per percentage increase in the FRS. The HR of the TVC was 0.99 (95%CI; 0.99-1.00, P=0.002), indicating that the risk decreases by 1% per year. Adjusting for Hba1c and concentration of insulin (model 2, HR: 1.03, 95%CI 1.01-1.05 P=0.001), the underlying primary kidney disease (model 3), hs-CRP (model 4) or use of calcineurin inhibitors, proliferation inhibitors and daily prednisolone dose (model 5) did not considerably alter this association.

Adjusting for time on haemodialysis, proteinuria, time between transplantation and baseline, acute rejection, type of renal transplantation and HLA-ab mismatch (model 6, HR: 1.03, 95%CI 1.01-1.05 P=0.001) or eGFR (model 7, HR: 1.03, 95%CI 1.01-1.05), P=0.006) did also not considerably alter the association. The association of the FRS with chronic graft failure remained significant in a fully adjusted model (HR: 1.02, 95%CI 1.00-1.05), P=0.026). These results show that even when considering a substantial number of prevailing risk factors, the FRS remained to be prospectively associated with graft failure.

Next, ROC curves for the FRS, eGFR, and a combination of eGFR and FRS were computed. The FRS ROC curve showed a fair degree of separability, and can therefore serve as a suitable predictor for graft failure (AUC = 0.66, P<0.001, Figure 1A). eGFR is currently the standard biomarker in clinical use and indeed its ROC curve also showed a good degree of separability, and a larger area under the ROC curve (AUC = 0.75, P<0.001, Figure 1B). Combining the FRS and eGFR ROC curves suggested that the FRS would add to the predictive value of eGFR (AUC = 0.79, P<0.001, Figure 1C). Furthermore, when adding the FRS to eGFR the model likelihood ratio statistic increases from 67.60 to 88.39, with a highly significant likelihood-ratio test (p<0.001). These results indicate that the FRS used together with eGFR significantly improves risk prediction of chronic graft failure.

Table 2. Hazard ratios for chronic graft failure per one percent increase of the Framingham risk score

	Hazard ratio main effect	95% CI	P value	Hazard ratio TVC	95% CI	P value
Model 1	1.04	1.02-1.06	<0.001	0.99	0.99-1.00	0.002
Model 2	1.03	1.01-1.05	0.001	0.99	0.99-1.00	0.002
Model 3	1.04	1.02-1.06	<0.001	0.99	0.99-1.00	0.002
Model 4	1.04	1.02-1.06	<0.001	0.99	0.99-1.00	0.002
Model 5	1.04	1.02-1.06	<0.001	0.99	0.99-1.00	0.002
Model 6	1.03	1.01-1.05	0.001	0.99	0.99-1.00	0.002
Model 7	1.03	1.01-1.05	0.006	0.99	0.99-1.00	0.002
Model 8	1.02	1.00-1.05	0.026	0.99	0.99-1.00	0.007

Model 1: crude; model 2: adjustment for Hba1c and concentration on insulin; model 3: adjustment for primary kidney disease; model 4: adjustment for C-reactive protein concentration; model 5: adjustment for use of calcineurin inhibitors, proliferation inhibitors, and dose of prednisolone; model 6: adjustment for time on haemodialysis, proteinuria, time between renal transplant and baseline, acute rejection, type of renal transplantation, and number of human leukocyte antigen mismatches; model 7: adjustment for eGFR; model 8: fully adjusted.
Abbreviations: CI, confidence interval; TVC, time varying covariate.

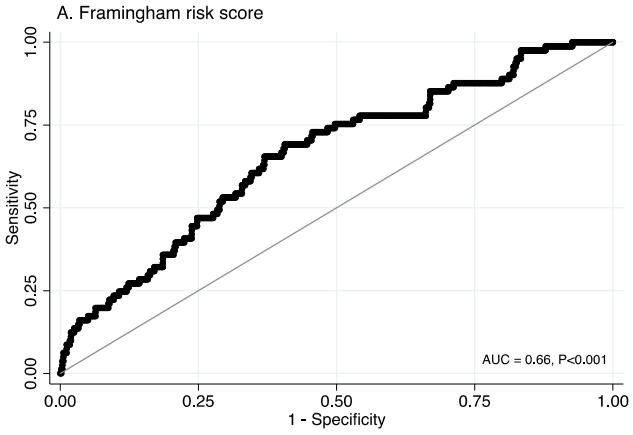

Figure 1. Receiver operating curves
Receiver operating curves for A. the Framingham risk score, B. eGFR and C. the Framingham risk score and eGFR combined. Abbreviations: eGFR, estimated glomerular filtration rate; AUC, area under the curve.

DISCUSSION

Our results show that the 10-year cardiovascular FRS is significantly associated with chronic graft failure, a condition believed to be largely driven by de novo atherosclerotic lesion formation, even when adjusting for other relevant risk factors. The data indicate that every percent increase in the FRS leads to a 4% increased risk in developing chronic graft failure over the course of 10 years. In the adjusted model, taking all of the most common risk factors into account this association drops to 3%. Furthermore, the hazard decreases by 1% every year. When combining eGFR and the FRS in a ROC curve, the predictive value increases substantially.

Currently, there is no consensus on clinical risk prediction for chronic graft failure in RTR. There have been studies that looked at a variety of potentially useful predictors of graft failure such as serum creatinine, eGFR, proteinuria, acute rejection, acute tubular necrosis, carotid-femoral pulse wave velocity, and use of immunosuppressants. However, most of the conducted research is not readily clinically implementable.[20]

A major contributing factor to chronic graft failure is transplant vasculopathy, where lesions closely resembling classical atherosclerosis damage the vascular make-up of the transplanted graft. [6,7] The FRS is to date the best researched CVD risk prediction model and is used worldwide. It includes the risk factors gender, age, smoking, systolic blood pressure, total cholesterol, and HDL-C, all key metrics thought to pathophysiologically contribute to the formation of atherosclerotic plaques. [11,21] Hypothetically, the FRS could be an adequate

fit for chronic graft failure risk stratification, an assumption confirmed by the present study.

A well-established risk factor to predict chronic graft failure is eGFR. [22] For this reason, it is interesting that adding the FRS to eGFR as a predictor for chronic graft failure resulted in an even stronger predictive model. It would be recommended to replicate this study in larger, ideally multi-centric cohorts and to integrate eGFR in the FRS. With respect to other potential limitations of this study, it is important to consider the homogeneity of the population. This study was conducted in a single centre in the Northern part of the Netherlands, and all participants were Caucasian. However, the Framingham heart study was also primarily carried out in an all-Caucasian North American city, therefore making it applicable to the study population. Furthermore, although our work included enough renal transplant recipients to be sufficiently powered, the number of patients which experienced graft failure was somewhat limited. However, still the data reveal a strong enough association to implicate clinical value.

An advantage of using the FRS is that in general all components included in the FRS are susceptible to pharmacotherapy. The dependency of sustained graft functioning on the FRS indicates that to a certain extent modifiable risk factors underlie chronic graft failure. Intervention trials would be required to confirm such reasoning. Proper risk stratification has the potential to extend the average 10-year survival of renal grafts, putting less patients in need to consequently being re-transplanted, thereby overall contributing to an increased availability of donor kidneys.

Taken together, the results of this study demonstrate that the FRS has clinical potential as a predictive tool to identify renal transplant recipients at risk of chronic graft failure . Due to the low costs and broad availability of the measurements included in the FRS, implementing the use of the FRS in daily clinical practice is realistic. Effective risk stratification combined with the potential for therapeutic intervention could conceivably contribute to prolonged survival of renal donor grafts.

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6

STATIN USE AND INCIDENT CARDIOVASCULAR EVENTS IN RENAL TRANSPLANT RECIPIENTS

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European Journal of Clinical Investigation, 2021

ABSTRACT

BACKGROUND

Statins achieve potent LDL lowering in the general population leading to a significant cardiovascular (CV) risk reduction. In renal transplant recipients (RTR) statins are included in treatment guidelines, however, conclusive evidence of improved cardiovascular outcomes has not been uniformly provided and concerns have been raised about simultaneous use of statins and the immunosuppressant cyclosporine. This study aimed to elucidate the effect of statins on a compound CV endpoint, comprised of ischemic CV events and CV mortality in RTR, with subgroup analysis focussing on cyclosporine users.

METHOD

622 included RTR (follow-up 5.4 years) were matched based on propensity scores and dichotomized by statin use. Survival analysis was conducted.

RESULTS

Cox regression showed that statin use was not significantly associated with the compound CV endpoint in a fully adjusted model (HR=0.81,95%CI =0.53-1.24, p=0.33). Subgroup analyses in RTR using cyclosporine revealed a strong positive association of statin use with the CV compound outcome in a fully adjusted model (HR=6.60, 95%CI 1.75-24.9, p=0.005). Furthermore, statin use was positively correlated with cyclosporine trough levels (correlation coefficient 0.11, p=0.04).

CONCLUSION

In conclusion, statin use does not significantly decrease incident CV events in an overall RTR cohort, but is independently associated with CV specific mortality and events in cyclosporine using RTR, possibly due to a bilateral pharmacological interaction.

INTRODUCTION

HMG-CoA reductase inhibitors, commonly referred to as statins, are among the world's most widely prescribed medications. Atorvastatin became the best-selling pharmaceutical in history in 2003, under the brand name Lipitor, with a yearly revenue of above 10 billion US dollars.[1,2] Statins potently reduce circulating low density lipoprotein cholesterol (LDL-C) levels, a prime risk factor for atherosclerotic cardiovascular disease (CVD). Statins are thus a mainstay of anti-atherosclerotic therapy, with proven efficacy in primary, as well as secondary, CVD prevention.[3] Overall, the introduction of statins has resulted in a 15-30% decrease in the incidence of cardiovascular mortality in the general population.[4–6]

However, in selected high-risk populations most in need of CVD risk management the effect of statins is less evident. End stage renal disease (ESRD) patients for example suffer from an age adjusted 30-fold increase in CVD mortality that is not substantially reduced by statins.[7] Another patient population particularly vulnerable for development of CVD are renal transplant recipients (RTR). Due to the increasing success of renal transplantations the number of RTR is ever increasing, in several countries even surpassing that of patients on maintenance dialysis.[8] However, RTR still suffer an exceptionally high, but (patho) physiologically still poorly understood, high mortality burden.[9] The biggest threat to this patient group is a vast increase in CVD mortality, translating to a 4-6 times higher age-adjusted risk compared to the general population.[10,11] Also in RTR the effect of statins is uncertain since evidence is sparse and has limitations, such as incomplete follow-up, leading to low quality of evidence.[12]

Furthermore, concern has been raised about the combination of statins with immunosuppressive regimen. In particular, it has been suggested that simultaneous use of statins and the immunosuppressive drug cyclosporine leads to an increased unbound fraction of serum cyclosporine, with imaginable adverse consequences.[13]

Nonetheless statins were included in treatment guidelines for RTR, due to the fact that 'it was assumed that similar treatment efficacy to that reported in the general population would be found if the trials were carried out in kidney transplant patients.'[14] Studies directly proving this assumption are thus far not available. The aim of this study is therefore to assess the effect of statins on incident CVD in a well characterised cohort of RTR, with subgroup analysis in cyclosporine using RTR.

METHODS

PATIENT POPULATION

For this follow-up study all renal transplant recipients who visited the University Medical Centre Groningen (UMCG) outpatient clinic between November 2008 and March 2011, with a functioning allograft for at least 1 year, were invited to participate. Patients diagnosed with overt congestive heart failure, endocrine abnormalities except diabetes or malignant disease other than cured skin cancer were not eligible for inclusion. Of the 707 patients that gave written informed consent, 85 were excluded due to a suspected acute infection, as indicated by a CRP value of >15mg/l (figure 1). The remaining 622 patients were followed for a median of 5.4 years (25th–75th interquartile range (IQR) 4.9–6.0 years) and no patients were lost during follow-up. All relevant patient characteristics were obtained from the "Groningen Renal Transplant Database". More detailed definitions of the characteristics of the database, patients' baseline characteristics, as well as routine laboratory methods used have been previously reported.[15] The study protocol was approved by the University Medical Centre Groningen Institutional Review Board (METc 2008/186) and is in accordance with the Declaration of Helsinki. The "TransplantLines Food and Nutrition Biobank and Cohort Study" is registered at clinicaltrials.gov as NCT02811835.

IMMUNOSUPPRESSIVE MEDICATION

RTRs all received standard immunosuppressive therapy. Standard immunosuppression consisted of the following: cyclosporine (target trough levels 175–200 mg/L in the first 3 months, 100 mg/L thereafter) and prednisolone (starting with 20 mg/day and tapering to 10 mg/day) from 1989 to 1996. In 1997 mycophenolate mofetil (2 g/day) was added to the standard immunosuppressive regimen. In 2012 cyclosporine was replaced by tacrolimus, and RTRs continued triple-immunosuppressive therapy with prednisolone (20 mg/day, tapering to 5 mg/day), tacrolimus (target trough levels 8–12 µg/L in the first 3 months, 6–10 µg/L until month 6, and 4–6 µg/L from 6 months onward), and mycophenolate mofetil (starting with 2 g/day, tapering to 1 g/day). A cyclosporine-based regime is advised if side effects of tacrolimus occur and in post-transplantation DM patients.

END POINT

The main outcome measure in this study was the use of any type of statin at the time of inclusion. The primary end point of this study was a compound CVD endpoint, consisting of the first occurrence of an ischemic CV event or CVD death. The following events were considered ischemic in nature and were included: myocardial infarction (MI), angina pectoris, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA), and ischemic cerebral infarction. Patients were censored for non-CV causes of mortality. Cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics. Cause

of death was coded according to the International Classification of Disease, 9th revision (ICD-9). CVD mortality was defined as the principal cause of death being cardiovascular in origin, namely ICD-9 codes 410-447.

MEASUREMENTS AND DEFINITIONS

Information regarding medication was extracted from patients' medical records. Blood samples were drawn after a 8–12 h fasting period, prior to medication intake. Serum high-sensitivity C-reactive protein (hsCRP), glycated hemoglobin (HbA1C), triglycerides, total cholesterol, LDL-C and high-density lipoprotein cholesterol (HDL-C) were measured using routine laboratory methods. A modified version of the Jaffé method was used to determine serum creatinine. (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). All participants were instructed to collect a 24-h urine sample the day before their visit to the outpatient clinic. Total urinary protein concentration was determined using the Biuret reaction (MEGA AU 150, Merck Diagnostica, Darmstadt, Germany).

Diabetes was defined as use of antidiabetic medication, fasting plasma glucose ≥7.0 mmol/L or HbA1C higher than 6.5%.[16] Proteinuria was present when urinary protein excretion was ≥0.5 g/24 h. A history of an atherosclerotic CV event was defined as the occurrence of an MI, angina pectoris, CABG, PTCA or ischemic cerebral infarction.

STUDY DESIGN

To address potential confounders for the primary prespecified analysis, we used propensity-score matching to compare the CVD compound endpoint between subjects receiving statin therapy and those who did not. A logistic regression was fitted for use of statins, including variables that, based on literature, are related to the outcome. This included patient demographics (age, sex), lifestyle factors (smoking, alcohol use), lipid biomarkers (HDL-C, LDL-C, triglycerides), kidney function parameters (serum creatinine, urinary protein excretion), CVD risk factors (history of MI, CVA or coronary intervention, systolic blood pressure, number of antihypertensives), medication use (prednisolone dose, use of proliferation inhibitors, use of tacrolimus, use of cyclosporine) and co-morbidities (primary renal disease, hsCRP, dialysis time, diabetes mellitus (DM), glucose levels, metabolic syndrome).[17] Propensity scores were obtained from the outcome of the logistic regression. Statin users were matched to non-statin users by one to one nearest-neighbor matching with replacement, meaning that a control subject could be used in multiple case-control pairs, allowing for more optimal matching.[18] Quality of matching was graphically evaluated (supplemental figure) and the reduction of bias assessed using a t-test for equality of means, the standardized percentage bias and the variance ratio (supplemental table). Survival analysis was conducted with weighing for the propensity score.

STATISTICAL ANALYSIS

Differences in baseline characteristics were assessed between statin users and non statin users in the unmatched entire cohort, as well as in the propensity score matched subset. Continuous, normally distributed variables are presented as mean (± SD) and differences tested with a one-way ANOVA. Continuous variables with a skewed distribution are given as median (25^{th}, 75^{th} percentile) and differences tested by Mann-Whitney U test. Categorical data are summarized by n (%) and differences tested by the chi-squared test.

Cox proportional hazards regression was performed in the propensity matched cohort, using weighted estimations based on the frequency with which a single observation was used as a match. Cumulative hazards were computed for the endpoints. Due to the fact that matching was done with replacement and the analysis weighted based on this, one participant counts as a control subject for a variable number of times. This accounts for sudden increases in the cumulative hazard rate, as seen in figures 2 and figure 3. Analyses were performed both crude, as well as with further adjustment for covariates for which balance was not achieved with matching, indicated by significant differences between groups. This included age, metabolic syndrome, total cholesterol, LDL-C, HbA1c, use of cyclosporine and living donor kidney donors. Subgroup analysis was conducted in subjects receiving cyclosporine treatment, subjects receiving tacrolimus treatment and subject that did not receive treatment with a calcineurin inhibitor. The proportional hazards assumption was tested using log-log graphs, and was found not to be violated.

A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using STATA version 15 (2017, StataCorp, College Station, TX, USA). Reporting of the study conforms to broad EQUATOR guidelines.[19]

RESULTS

BASELINE DEMOGRAPHIC CHARACTERISTICS

A total of 707 subjects from the “TransplantLines Food and Nutrition Biobank and Cohort Study” were assessed for eligibility. After exclusion due to suspected acute infection, as determined by a hsCRP >15 mg/l, 622 subjects were eligible for inclusion in the cohort (figure 1). The matching procedure matched 250 statin users to 90 non-statin users. Due to matching with replacement, 250 case-control pairs were matched. Standardized percentage bias and the variance ratio are shown in supplemental table 1.

Of the 622 RTR originally included in the study, 332 (53%) RTR received statins. In the propensity matched cohort 250 subjects used statins, of which 163 (48% of statin users) used atorvastatin, 52 (15% of statin users) used simvastatin, 13 (4% of statin users) used fluvastatin,

12 used rosuvastatin (4% of statin users) and 11 (3% of statin users) used pravastatin.

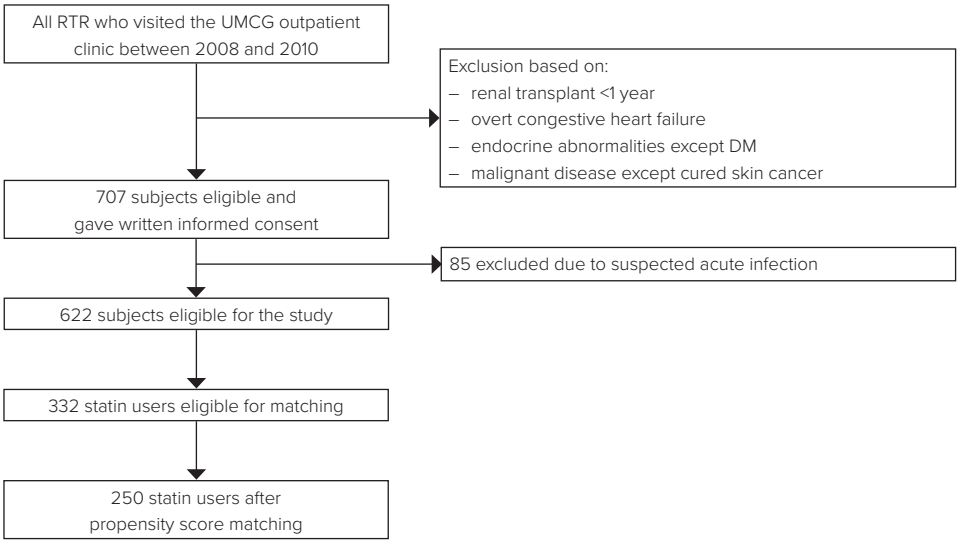

Figure 1. Inclusion of renal transplant recipients
RTR, renal transplant recipients; UMCG, University Medical Centre Groningen; DM, diabetes mellitus

Baseline characteristics for patients receiving statins and those not receiving statins in the entire cohort are summarized in table 1. As expected, significant differences between the groups were found for known CVD risk factors, components of the metabolic syndrome, cholesterol measures, including significantly lower LDL-C levels in statin users, history of ischemic CV events, use of antidiabetic medication and markers of insulin resistance. After matching, considerable improvement in balance was achieved for most patient characteristics (table 2). Age, presence of metabolic syndrome, total cholesterol, HbA1c and living kidney donor remained significantly higher in the statin group, while LDL-C remained significantly lower.

Table 1. Baseline characteristics according to use of statins in the entire unmatched cohort (n=622)

Characteristic	No use of statins (n=290)	Use of statins (n=332)	P value
Recipient demographics			
Age, years	50.2 (39.6, 61.7)	57.1 (49.0, 64.0)	<0.001
Male gender, *n* (%)	167 (58%)	189 (57%)	0.87
Current smoking, *n* (%)	34 (12%)	39 (13%)	0.93
Former smoking, *n* (%)	110 (40%)	160 (52%)	0.004
Never smoking, *n* (%)	131 (48%)	110 (36%)	0.003
Metabolic syndrome, *n* (%)	126 (43%)	260 (78%)	<0.001
Body composition			
BMI	26.1 ± 4.9	27.1 ± 4.7	0.008
Lipid Profile			
Total cholesterol, mmol/l	5.4 ± 1.1	4.9 ± 1.1	<0.001
LDL cholesterol, mmol/l	3.3 ± 0.9	2.7 ± 0.9	<0.001
HDL cholesterol, mmol/l	1.4 ± 0.5	1.4 + 0.5	0.92
Triglycerides, mmol/l	1.6 (1.2, 2.2)	1.7 (1.3, 2.4)	0.003

Table 1 continued.

Characteristic	No use of statins (n=290)	Use of statins (n=332)	P value
Cardiovascular disease history			
History of MI, CVA or coronary intervention, *n* (%)	29 (10%)	62 (19%)	0.003
Blood pressure			
Systolic blood pressure, mmHg	134.8 ± 16.5	137.0 ± 18.3	0.09
Diastolic blood pressure, mmHg	83.0 ± 11.7	82.1 ± 10.4	0.31
Use of ACE inhibitors, *n* (%)	87 (30%)	117 (35%)	0.17
Use of *β*-blockers, *n* (%)	160 (55%)	225 (68%)	0.001
Use of diuretics, *n* (%)	96 (33%)	150 (45%)	0.002
Number of antihypertensive drugs, *n*	2 (1, 2)	2 (1, 3)	<0.001
Glucose homeostasis			
Glucose, mmol/l	5.2 (4.7, 5.8)	5.3 (4.8, 6.2)	0.016
HbA1c, %	5.6 (5.4, 6)	5.9 (5.6, 6.4)	<0.001
Diabetes mellitus, *n* (%)	50 (17%)	94 (28%)	0.001
Use of anti-diabetic drugs, *n* (%)	28 (10%)	66 (20%)	<0.001
Use of insulin, *n* (%)	17 (6%)	39 (12%)	0.011
Inflammation			
hsCRP, mg/l	1.7 (0.8, 5.1)	1.5 (0.6, 4.0)	0.05
Donor demographics			
Age, years	47 (33, 55)	46 (32, 54)	0.85
Male gender, *n* (%)	148 (52%)	170 (52%)	1.0
Living kidney donor, *n* (%)	108 (37%)	112 (34%)	0.36
(Pre)transplant history			
Dialysis time, months	25 (7, 48)	28 (13, 54)	0.03
HLA mismatches	2 (1, 3)	2 (1, 3)	0.63
Time between tx and baseline, years	5.0 (1.4, 10.1)	5.6 (2.1, 12.4)	0.11
Primary renal disease			
Primary glomerular disease, *n* (%)	79 (27%)	95 (29%)	0.70
Glomerulonephritis, *n* (%)	19 (7%)	30 (9%)	0.25
Tubulo-interstitial disease, *n* (%)	37 (13%)	33 (10%)	0.27
Polycystic renal disease, *n* (%)	64 (22%)	63 (19%)	0.03
Dysplasia and hypoplasia, *n* (%)	13 (4%)	13 (4%)	0.72
Renovascular disease, *n* (%)	18 (6%)	20 (6%)	0.92
Diabetic nephropathy, *n* (%)	12 (4%)	20 (6%)	0.29
Other or unknown cause, *n* (%)	48 (17%)	58 (17%)	0.76
Immunosuppressive medication			
Daily prednisolone dose, mg	10 (7.5, 10)	10 (7.5, 10)	0.49
Calcineurin inhibitors, *n* (%)	172 (59%)	190 (57%)	0.60
Tacrolimus, *n* (%)	67 (23%)	50 (15%)	0.01
Cyclosporine, *n* (%)	106 (37%)	140 (42%)	0.15
Proliferation inhibitors, *n* (%)	242 (83%)	276 (83%)	0.01
Azathioprine, *n* (%)	42 (15%)	59 (18%)	0.27
Mycophenolate mofetil, *n* (%)	200 (69%)	217 (65%)	0.34
Renal allograft function			
Serum creatinine, µmol/l	122 (118, 129)	123 (99, 160)	0.74
Urinary protein excretion, g/24 h	0.2 (0, 0.4)	0.2 (0, 0.4)	0.33

Normally distributed continuous variables are presented as mean±SD, and differences were tested with one-way ANOVA. Continuous variables with a skewed distribution are presented as median (25^{th}, 75th percentile), and differences were tested by Mann Whitney test. Categorical data are summarized by n (%), and differences were tested by chi-squared test. BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; MI, myocardial infarction, CVA, cerebrovascular event; ACE, angiotensin-converting enzyme; HbA1C, glycated hemoglobin; hsCRP, high sensitivity C-reactive protein; HLA, human leukocyte antigen; tx, transplantation.

Table 2. Baseline characteristics according to use of statins in the propensity score matched cohort (n=340)

Characteristic	No use of statins (n=90)	Use of statins (n=250)	P value
Recipient demographics			
Age, years	53.1 (44.5, 64.3)	56.7 (49.4, 63.6)	0.036
Male gender, *n* (%)	41 (45%)	105 (42%)	0.56
Current smoking, *n* (%)	13 (14%)	33 (13%)	0.77
Former smoking, *n* (%)	43 (48%)	129 (52%)	0.53
Never smoking, *n* (%)			
Metabolic syndrome, *n* (%)	59 (66%)	191 (76%)	0.046
Body composition			
BMI	26.2 ± 4.5	26.9 ± 4.6	0.22
Lipid Profile			
Total cholesterol, mmol/l	5.3 ± 1.2	5.0 ± 1.1	0.011
LDL cholesterol, mmol/l	3.1 ±1.0	2.8 ± 0.9	0.001
HDL cholesterol, mmol/l	1.4 ± 0.5	1.4 ± 0.5	0.92
Triglycerides, mmol/l	1.9 (1.3, 2.6)	1.7 (1.3, 2.5)	0.70
Cardiovascular disease history			
History of MI, CVA or coronary intervention, *n* (%)	14 (16%)	45 (18%)	0.60
Blood pressure			
Systolic blood pressure, mmHg	135.0 ± 15.4	137.0 ± 18.1	0.34
Diastolic blood pressure, mmHg	83.1 ± 10.9	82.4 ± 10.8	0.59
Use of ACE inhibitors, *n* (%)	29 (32%)	91 (36%)	0.48
Use of *β*-blockers, *n* (%)	61 (68%)	169 (68%)	0.98
Use of diuretics, *n* (%)	41 (46%)	112 (45%)	0.90
Number of antihypertensive drugs, *n*	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	0.56
Glucose homeostasis			
Glucose, mmol/l	5.2 (4.7, 5.9)	5.3 (4.7, 6.0)	0.58
HbA1c, %	5.7 (5.5, 6.1)	5.9 (5.6, 6.3)	0.016
Diabetes mellitus, *n* (%)	19 (21%)	63 (25%)	0.44
Use of anti-diabetic drugs, *n* (%)	13 (14%)	44 (18%)	0.49
Use of insulin, *n* (%)	6 (7%)	26 (10%)	0.30
Inflammation			
hsCRP, mg/l	1.4 (0.8, 2.9)	1.4 (0.6, 3.2)	0.66
Donor demographics			
Age, years	44.5 (29.0, 55.0)	46.0 (34.0, 54.0)	0.46
Male gender, *n* (%)	37 (41%)	136 (54%)	0.031
Living kidney donor, *n* (%)	19 (21%)	90 (36%)	0.009
(Pre)transplant history			
Dialysis time, months	30.0 (9.0, 50.0)	25.5 (10.0, 52.0)	0.80
HLA mismatches	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	0.15
Time between tx and baseline, years	6.1 (2.5, 11.4)	5.4 (2.2, 12.5)	0.76
Primary renal disease			
Primary glomerular disease, *n* (%)	26 (29%)	79 (32%)	0.63
Glomerulonephritis, *n* (%)	5 (6%)	16 (6.4%)	0.78
Tubulo-interstitial disease, *n* (%)	13 (14%)	24 (10%)	0.21
Polycystic renal disease, *n* (%)	17 (19%)	48 (19%)	0.95
Dysplasia and hypoplasia, *n* (%)	3 (3%)	8 (3%)	0.95
Renovascular disease, *n* (%)	4 (4%)	18 (7%)	0.36
Diabetic nephropathy, *n* (%)	6 (7%)	13 (5%)	0.60
Other or unknown cause, *n* (%)	16 (18%)	44 (18%)	0.97

Table 2 continued

Characteristic	No use of statins (n=90)	Use of statins (n=250)	P value
Immunosuppressive medication			
Daily prednisolone dose, mg	10.0 (7.5, 10.0)	10.0 (7.5, 10.0)	0.99
Calcineurin inhibitors, *n* (%)	50 (56%)	137 (55%)	0.95
Tacrolimus, *n* (%)	19 (21%)	32 (13%)	0.21
Cyclosporine, *n* (%)	31 (34%)	105 (42%)	0.058
Proliferation inhibitors, *n* (%)	71 (79%)	207 (83%)	0.41
Azathioprine, *n* (%)	10 (11%)	43 (17%)	0.17
Mycophenolate mofetil, *n* (%)	61 (68%)	164 (66%)	0.71
Renal allograft function			
Serum creatinine, µmol/l	123.5 (97.0, 166.0)	122.5 (100.0, 156.0)	0.93
Urinary protein excretion, g/24 h	0.2 (0.0, 0.4)	0.2 (0.0, 0.3)	0.75

Normally distributed continuous variables are presented as mean±SD, and differences were tested with one-way ANOVA. Continuous variables with a skewed distribution are presented as median (25th, 75th percentile), and differences were tested by Mann Whitney test. Categorical data are summarized by n (%), and differences were tested by chi-squared test. BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; MI, myocardial infarction, CVA, cerebrovascular event; ACE, angiotensin-converting enzyme; HbA1C, glycated hemoglobin; hsCRP, high sensitivity C-reactive protein; HLA, human leukocyte antigen; tx, transplantation.

TIME TO EVENT ANALYSIS

The CV compound endpoint was reached in 56 (16.5%) of the included RTR in the propensity matched cohort, of which 44 received statin therapy and 12 did not (p=0.35). Cumulative hazard ratios were computed for the CV compound endpoint. In a crude analysis the use of statins was not significantly associated with the endpoint (HR=0.93, 95% confidence interval [CI] = 0.62-1.40, p=0.72) (figure 2A). Adjustment for variables for which balance was not achieved with matching, namely age, metabolic syndrome, total cholesterol, HbA1c, LDL-C and living kidney donation, did not impact this association (HR=0.81,95%CI =0.53-1.24, p=0.33). Previous work suggested a potential interaction between statins and cyclosporin.[13]

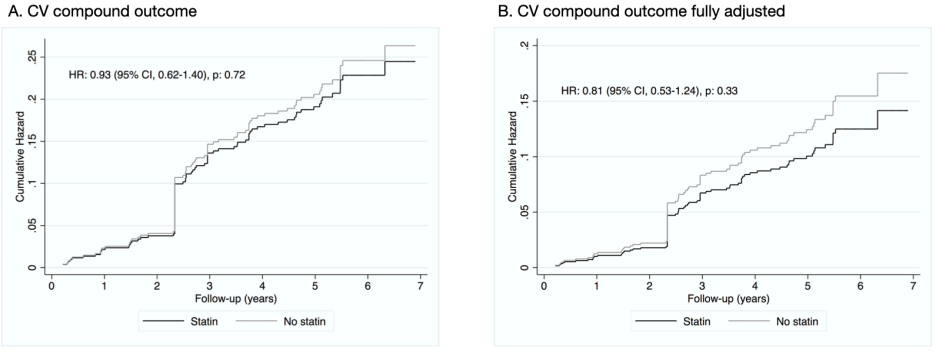

Figure 2. Comparison of the Cumulative Hazard of the cardiovascular (CV) compound endpoint of statin use versus no statin use in the propensity matched cohort

Hazard ratios were obtained using weighted cox proportional hazard regressions. Fully adjusted models were adjusted for age, metabolic syndrome, total cholesterol, LDL-C, HbA1c, use of cyclosporine and living kidney donors. CV, cardiovascular; HR, Hazard Ratio; 95%CI, 95% confidence interval.

Indeed, in our cohort a significant correlation existed between the use of statins and blood levels of cyclosporine, with a correlation coefficient of 0.11 (p=0.04), meaning that use of statins accounts for 11% of the variability of cyclosporine levels.

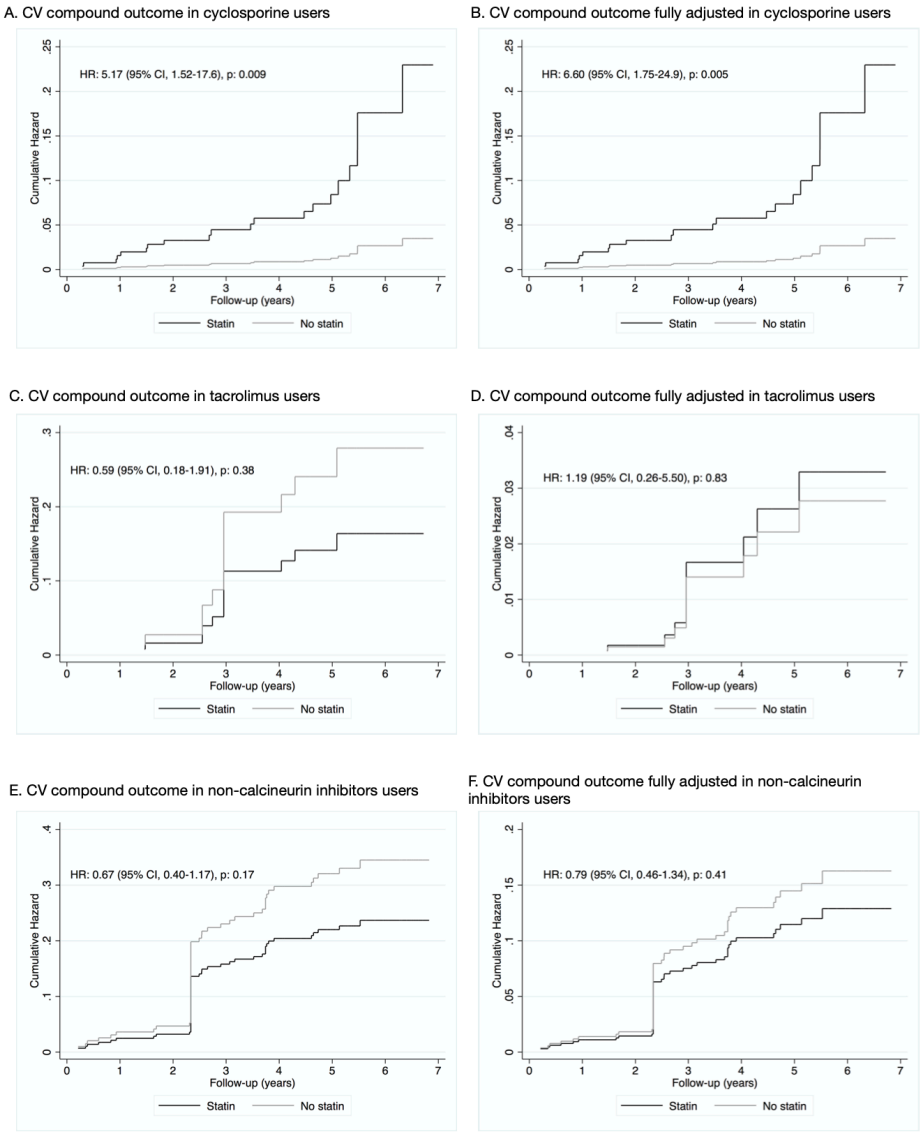

Figure 3. Comparison of the Cumulative Hazard of the cardiovascular compound endpoint of statin use versus no statin use by cyclosporine use, tacrolimus use and no use of calcineurin inhibitors in the propensity matched cohort

Hazard ratios were obtained using weighted cox proportional hazard regressions. Fully adjusted models were adjusted for age, metabolic syndrome, total cholesterol, LDL-C, HbA1c, use of cyclosporine and living kidney donors. CV, cardiovascular; HR, Hazard Ratio; 95%CI, 95% confidence interval.

In order to better understand the effect of simultaneous use of statins and cyclosporine subgroup analysis was performed in RTR receiving cyclosporine treatment, those receiving tacrolimus treatment and those who did not receive treatment with a calcineurin inhibitor. Interestingly, use of statins was strongly positively associated with the CV compound endpoint in cyclosporine users (HR=5.17, 95%CI=1.52-17.6, p=0.009), translating to a 5-fold increased risk in reaching the endpoint in statin users. Again, a fully adjusted analysis was performed, which did not substantially alter the association (HR=6.6, 95%CIs 1.75-24.9, p=0.005). On the other hand, in RTR receiving tacrolimus therapy (HR=1.19, 95%CI=0.26-5.50, p=0.83) and in RTR not receiving calcineurin therapy (HR=0.79, 95%CI=0.46-1.34, p=0.41) use of statins had no effect on the CV compound endpoint.

DISCUSSION

The results of this study demonstrate that use of statins is not associated with CV events or mortality in renal transplant recipients meaning that no overall protective effect of statin therapy was discernible in this patient group. In fact, use of statins in cyclosporine treated patients had a strong positive association with incident CV events and mortality.

Statins are a standard treatment modality in RTR, with the goal of reducing CVD risk through lowering of LDL-C. The topic is addressed in the most recent version of the guidelines from the National Kidney Foundation, Kidney Disease: Developing Global Guidelines (KDIGO) organisation about lipid management in chronic kidney disease (CKD).[20] By definition RTR are considered to have CKD, but are additionally also specifically referred to in the guidelines, which state that all RTR, irrespective of age and LDL-C levels, should receive statin therapy.[20] The rationale behind this recommendation is based on a single randomised trial, namely the Assessment of LEscol in Renal Transplantation (ALERT) trial. ALERT investigated the effect of statins in 2102 RTR, with an age range of 30 – 75 years, who were followed for a mean of 5.1 years.[21] However, no significant association was seen with the primary endpoint, which was the first occurrence of a major adverse cardiac event, defined as cardiac death, non-fatal MI or coronary-artery bypass.[21] A significant risk reduction was found in secondary endpoints, however, the authors themselves stated that the results should be interpreted with caution due to the absence of a significant primary endpoint and lack of correction for multiple comparisons.[21] A later post-hoc subgroup analysis reached the conclusion that cardiac death and nonfatal myocardial infarction were prevented in RTR.[22]

Several possible explanations exist for not only the lack of efficacy of statins in RTR, but the consequent increase in the CVD endpoint specifically in cyclosporine users. These include i.) a different underlying pathophysiological basis of CVD in RTR, ii.) reduced efficacy of statins due to reduced kidney function, and iii.) concurrent use of immunosuppressive medication.

LDL-C is widely applied in estimating future risk in CVD in the general population, due to its causal contribution to atherosclerotic disease, which provides the rationale for the use of statins in RTR. However, in RTR traditional CVD risk factors, including LDL-C, do not uniformly predict CVD mortality to the same extent as in the general population,[23] therefore suggesting that a different pathophysiological mechanism contributes to CVD. Furthermore, atherosclerosis might be less frequently the underlying cause of CVD mortality in CKD patients, and consequently as well in RTR, with a higher proportion of sudden death, arrhythmia and heart failure.[10,24] This potentially explains why LDL-C lowering in the form of statins does not decrease CVD mortality in patients with impaired renal function.[7]

Furthermore, RTR have a lower kidney function due to various factors, including ischemic and reperfusion injuries, revascularisation and nephrotoxicity of immunosuppressants.[25] In addition, most RTR have a history of ESRD and haemodialysis. CKD has previously been linked to a reduced efficacy of statins.[26] The efficacy seems to be further reduced with deteriorating renal function. Two trials have shown that in haemodialysis patients statins do not have an effect on CVD related end points. [7,27] Although not widely supported by actual data, it is plausible that the efficacy of statins is reduced in RTR as well.

RTR are a unique patient population due to their use of immunosuppressive medication. Standard immunosuppressive regimen include a calcineurin inhibitor, either cyclosporine or tacrolimus, prednisolone and additional use of either the proliferation inhibitor mycophenolate mofetil (MMF), in case of RTR at high immunological risk, or an mTOR inhibitor.[28] Cyclosporine has numerous dose dependent adverse effects, including nephrotoxicity and induction of haemolytic-uremic syndrome.[29] Cyclosporine is also known to increase CVD risk, at least partially attributable to increased circulating LDL particle numbers and increased oxidisability of LDL.[29–31] Furthermore, increased homocysteine levels have been reported, as well as unfavourable effects on the fibrinolytic system.[32] Use of cyclosporine is also associated with an elevated blood pressure, as well as increased risk of infections.[33–35]

Although the different statins share a similar mechanism of action they vary in their bioavailability, excretion and protein binding.[26] Simvastatin, lovastatin and atorvastatin are metabolized in the liver primarily by cytochrome P450 3A4.[36] Cyclosporine is also metabolized by cytochrome P450 3A4[37], making a bilateral pharmacological interaction plausible. And indeed, ample evidence shows that plasma levels of statins increase with concurrent administration of cyclosporine.[36,38–41] However, a rise of statin plasma levels in cyclosporine treated patients was not only found in patients receiving statins metabolized by cytochrome P450 3A4, but as well in patients receiving fluvastatin, which is metabolized by cytochrome P450 2C9, therefore indicating that the mechanism of the interaction might not be restricted to competition at the level of the cytochrome P450 3A4 pathway.[37]

Interestingly, the increased systemic statin exposure does not lead to an increased lipid lowering effect.[38] Furthermore, the incidence of myopathy was reported to be significantly higher with combined administration of cyclosporine and all statins except fluvastatin, ascribed to reduced clearance of statins and consequent higher serum concentrations.[42,43]

On the other hand, less information is available about the effect of statins on cyclosporine levels. These are difficult to assess retrospectively as cyclosporine dosages are continuously adjusted based on serum levels. One study showed that both cyclosporine and pravastatin are excreted by P-glycoprotein and multidrug resistant protein (MRP) 2 and that simultaneous administration of these drugs causes both a rise of cyclosporine in vivo[44], as well as pravastatin, due to competitive inhibition of the MRP2 transporter. It is therefore plausible to believe that simultaneous administration of cyclosporine and statins increases the bioavailability of both drugs due to inhibited metabolic clearance, therefore increasing the toxic and adverse cardiovascular effects of cyclosporine. Indeed, this is in line with our results, which show a higher risk on cardiovascular endpoints in cyclosporine treated patients.

Several limitations of our study need to be considered. It was carried out in a single centre and analysis was conducted retrospectively. It is plausible that some form of prescription bias exists, despite propensity score matching. Further prospective research is warranted to validate our findings. Furthermore, although the study was sufficiently powered, the number of events was too small to be able to sub-divide the primary CVD endpoint into different events. For the same reason we were not able to assess the effect of different types of statins with sufficient certainty. Furthermore, the subgroup analysis of tacrolimus users consisted of a rather small number of subjects. Also, the studied population was predominantly Caucasian, creating difficulties in extrapolation our findings to other ethnicities. Throughout recent years a shift away from cyclosporine and towards tacrolimus as initial immunosuppressive regimen has taken place. However, in two groups use of cyclosporine is still favoured, namely in those where (i) treatment was initiated with cyclosporine and (ii) patients who are predisposed to tacrolimus related toxicity, such as new onset diabetes after transplantation (NODAT).[28]

In conclusion, to the best of our knowledge we are the first to evaluate the efficacy of stain use in RTR outside of the ALERT trial. Interestingly, our data indicate no obvious protective effect of statins with respect to lowering risk of CV events and CV mortality in RTR. Furthermore, statin use is potentially harmful in cyclosporine using RTR. The pathophysiological basis of these observations remains to be clarified, but it is plausible to assume a drug-drug interaction of statins with cyclosporine, which leads to increased bioavailability of both drugs and a subsequent increase of adverse effects. Based on these data we suggest that there is an apparent clinical need for prospective randomised controlled trials testing the impact of LDL-C lowering with different therapeutic modalities on CVD outcomes in RTR.

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7

PROTEOGLYCAN-BINDING AS PRO-ATHEROGENIC FUNCTION METRIC OF APOB-CONTAINING LIPOPROTEINS AND CHRONIC GRAFT FAILURE IN RENAL TRANSPLANT RECIPIENTS

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Journal of Lipid Research, 2021

ABSTRACT

BACKGROUND

Lipoprotein-proteoglycan binding is an early key event in atherosclerotic lesion formation and thus conceivably could play a major role in vasculopathy driven chronic graft failure and cardiovascular mortality in renal transplant recipients.

METHOD

The present study investigated whether lipoprotein-proteoglycan binding susceptibility (LPBS), and the classical biomarker low-density lipoprotein cholesterol (LDL-C) are associated with cardiovascular mortality (n=130) and graft failure (n=73) in 589 renal transplant recipients who were included at least 1-year post-transplantation and followed for 9.5 years.

RESULTS

At baseline, LPBS was significantly higher in patients who subsequently developed graft failure compared to those with a surviving graft (1.68 ± 0.93 versus 1.46 ± 0.49 nmol/mmol, p=0.001). Cox regression analysis showed a prospective association between LPBS and chronic graft failure in an age- and sex-adjusted model (hazard ratio: 1.45; 95% CI, 1.14-1.85; p=0.002), however not with cardiovascular mortality. LDL-C levels were neither associated with graft failure nor with cardiovascular mortality.

CONCLUSION

This study shows that a measure of cholesterol retention outperformed the traditionally used quantitative parameter of LDL cholesterol levels, suggesting a higher relevance of pro-atherogenic function compared to quantity of lipoproteins in chronic graft failure.

INTRODUCTION

Atherosclerosis negatively impacts the prognosis of renal transplant recipients (RTR) in two ways, (i) in the form of preexisting, mostly complex atherosclerotic lesions, which are the underlying pathology for cardiovascular disease (CVD), and (ii) as *de novo* atherosclerotic lesion formation in the graft, known as transplant vasculopathy (TV), the single major cause for chronic graft failure (GF) [1–3]. Patients receiving kidney grafts often have a long-standing history of end-stage renal disease (ESRD) with dialysis treatment, which is *per se* associated with a 30 to 40-fold increase in age-adjusted CVD mortality[4]. After transplantation CVD risk is still 4 to 6-fold higher and is the leading cause of death in RTR[4]. A decrease in renal function over time further contributes to the increased risk. The substrate for this chronic graft functional decline is TV, an atherosclerotic process in the vasculature of the transplanted organ, affecting 50% of allografts after five years and 90% after ten years[2,3]. Importantly, in RTR classical CVD risk factors, such as levels of low density lipoprotein cholesterol (LDL-C) or high density lipoprotein (HDL)-C, fail to serve as predictive biomarkers either for CVD events or for *de novo* atherosclerosis leading to TV-mediated chronic GF[1,3,5]. Thus, the identification of predictive biomarkers represents an unmet clinical need in this patient population. Recent studies indicated that assays capturing the functional properties of HDL lipoproteins might provide clinical information beyond cholesterol levels within these, exemplified by HDL cholesterol efflux capacity being able to predict CVD in the general population [6,7] and chronic GF in RTR[8], independent of HDL-C levels. However, throughout the atherogenic process especially LDL particles play a central and pivotal role[9]. Specifically, binding of LDL particles to proteoglycans in the vessel wall is an early key event in the initiation of atherosclerotic lesions, as summarized in the now widely accepted response-to-retention hypothesis of atherogenesis [10]. However, thus far the concept that measures of LDL functionality can be used as predictive biomarkers has not been widely explored. Especially the susceptibility of LDL to bind to vascular proteoglycans appears promising in this respect. Therefore, in the present work we investigated, whether in comparison to LDL-C levels, the lipoprotein-proteoglycan binding susceptibility (LPBS) of apoB-containing lipoproteins is prospectively associated with the two clinically relevant atherosclerosis-related outcomes in RTR, CVD mortality on the one hand and chronic GF on the other.

MATERIALS AND METHODS

STUDY DESIGN AND STUDY POPULATION

For this prospective study an established and well-characterized patient cohort of adult RTR (TransplantLines) was used[8,11]. Patients were recruited at the outpatient clinic of the University Medical Center Groningen between August 2001 and July 2003. In order to lessen the potential confounding effect of early immune-mediated rejection, patients were

required to have, at inclusion, a functioning graft for at least one year after transplantation. Exclusion criteria were overt congestive heart failure, endocrine abnormalities other than diabetes mellitus, malignancies other than cured skin cancer and suspected acute infection. 606 out of 847 eligible patients volunteered to participate and were included in the cohort (72% consent rate). Non-participants were compared to participants with regards to age, gender, body mass index (BMI), plasma creatinine, creatinine clearance, and proteinuria and no significant differences were found[8,11]. Written informed consent was obtained from all participants. The Institutional Review Board approved the study protocol (METc2001/039), which complied with the Declaration of Helsinki. The study is registered at ClinicalTrials.gov with identifier NCT03272854 under the name "The TransplantLines Insulin Resistance and Inflammation Biobank and Cohort Study".

OUTCOME MEASURES AND END POINTS OF THE STUDY

The main outcome measure of this study is LPBS. Primary end points are i. death-censored GF, defined as return to dialysis therapy or re-transplantation, and ii. cardiovascular mortality, defined as deaths with the principal cause of death being CV in nature, using codes 410–447 of the International Classification of Diseases, ninth revision (ICD-9) [12]. If the status of a patient was unknown, general practitioners or referring nephrologists were contacted. Subjects' status regarding survival and GF were recorded until April 2012. Causes of death were available until May 2009. One subject was lost to follow-up.

BASELINE MEASUREMENTS AND DEFINITION*s*

Transplant characteristics, such as subject demographics, previous history as well as date and type of transplantation, were extracted from the Groningen Renal Transplant Database. Smoking status and CVD history (considered positive if participants previously had a myocardial infarction, transient ischemic attack, or cerebrovascular accident) were obtained using a self-report questionnaire at inclusion. Medical records were consulted for information on use of medication. Standard immunosuppressive regimen changed over the years: from 1968 to 1989, prednisolone and azathioprine (100 mg/day); from January 1989 to February 1993, cyclosporine standard formulation (10 mg/kg; trough levels of 175 to 200 µg/L in the first 3 months, 150 µg/L between 3 and 12 months post-transplantation, and 100 µg/L thereafter) and prednisolone (initially 20 mg/day, rapidly tapered to 10 mg/day); from March 1993 to May 1997, cyclosporine microemulsion (10 mg/kg, trough levels as before) and prednisolone; from May 1997 onwards, mycophenolate mofetil (2 g/day) was added[13].

Blood samples were drawn at time of inclusion after an 8 to 12 hour overnight fasting period. No specific anti-oxidant was added at the time of blood draw, however, all samples contained EDTA, a known anti-oxidant, and were handled in an identical fashion. All standard laboratory measures, including HbA1c, were performed at time of inclusion. Concentrations of total cholesterol (TC) and HDL-C were determined using the cholesterol

oxidase-phenol aminophenazone method. Concentration of LDL-C was calculated using the Martin Hopkins equation[14]. Apolipoprotein A-I, apolipoprotein B and lipoprotein (a) were determined by nephelometry with reagents for Dade Behring nephelometer systems (BNII, Siemens, Marburg, Germany). The Glycerol-3-phosphate oxidase-phenol aminophenazone method was used to measure plasma triglycerides. Levels of plasma high sensitive C-reactive protein were assessed by enzyme-linked immunosorbent assay (ELISA)[8]. Plasma glucose levels were determined using the glucose-oxidase method and plasma insulin was measured using an AxSym autoanalyzer. Levels of glycosylated hemoglobin, type A1C (HbA1c) were assessed by high-performance liquid chromatography[8]. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was used to determine insulin resistance by multiplying glucose (mmol/l) with insulin (µU/ml) and dividing the result by 22.5 [15]. Creatinine concentrations in plasma and urine were determined using a modified version of the Jaffé method. eGFR was calculated using the CKD-EPI formula [16]; all values included in the analyses were obtained at time point of inclusion. Creatinine clearance was calculated from 24-hour urinary creatinine excretion and plasma creatinine. Total urinary protein concentration was analyzed using the Biuret reaction.

Proteinuria was defined as urinary protein excretion ≥ 0.5 g per 24 hours. The diagnosis of diabetes mellitus was made if anti-diabetic medication was used or fasting plasma glucose concentration was ≥ 7.0 mmol/l or HbA1c was > 6.5% [17]. The body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Blood pressure was measured automatically in 1 min intervals after a 6 min rest in supine position (Omron M4; Omron Europe BV, The Netherlands) and the mean of three measurements was taken.

LDL particle number and average size were determined in EDTA plasma (n=158 participants) by ^{1}H-NMR spectroscopy using a Vantera® NMR Clinical Analyzer as previously described (LabCorp, Raleigh, USA) [18]. Plasma sPLA2-IIA was assessed in n=40 participants with an enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Conjugated dienes were determined in apoB-containing lipoproteins (n=40 participants) by absorbance at 233nm following lipid extraction with dichloromethane/methanol (2:1, v/v)[19] using precipitates generated by polyethylene glycol-6000 precipitation of apoB-containing lipoproteins as described[6]. Values were normalized for LDL-cholesterol levels.

LABORATORY ANALYSIS OF LIPOPROTEIN BINDING TO PROTEOGLYCANS

EDTA plasma samples of 589 participants were available for the laboratory analysis of this study. The plasma samples were collected at baseline, placed on ice, centrifuged at 4°C, immediately stored at −80°C, and left unthawed until analysis. The samples were stored from time of inclusion until 2019.

To determine the binding susceptibility of lipoproteins to proteoglycans human aortic proteoglycans were isolated from the intima-media of atherosclerotic human aortas[20], and the glycosaminoglycan content of proteoglycans was quantified as overall marker of proteoglycans[21]. Then wells of polystyrene 96-well plates (Thermo Fisher Scientific) were coated with 100µL of proteoglycans (50µg/mL in PBS) by incubation at 4°C overnight. Wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1h at 37°C. Wells without proteoglycan coating served as controls for unspecific binding. To measure lipoprotein binding to the immobilized proteoglycans, 1µL of plasma (derived from RTR at baseline) was added to the wells in a buffer containing 140mmol/L NaCl, 2mmol/L MgCl2, 5mmol/L CaCl2 and 10mmol/L MES, pH5.5 and incubated for 1h at 37°C. The wells were washed with 10mmol/L MES-50 mmol/L NaCl, pH5.5, and the amount of bound TC was determined using the Amplex Red cholesterol kit (Molecular Probes). Each sample was analyzed in duplicate and the non-specific binding in a single well had been blocked by the blocking buffer. The non-specific binding consistently accounts for about 5% of the binding to the PG-coated wells. The assay was performed over a duration of several weeks. The day-to-day variation of the measurement is <15% and to control for this, a control plasma sample is analyzed in each plate. However, no drift in the assay was noted. The variation between duplicate measurements carried out in separate microtiter well plates is 2.3%. In order to correct for interindividual differences in pro-atherogenic lipoproteins in each individual sample, the amount of bound TC was divided by the concentration of plasma LDL-C. Results are thus expressed as nmol bound TC/mmol plasma LDL-C. This measure gives a realistic reflection of the binding susceptibility of plasma pro-atherosclerotic lipoproteins to proteoglycans in the arterial vessel wall. More than 90% of TC that binds to lipoproteins from plasma has been shown to be associated with LDL particles[21,22].

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STATISTICAL ANALYSIS

Baseline characteristics of the study population were analyzed for gender-stratified tertiles of levels of bound TC/plasma LDL-C (low, medium, and high). Normally distributed continuous variables are depicted as mean ± standard deviation, whereas continuous variables with a skewed distribution are given as median [25th–75th percentile]. Categorical variables are summarized by absolute numbers (percentages).

Baseline characteristics were tested for differences among groups with low, medium, and high LPBS based on gender-stratified tertiles. Baseline characteristics for normally distributed continuous variables were tested for differences among groups with one-way analysis of variance (ANOVA). Kruskal–Wallis test was used to assess differences between groups for continuous variables with a skewed distribution. Group differences in categorical data were tested with Pearson chi-squared test.

LPBS of subjects who reached the respective end points was compared to values of subjects not reaching the end points of the study independent samples t-test. Similarly, the LPBS

for males and females was computed using independent samples t-test. Subsequently, all characteristics with a P<0.10 across gender-stratified tertiles of LPBS were entered into a step-wise multivariable linear regression model with backward elimination (P<0.0.5) in order to identify variables independently associated with LPBS.

Multivariable Cox regression was used to calculate hazard ratios (HR) and 95% confidence intervals (95%CI) for the primary end points. Adjustment of potential confounders was used to assess the independent association of LPBS with the endpoints chronic GF and cardiovascular mortality. Potential confounders were determined as known risk factors of chronic GF and CVD in RTR and included age, gender, eGFR, periods of acute rejection, number of human leukocyte antigen mismatches, primary renal disease, diabetes mellitus, BMI, dialysis time, type of transplantation, use of calcineurin inhibitors, use of proliferation inhibitors, use of statins, time between transplantation and baseline and donor age. Validity of proportional hazards assumptions was tested using Schoenfeld residuals. Furthermore, subgroup analysis using interaction tests were performed in which HR were determined across categories of baseline characteristics. For continuous variables the median value was used as cutoff. For the end point chronic GF the subject characteristics were sex (male versus female), age (<52.1 versus >52.1 years), use of statins (yes versus no), eGFR (<46.7 and ≥46.7 mL/min per 1.73 m^2) and period of acute rejection (yes versus no).

To compare the relevance of the proposed novel functionality parameter with a traditional quantitative parameter, statistical analyses were repeated for plasma concentration of LDL-C at baseline and results were compared to those of LPBS.

Two-sided p-values <0.05 were considered to indicate statistical significance. All statistical analyses and visualization of data were conducted using STATA® Statistical Software, Release 15.1 (StataCorp, College Station, TX).

RESULTS

In this longitudinal follow-up study the LPBS was measured in 589 RTR. Individual LPBS values were expressed as ratio between proteoglycan-bound cholesterol and plasma LDL-C levels (nmol/mmol). Baseline characteristics according to gender-stratified tertiles of LPBS are summarized in Table 1. The concentrations of total cholesterol (TC), LDL-C, apolipoprotein (apo) A-I (each p<0.001), triglycerides (p=0.004), apoB (p=0.012) and the LDL-C/apoB ratio (p<0.001) decreased significantly with increasing tertiles of LPBS. Systolic blood pressure (p=0.04) and BMI (p=0.015) showed a significant inverse association with binding susceptibility, but for other potential cardiovascular risk factors including age, history of cardiovascular events, diabetes mellitus, tobacco abuse, plasma triglycerides and HDL-C no relationship with LPBS was evident.

Table 1. Baseline characteristics according to gender-stratified tertiles of lipoprotein-proteoglycan binding susceptibility

Tertiles of lipoprotein-proteoglycan binding susceptibility (bound TC / LDL-C)				
	First (n=197)	Second (n=196)	Third (n=196)	*p*
Outcome parameter				
Lp-PG binding susceptibility, nmol/mmol	1.04 (0.94, 1.01)	1.29 (1.21, 1.34)	1.72 (1.64, 1.88)	<0.001
Recipient demographics				
Age, years	54.1 (43.9, 60.9)	52.8 (42.3, 61.0)	50.7 (41.5, 59.8)	0.099
Male gender, n (%)	109 (55.1%)	108 (55.1%)	108 (55.1%)	1.00
Current smoking, n (%)	71 (35.9%)	82 (42.5%)	81 (41.3%)	0.36
Body composition				
BMI, kg/m^2	26.8 ±4.4	25.8 ±4.2	25.6 ±4.2	0.015
Waist circumference, men, cm	101.4 ±11.8	99.0 ±12.6	98.9 ±12.8	0.23
Waist circumference, women, cm	95.0 (85.5, 104.5)	91.5 (81.0, 101.0)	93.5 (82.0, 104.0)	0.21
Waist-hip ratio, women, cm	0.91 (0.85, 1.00)	0.90 (0.85, 0.97)	0.94 (0.85, 1.02)	0.068
Waist-hip ratio, men, cm	1.02 (0.96, 1.07)	1.00 (0.95, 1.08)	1.02 (0.96, 1.08)	0.25
Lipids				
TC, mmol/l	5.89 ±1.10	5.69 ±1.07	5.28 ±1.00	<0.001
LDL-C, mmol/l	3.72 ±0.97	3.64 ±0.94	3.29 ±0.96	<0.001
HDL-C, mmol/l	1.12 ±0.34	1.11 ±0.30	1.05 ±0.33	0.054
Apolipoprotein A-I, g/l	1.57 (1.39, 1.78)	1.59 (1.41,1.78)	1.45 (1.29,1.65)	<0.001
Apolipoprotein B, g/l	1.10 (0.96, 1.24)	1.06 (0.93, 1.23)	1.01 (0.90, 1.21)	0.012
Triglycerides, mmol/l	2.21 (1.57, 2.92)	1.87 (1.41, 2.40)	1.82 (1.29, 2.41)	0.004
LDL-C/Apolipoprotein B	3.52±0.94	3.35±0.56	3.08±0.71	<0.001
Cardiovascular disease history				
Myocardial infarction, n (%)	14 (7.1%)	14 (7.2%)	20 (10.3%)	0.43
TIA/CVA, n (%)	14 (7.1%)	9 (4.6%)	9 (4.6%)	0.47
Systolic blood pressure, mmHg	152.5 (139.0, 167.0)	150.0 (137.0, 167.0)	149.0 (132.5, 163.0)	0.037
Medication				
Antihypertensive drugs, n (%)	177 (89.4%)	166 (84.7%)	171 (87.2%)	0.38
Calcineurin inhibitors, n (%)	151 (76.3%)	160 (81.6%)	151 (77.0%)	0.38
Proliferation inhibitors, n (%)	142 (71.7%)	140 (71.4%)	152 (77.6%)	0.30
Use of statins, n (%)	100 (50.5%)	105 (53.6%)	91 (46.4%)	0.37
Glucose homeostasis				
Glucose, mmol/l	4.65 (4.20, 5.14)	4.45 (4.08, 5.02)	4.52 (4.10, 4.88)	0.22
HbA1c, %	6.30 (5.90, 7.10)	6.40 (5.80, 6.90)	6.40 (5.80, 7.003)	0.44
HOMA-IR	2.31 (1.62, 3.45)	2.33 (1.64, 3.31)	2.29 (1.55, 3.80)	0.89
Diabetes mellitus, n (%)	34 (18.5%)	30 (16.5%)	30 (16.0%)	0.79
C-reactive protein, mg/l	2.08 (0.95, 4.76)	1.82 (0.68, 4.49)	2.32 (0.76, 5.43)	0.55
Donor demographics				
Age, years	37.0 (22.0, 49.0)	35.5 (23.5, 48.0)	41.0 (24.0, 52.0)	0.27
Male gender, n (%)	109 (55.3%)	110 (56.4%)	102 (52.3%)	0.70
Living kidney donor, n (%)	26 (13.1%)	25 (12.8%)	27 (13.8%)	0.96
Number of HLA mismatches	1.00 (0.00, 2.00)	1.00 (0.00, 2.00)	1.00 (0.00, 2.00)	0.64
(Pre)transplant history				
Acute rejection, n (%)	92 (46.5%)	90 (45.9%)	83 (42.3%)	0.67
Dialysis time, month	26.0 (13.0, 47.0)	30.0 (17.0, 48.0)	27.0 (12.0, 52.0)	0.35
Time between Tx and inclusion, month	80.5 (37.0, 149.0)	70.0 (28.5, 134.0)	67.0 (34.0, 117.0)	0.11
Renal allograft function				
eGFR, mL/min/1.73 m^2	46.3 ±15.2	47.4 ±15.2	47.1 ±16.9	0.78
Urinary protein excretion, g/24h	0.20 (0.00, 0.50)	0.20 (0.00, 0.50)	0.30 (0.20, 0.60)	0.13
Proteinuria ≥0.5 g/24 h, n (%)	50 (25.3%)	54 (27.8%)	62 (31.6%)	0.37

Normally distributed continuous variables are depicted as mean ± standard deviation, continuous variables with a skewed distribution are given as median [25th–75th percentile] and categorical variables are summarized by absolute numbers (percentages). Differences between tertiles of lipoprotein-proteoglycan binding susceptibility were tested

using one-way ANOVA for normally distributed continuous variables, Kruskal-Wallis test for continuous variables with a skewed distribution, and Pearson's chi-squared test for categorical variables.
(Abbreviations: Lp, lipoprotein; PG, proteoglycan; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; IQR, interquartile range; BMI, body mass index; ANOVA, analysis of variance; HDL-C, high-density lipoprotein-cholesterol, TIA, transient ischemic attack; CVA, cerebrovascular accident; HbA1c, hemoglobin type A1C; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HLA, human leukocyte antigen; Tx, transplantation; eGFR, estimated glomerular filtration rate.)

Subsequently, backward multiple linear regression analysis was used to assess which variables are determinants of LPBS in RTRs (Table 2). Concentration of total cholesterol (standardized β =-0.24, p<0.001) and apolipoprotein A-I (standardized β =-0.16, p<0.001) were inversely associated with LPBS. Model R^2 was 0.10.

To further explore factors associated with LPBS, we first correlated the LDL-C/apoB ratio as an, allowedly, relatively crude but easy to calculate measure of LDL size with LPBS. Previously a smaller size of LDL particles had been identified as a determinant of increased binding to proteoglycans [23]. Surprisingly an overall significant positive correlation was observed in the RTR (r=0.159 p<0.001). In order to gain more insight, we used NMR lipoprotein particle number and sizing data that were available for n=158 participants in our study. Interestingly, only small LDL had a positive correlation with LPBS (r=0.20, p=0.01), while medium (r=-0.07, n.s.) or large sized LDL particles (r=0.03, p=n.s.) did not correlate with LPBS. Since oxidative modification had been shown to decrease binding of apoB-containing lipoproteins to proteoglycans[24]we measured in a subset of our cohort (n=40) the conjugated diene content in these particles and correlated the result with LPBS. Consistent with previous reports, also in RTR a significant negative correlation between conjugated dienes and LPBS was observed (r=-0.46, p=0.003). In addition, enzymatic modification by group IIA phospholipases (sPLA2-IIA) were reported to increase binding of LDL to proteoglycans [23]. Also, this result could be replicated in our cohort, since plasma levels of sPLA2-IIA correlated positively with LPBS (r=0.42, p=0.016, n=40). Since it has been previously demonstrated that lipoprotein(a) (lp(a)) is increased among patients with low eGFR,[25] which in turn leads to a higher binding affinity of proteoglycans[26] we measured lp(a) levels in our study population. Lp(a) was not correlated with LPBS (r=-0.06, p=0.13). Furthermore, an adjusted Cox regression with lp(a) as independent variable showed that there is no significant association between lp(a) levels and GF (HR=1.11, p=0.25).

Table 2. Predictors of lipoprotein binding susceptibility

	β	95% CI	Standardized β	*p*
Total cholesterol	-0.94	-0.12, -0.06	-0.24	<0.001
Apolipoprotein A-I	-0.24	-0.35, -0.12	-0.16	<0.001

All variables with p<0.1 between tertiles were entered into a stepwise linear regression with backward elimination. (Abbreviations: CI, confidence interval)

Data concerning specific causes of death were available for a median follow-up of 7 years. Out of 130 (22%) patients that died in this period, 68 did so due to confirmed cardiovascular causes (12% of the total study population, 52% of the deceased patients). Furthermore, 29 (5% of the total study population, 22% of the deceased patients) patients died from malignancy, 23 (4% of the total study population, 18% of the deceased patients) from an infectious death and 10 (2% of the total study population, 7% of the deceased patients) from other causes. During the median follow-up of 9.5 years for GF, a total of 73 (13%) subjects experienced this end point.

At baseline, LDL-C, as well as LPBS, were comparable between survivors and deceased RTR, with respect to cardiovascular mortality (LDL-C: 3.90 ± 1.0 versus 3.88 ± 1.0 mmol/l, p=0.87; LPBS: 1.34 ± 0.42 versus 1.29 ± 0.5 nmol/mmol, p=0.33). While baseline LDL-C levels were also similar in patients developing GF or not (4.03 ± 1.38 versus 3.88 ± 0.91 mmol/l, p=0.22), LPBS was significantly higher in patients who subsequently developed GF compared to those with a surviving graft (1.47 ± 0.63 versus 1.32 ± 0.39 nmol/mmol, p=0.003).

Cox regression analysis revealed that neither LPBS nor the classical biomarker LDL-C were associated with CVD mortality (Table 3A); this conclusion remained valid after adjustment for a number of potential confounding factors in different statistical models. However, Cox regression analysis showed a prospective association between LPBS and chronic GF (hazard ratio [HR], 1.87; [95% confidence interval [95% CI], 1.24-2.84; p=0.003, Table 3B, model 1). Adjusting for age and gender did not considerably reduce this association (HR, 1.84; 95% CI, 1.21-2.81; p=0.004, Table 3B, model 2).

Comparably, adjustment for a number of other potentially impacting factors, namely use of statins, periods of acute rejection, number of HLA mismatches, primary renal disease, dialysis time, time between transplantation and inclusion, type of transplantation and donor age did not appreciatively change the significance of the prospective association. However, following additional adjustments for eGFR, significance was lost (HR, 1.25, 95% CI, 0.85-1.82; p=0.25, Table 3B, model 7). We attempted to further delineate the relationship of LPBS and eGFR. Pearson's correlation coefficients showed that there is no significant correlation between eGFR and LPBS (r=-0.03, p=0.52). Then an interaction term was computed and the association with graft failure was assessed (HR=0.99, p=0.40). This showed that there is no significant interaction between eGFR and LPBS.

In contrast to these findings with respect to the functional read-out of LPBS, LDL-C levels were not associated with GF, neither in univariate nor in all computed multivariable Cox regression models (Table 3B).

Table 3A. Comparison between the association of either LDL function (LPBS) or mass levels of LDL-C with cardiovascular mortality

	LPBS		LDL-C concentration	
	HR [95% CI]	*p*	HR [95% CI]	*p*
Model 1	0.70 [0.38 – 1.30]	0.26	0.97 [0.75 – 1.25]	0.81
Model 2	0.74 [0.41 – 1.34]	0.32	0.98 [0.76 – 1.27]	0.89
Model 3	0.76 [0.41 – 1.39]	0.37	1.03 [0.80 – 1.32]	0.82
Model 4	0.74 [0.41 – 1.34]	0.32	0.97 [0.75 – 1.25]	0.80
Model 5	0.76 [0.42 – 1.38]	0.36	1.00 [0.77 – 1.29]	0.98
Model 6	0.76 [0.41 – 1.37]	0.36	1.00 [0.78 – 1.30]	0.97
Model 7	0.73 [0.40 – 1.35]	0.33	0.99 [0.76 – 1.28]	0.92
Model 8	0.72 [0.40 – 1.32]	0.29	0.99 [0.76 – 1.28]	0.93

Model 1: crude; model 2: adjusted for age and gender; model 3: model 2+ adjustment diabetes mellitus; model 4: model 2+ adjustment for body mass index; model 5: model 2+ adjustment for dialysis time and time between transplantation and inclusion; model 6: model 2+ adjustment for type of transplantation and donor age; model 7: model 2+ adjustment for use of calcineurin inhibitors and proliferation inhibitors; model 8: model 2+ adjustment for use of statins. Abbreviations: LPBS, lipoprotein-proteoglycan binding susceptibility; LDL-C, low-density lipoprotein; HR, hazard ratio; CI, confidence interval.

Table 3B. Comparison between the association of either LDL function (LPBS) or mass levels of LDL-C with chronic graft failure.

	LPBS		LDL-C concentration	
	HR [95% CI]	*p*	HR [95% CI]	*p*
Model 1	1.87 [1.24 – 2.84]	0.003	1.13 [0.91 – 1.41]	0.25
Model 2	1.84 [1.21 – 2.81]	0.004	1.13 [0.91 – 1.41]	0.26
Model 3	1.85 [1.22 – 2.83]	0.003	1.15 [0.93 – 1.42]	0.20
Model 4	1.25 [0.85 – 1.82]	0.25	0.97 [0.78 – 1.20]	0.76
Model 5	1.85 [1.22 – 2.81]	0.004	1.14 [0.91 – 1.43]	0.24
Model 6	1.86 [1.23 – 2.83]	0.003	1.13 [0.90 – 1.42]	0.28
Model 7	1.89 [1.24 – 2.89]	0.003	1.13 [0.90 – 1.41]	0.29
Model 8	1.75 [1.15 – 2.69]	0.010	1.14 [0.90 – 1.45]	0.27

Model 1: crude; model 2: adjusted for age and gender; model 3: model 2 + adjustment for use of statins; model 4: model 2 + adjustment for estimated glomerular filtration rate; model 5: model 2 + adjustment for period of acute rejection; model 6: model 2 + adjustment for number of human leukocyte antigen mismatches, primary renal disease and period of acute rejection; model 7: model 2+ adjustment for dialysis time and time between transplantation and baseline; model 8: model 2+ adjustment for type of transplantation and donor age. (Abbreviations: LPBS, lipoprotein-proteoglycan binding susceptibility; LDL-C, low-density lipoprotein; HR, hazard ratio; CI, confidence interval.)

Cox regression analyses were repeated with crude proteoglycan binding. The results were not substantially different with regards to the normalized marker of LPBS (supplemental tables 1S and S2).

As shown in figure 1, the association of LPBS with chronic GF was not different for males versus females (p for interaction=0.12), subject with high versus low age (p for interaction=0.38), use of statins versus no use of statins (p for interaction=0.67), high versus low eGFR (p for interaction=0.17) or period of acute rejection versus no period of acute rejection (p for interaction=0.45). However, for the association of LPBS with CVD mortality there was an interaction with use of calcineurin inhibitors versus no use of calcineurin inhibitors (p for interaction = 0.03), indicating that the relationship of LPBS with CVD risk is stronger in

subjects that use calcineurin inhibitors.

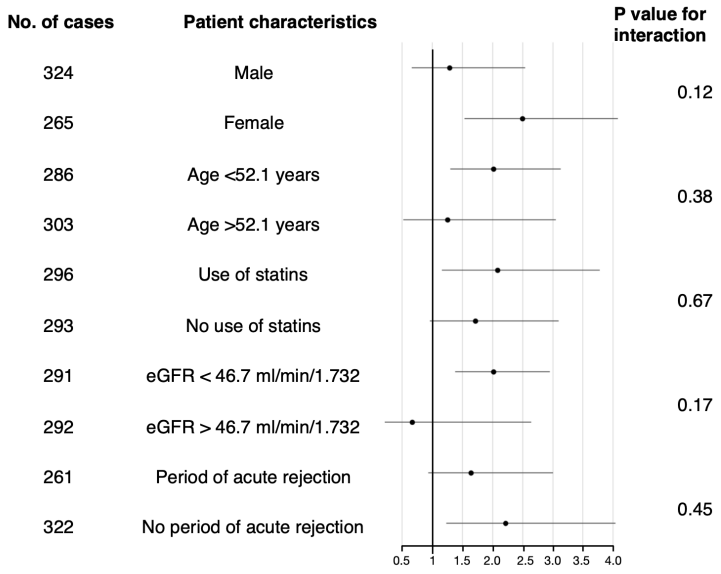

Figure 1. Hazard ratios for the association of LDL function (LPBS) with incident graft failure, by several participant level characteristics.

DISCUSSION

The results of this study demonstrate that beyond the static measurement of circulating LDL-C levels, functional metrics determining lipoprotein retention to proteoglycans such as LPBS can provide useful clinical information. Specifically, we show that in RTR LPBS was prospectively associated with chronic GF, a manifestation of de novo atherosclerosis, but not with CVD death, largely a consequence of preexisting, complex atherosclerotic lesions. We interpret these findings as being consistent with the response-to-retention hypothesis of atherosclerotic lesion formation[10]. In contrast, LDL-C was neither associated with incident CVD mortality nor with chronic GF.

Although RTR have a substantially elevated risk for atherosclerosis-related disease, classical biomarkers such as LDL-C or HDL-C levels fail to serve as predictors[1,3,5]. Thus, the identification of patients at risk to develop either CVD events or accelerated chronic GF represents an unmet clinical need. Dynamic functional tests for pro-atherogenic properties of apoB-containing lipoproteins might offer a realistic chance to fill this current

gap. Mechanistically, RTR have an increased proinflammatory and oxidative stress load[27–29] that could conceivably contribute to modify apoB-containing lipoproteins in a way that they bind with higher affinity to vascular proteoglycans. However, the precise molecular mechanisms of this deserves further research. Although we consistently observed that a high LPBS is associated with measures of small LDL particles (NMR as well as LDL-C/apoB ratio), high LPBS was also associated with lower triglycerides. Given the inverse correlation between triglycerides and small LDL in the general population, especially the determinants of triglyceride metabolism in the setting of RTR need to be better understood, since this specific patient group potentially experiences the combined impact of kidney (dys)function, pre-existing disturbances in metabolism such as insulin resistance and immunosuppressive medication. It also appears relevant to include in such studies measures of oxidation in lipoproteins of different size, since small LDL bind better to proteoglycans, but are on the other hand also more susceptible to oxidative modification [30], which would inhibit such binding[24]. A multimodal approach covering a multitude of different potentially impacting factors could possibly help to elucidate the perceived complex interaction of different processes and modifications relevant for the binding of lipoproteins to proteoglycans in RTR. Conversely, and not addressed in the present work, the function and/or quantity (and hence atherogenicity) of proteoglycans could be altered as well in RTR. Also here, important impacting factors are inflammation and oxidative stress. Oxidized LDL particles stimulate the production of modified proteoglycans with elongated chains, which consequently have a higher ability to retain LDL [31,32]. It is conceivable that a multiplicative effect of modifications in both lipoproteins and proteoglycans will lead to an even further enhanced binding susceptibility, and subsequently increased atherosclerotic lesion formation. Research in rats found that expression of perlecan, a basement membrane-type heparan sulfate proteoglycan, is significantly increased in renal allografts after experimental transplantation, compared to non-transplanted control kidneys and isografts [33]. The proteoglycan expression correlated with the severity of tissue remodeling and impaired graft function [33]. This underlines, although challenging in clinical routine, the potential benefits of including proteoglycans present in the allograft's extracellular matrix in individual TV risk analyses.

Potential therapeutic intervention options to decrease the interaction between LDL and proteoglycans are thus far limited. In vitro, glucosamine has been indicated to result in the production of proteoglycans by smooth muscle cells with a reduced binding capacity for LDL [34]. However, dietary glucosamine supplementation resulted in increased atherosclerotic lesion formation in preclinical models limiting the applicability of such an approach [35,36]. Statins were shown to have a dual effect, decreasing LDL-proteoglycan binding [21], as well as the production of proteoglycans with reduced affinity to LDL [37]. However, in our patients statin use was not associated with any change in LPBS. Finally, measures to enrich LDL in cholesteryl oleate such as canola oil appear to reduce the binding of LDL to proteoglycans [38], but prospective clinical studies addressing this concept are not available.

A potential limitation of the current work is that this study is from a single center in the North of the Netherlands, thus representing a population with a relatively homogenous and also narrow Caucasian genetic background. Further replication would be required to inform, if our results are generalizable. In addition, although TransplantLines is one of the largest prospective renal transplantation cohorts, the number of participants is still limited, thus impacting predictive power. Future longitudinal research also needs to address, if and in which direction the relationship of LPBS with graft function is causative. Next to our assumption that increased LPBS worsens graft function, it could mechanistically also be envisioned that a worsening graft function results in increased LDL modifications and thereby more LDL-proteoglycan binding [39]. After all, a decreased eGFR is to date the strongest predictor for incident GF [40]. To gain more mechanistic insights and distinguish lipid-driven from immune-mediated events (or elucidate an inter-dependency of these) it would be valuable to carry out serial histological evaluations of the vasculature of kidney grafts; however, due to the invasive nature of such work, respective studies are only possible to be carried out in preclinical models. Further, although EDTA (a known anti-oxidant in addition to being an anti-coagulant) plasma was used, sample generation and storage were similar for all patients and the assay was performed in an identical fashion for all samples using internal controls, a certain degree of oxidation cannot be formally excluded. In addition, despite differences in storage time being minor as opposed to time until the assay was performed, still samples were stored for a large number of years before analysis and different storage length of the samples might have a potential effect.. Furthermore, the clinical implementation of LPBS as a biomarker is at this point difficult due to logistic challenges and lack of standardization; overcoming these represents a future challenge. With respect to the chosen assay set-up we would like to point out that, although the use of isolated LDL in proteoglycan binding studies revealed important pathophysiological information [10,22,39], the aim of our present work was to set up an assay that can be performed in large cohorts and, if proven useful, be further developed for routine clinical chemistry laboratories. However, feasibility challenges of routine LPBS studies still include a lack of availability of sufficient amounts of standardized arterial proteoglycans, as well as using isolated LDL in the setting of a clinical test. To address the latter issue, we also used human plasma per unit volume. This procedure has the advantage that no previous information regarding the samples is required and no isolation of a specific class of lipoproteins needs to be carried out, that could potentially also impact lipoprotein composition or function. Importantly, another advantage of the chosen set-up is that any potential factors in plasma, which could influence the interaction of LDL with proteoglycans, are still present.

In summary, the current study indicates that LPBS as a dynamic test for the individual pro-atherogenicity of LDL particles is associated with incident GF. This association was particularly pronounced in patients with a low eGFR. Our work suggests that focusing on the interaction of lipoproteins with extracellular matrix components could lead to the

identification not only of useful personalized predictive biomarkers but also of potential pharmacological intervention targets. Thereby unmet clinical needs in the patient population of RTR could be successfully addressed. The goal would be to reduce lipid deposition in the vascular wall of coronary arteries and kidney grafts to prevent chronic GF and possibly also CVD events.

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PART II

STUDIES IN THE GENERAL POPULATION

8

HDL ANTI-INFLAMMATORY CAPACITY AND INCIDENT CARDIOVASCULAR EVENTS

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ABSTRACT

BACKGROUND

The role of high-density lipoprotein (HDL) function in cardiovascular disease represents an important emerging concept. The present study investigated whether HDL anti-inflammatory capacity is prospectively associated with first cardiovascular events in the general population.

METHODS

HDL anti-inflammatory capacity was determined as its ability to suppress tumor necrosis factor-α-induced vascular cell adhesion molecule-1 mRNA expression in endothelial cells *in vitro* (results expressed as achieved percent reduction by individual HDL related to the maximum tumor necrosis factor-α effect with no HDL present). In a nested case-control study design of the Prevention of REnal and Vascular End stage Disease study, 369 cases experiencing a first cardiovascular event (combined endpoint of death from cardiovascular causes, ischemic heart disease, nonfatal myocardial infarction and coronary revascularization) during a median of 10.5 years of follow-up were identified, and individually matched to 369 controls with respect to age, sex, smoking status and HDL cholesterol. Baseline samples were available in 340 cases and 340 matched controls.

RESULTS

HDL anti-inflammatory capacity was not correlated with HDL cholesterol or high sensitive C-reactive protein. HDL anti-inflammatory capacity was significantly lower in cases compared with controls (31.6% [15.7, 44.2] vs. 27.0% [7.4, 36.1], $p<0.001$), and was inversely associated with incident CVD in a fully adjusted model, (OR per 1SD=0.74, CI=0.61-0.90, $p=0.002$). Furthermore, this association was similar with all individual components of the CVD endpoint. The HDL anti-inflammatory was not correlated with cholesterol efflux capacity ($r=-0.02$, $p>0.05$). When combining both these HDL function metrics in one model both were significantly and independently associated with incident CVD in a fully adjusted model (efflux: OR per 1SD =0.74, $p=0.002$ and anti-inflammatory capacity: OR per 1SD =0.66 $p<0.001$). Adding HDL anti-inflammatory capacity improved risk prediction by the Framingham risk score, with a model likelihood ratio statistic increase from 10.50 to 20.40 ($p=0.002$).

CONCLUSIONS

The HDL anti-inflammatory capacity, reflecting vascular protection against key steps in atherogenesis, was inversely associated with incident cardiovascular events in a general population cohort, independent of HDL cholesterol and HDL cholesterol efflux capacity. Adding HDL anti-inflammatory capacity to the Framingham risk score improves risk prediction.

INTRODUCTION

Although circulating levels of high density lipoprotein (HDL) cholesterol are inversely associated with cardiovascular disease (CVD) risk in the general population[1], outcomes of pharmacological intervention trials aiming to increase plasma HDL cholesterol have been disappointing and largely negative [2–5]. Together with genetic studies showing that also lifelong low or high levels of HDL cholesterol do not relate as anticipated to CVD outcomes [6], focus has shifted from HDL cholesterol as biomarker towards the measurement of HDL function metrics [7]. A much-researched functionality of HDL particles is its ability to mediate cholesterol efflux from macrophage foam cells, the first step in the atheroprotective reverse cholesterol transport pathway [8,9]. It has been demonstrated that HDL cholesterol efflux capacity is indeed inversely associated with incident CVD events in the general population independent and even irrespective of HDL cholesterol levels [10–12]. However, cholesterol efflux still tracks considerably with HDL cholesterol and apolipoprotein A-1 levels [10–13], while an HDL function metric that is less dependent on the plasma HDL cholesterol concentration would be more interesting, both from the perspective of intervention and from the perspective of risk prediction. Since another key biological role of HDL is protection against inflammation[14], and given the inflammatory nature of the atherosclerotic process [15], particularly anti-inflammatory properties of HDL might have high clinical significance. In agreement with this hypothesis, a small study in patients who had experienced an acute myocardial infarction showed that a lower anti-inflammatory capacity of HDL relates to a higher incidence of new major cardiac events [16]. Whether a similar prospective association exists in the general population is currently not known. Therefore, the present study aimed to determine prospectively whether the anti-inflammatory capacity of HDL associates with incident CVD events in a general population cohort when taking established CVD risk factors into account. To be able to ascertain the relevance of this metric of HDL function irrespective of plasma HDL cholesterol, a nested case-control study design was chosen, matching participants not only for age and sex, but also for HDL cholesterol.

METHODS

STUDY POPULATION

The data that support the findings of this study are available from the corresponding author upon reasonable request. We performed a nested case-control study among participants in the Prevention of REnal and Vascular End stage Disease (PREVEND) study. PREVEND was initiated to investigate the association of renal damage with CV disease in a large cohort from inhabitants in the city of Groningen in the North of the Netherlands. Details of the study have been described elsewhere [10,17]. In the period between 1997-98, all inhabitants of the city of Groningen aged 28-75 years (a total of 85421 participants) were sent a questionnaire

containing information about the presence of CVD risk factors and morbidity and a vial to collect an early morning urine sample. In total, 40856 participants (47.8%) responded. The questionnaire collected information about the presence of risk factors of CVD and of CVD morbidity. Persons with diabetes using insulin and pregnant women were excluded. All participants with a urinary albumin concentration ≥ 10 mg/L were invited to the clinic together with randomly selected participants with a urinary albumin concentration < 10 mg/L. The study population comprised 8,592 predominantly Caucasian participants who completed the total screening program. The study was approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands (approval number: MEC96/01/022). All participants gave written informed consent.

STUDY DESIGN

First, those participants who had experienced a CVD event before the baseline evaluation were excluded. Cases were identified as participants who had a first CVD event before the end of follow-up (January 1, 2009). Cases were then divided into quartiles based on HDL cholesterol and stratified according to sex and current smoking behavior at baseline. Each case was matched to a control participant of the same sex, same smoking status, age (within 5 years) and HDL cholesterol (maximal difference 3.9 mg/dL [0.1 mmol/l]). Of the 8267 eligible participants all 369 cases that had been recorded during follow-up and 369 matched controls were initially identified. Blood samples for HDL anti-inflammatory capacity determinations were available in 357 controls and 352 cases. This resulted in 340 matched case-control pairs, which were included in the present analysis. The study questionnaire indicated that at the time of blood sampling none of the participants has experienced a recent acute illness, HIV infection, cancer or any other inflammatory condition.

OUTCOME MEASURES

The combined endpoint of our study was incident CVD, defined as death from CVD, hospitalization for myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), ischemic heart disease or coronary artery bypass graft (CABG). From the time of inclusion in the study, the vital status of the participants was checked through the municipal registry. The cause of death was obtained by linking the number of the death certificates to the primary cause of death, as coded by a physician from the Central Bureau of Statistics (CBS, Voorburg/Heerlen, The Netherlands). Causes of death were coded according to the 9th revision of the *International Classification of Diseases* (ICD-9. Information on MI (ICD-9 code 410), PTCA (code 45), ischemic heart disease (code 411) and CABG (code 414) was obtained from the national hospital information system (Prismant, Utrecht, The Netherlands). The censoring date was the date obtained from the municipal registry or date of death.

CLINICAL MEASURES, PROCEDURES AND DEFINITIONS

Body mass index (BMI) was calculated as the ratio between weight and height squared

(in kg/m^2). Blood pressure was measured using an automatic Dinamap XL model 9300 series device (Johnson-Johnson Medical, Tampa, FL, USA). Hypertension was defined as systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg or the use of anti-hypertensive drugs. Microalbuminuria was defined as urinary albumin excretion (UAE) between 30 and 300mg/24h based on two 24h urine collections. Type 2 diabetes mellitus (T2DM) was defined as a fasting glucose ≥ 126 mg/dL (7.0 mmol/L), a random glucose ≥ 200 mg/dL (11.1 mmol/L), self-report of a physician diagnosis or the use of glucose lowering drugs. Alcohol consumption was recorded assuming one drink to contain 10 grams of alcohol. Smoking was categorized into current, former and never. Estimated glomerular filtration rate (eGFR) was calculated applying the combined creatinine–cystatin C equation, which is considered to provide a more accurate estimate of GFR compared to the eGFR equation by creatinine alone [18,19]. Information on medication use was combined with data from a pharmacy-dispensing registry, which has complete information on drug use of > 95% of participants in the PREVEND study.

LABORATORY METHODS

Venous blood samples were obtained following 15 min rest after an overnight fast. Plasma glucose was measured directly after blood sampling. Plasma total cholesterol and glucose were assessed using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, New York, USA). Triglycerides were measured enzymatically. HDL cholesterol was measured with a homogeneous method (direct HDL, AEROSETTM System, Abbott Laboratories, Abbott Park, USA) [20]. Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Low density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula if triglycerides were ≤ 399 mg/dL (4.5 mmol/l). Apolipoprotein (apo) A1 and apoB were determined by nephelometry with reagents for Dade Behring nephelometer systems (BN II, Dade Behring Marburg, Germany). High sensitivity C-reactive protein (hs-CRP) was assayed by nephelometry with a lower limit of 0.175 mg/l (BNII N; Dade Behring, Marburg, Germany). Serum creatinine was measured by an enzymatic method on a Roche Modular analyzer (Roche Diagnostics, Mannheim, Germany), serum cystatin C by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). UAE was measured by nephelometry with a threshold of 2.3 mg/L (Dade Behring Diagnostic, Marburg, Germany). Cholesterol efflux capacity was quantified using human THP-1-derived macrophage foam cells and apolipoprotein B-depleted plasma as published previously[10].

DETERMINATION OF HDL ANTI-INFLAMMATORY CAPACITY

The HDL anti-inflammatory capacity was assessed in vitro as described previously (figure 1)[21]. Baseline EDTA plasma was obtained at time point of inclusion into PREVEND by centrifugation at 4°C, and samples were stored at -80°C until analysis. HDL was isolated from plasma by precipitation of ApoB-containing lipoproteins using 36% polyethylene glycol (PEG 6000, Sigma, St. Louis, MO, USA) exactly as published [11,12,21,22] and used directly for the HDL anti-inflammatory

assay. Human umbilical vein endothelial cells (HUVECs, provided by the Endothelial Cell Core Facility of the UMCG) were preincubated with either 2% individual apoB-depleted plasma samples or an equal volume of phosphate buffered saline (PBS) as a control for 30 min. Then, 10 ng/mL tumor necrosis factor α (TNF-α; R&D systems, Abingdon, UK) was added. After an additional incubation of 5 h, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and vascular cell adhesion molecule-1 (VCAM-1) mRNA expression levels were determined by quantitative real-time PCR (ABI-Prism 7700, Applied Biosystems, Carlsbad, CA) as described [21,22]. VCAM-1 expression levels were calculated relative to the average of the housekeeping gene cyclophilin and expressed as percent reduction compared with TNFα-stimulated cells without addition of HDL; thus, higher values indicate more efficient anti-inflammatory protection. The intra-assay coefficient of variation (CV) of this assay is 7.6%, the inter-assay CV is 8.8%. Previously we established that freezing of plasma does not impact the results and that the chosen concentration of HDL to be added was within the linear range of the assay [21,22]; sample storage of -80 °Celsius at least up to 4 years (n=20) does not affect the anti-inflammatory activity of HDL. Further, using actual samples from this study we determined that in our assay system less than 16.8% of the overall biological effect of the assay was not due to HDL and that it thus largely determines true effects of HDL. In apoB-depleted plasma samples (n=10) the average reduction of the maximum TNFα-induced VCAM-1 mRNA expression was 30.4±11.7 % (p=0.002); after removing HDL by ultracentrifugation from the apoB-depleted samples the remaining anti-inflammatory activity was 5.1±3.6 (n.s. compared with the maximum TNFα effect). To limit potential variation due to different assay conditions, measurements of the anti-inflammatory capacity of HDL were carried out at the same time using the same batch of pooled HUVECs and the same reagents. Measurements were performed in duplicate.

STATISTICAL ANALYSES

Differences in baseline characteristics were tested between participants who had experienced a cardiovascular event during follow up (cases) and those who had not (controls). Categorical variables are expressed as total numbers (%) and differences between groups were tested with chi^2 test. Normally distributed continuous variables were expressed as mean ± SD and differences tested using T-tests; skewed continuous variables were presented as median [25th, 75th quartile] and differences were assessed using Wilcoxon rank-sum test.

Spearman's rank correlation coefficients were used to assess relationships between baseline characteristics and anti-inflammatory capacity in crude analysis. Partial correlation coefficients were adjusted for age, sex and HDL cholesterol. Given the nested case-control design of the study (multivariable) conditional logistic regression analysis was used to assess the association of the HDL anti-inflammatory capacity with CVD outcome, with results being expressed as odds ratios (OR) with 95% confidence intervals (CI). OR were determined per 1 standard deviation (SD; 1SD= 22.2%) increase of anti-inflammatory capacity. Multivariable models were adjusted for established CVD risk factors. In sensitivity analyses the association

of the anti-inflammatory capacity with incident CVD was adjusted for potential confounders individually. Furthermore, the association of the anti-inflammatory capacity with individual CVD endpoints was assessed. The association of the cholesterol efflux capacity with incident CVD was shown as a comparison exposure. Additionally, a combination of both HDL anti-inflammatory capacity and HDL cholesterol efflux capacity was assessed.

Effect modification by gender, age, alcohol consumption, smoking status, BMI, diabetic status, hypertension, hsCRP, eGFR, UAE, total cholesterol, triglycerides and time between blood sampling and incident CVD was tested by inclusion of interaction terms. Subsequently subgroup analysis using interaction tests were performed in which OR were determined across categories of baseline characteristics. For continuous variables the median value was used as cutoff. The participant characteristics were sex (male versus female), age (<60.8 versus ≥ 60.8 years), alcohol consumption (<10 or ≥ 10 g/d), current or former smoking (yes versus no), BMI (<26.9 versus ≥ 26.9 kg/m^2), diabetes mellitus (yes versus no), hypertension (yes versus no), hsCRP <1.82 and ≥ 1.82 mg/L), eGFR (<90.0 and ≥ 90.0 mL/min per 1.73 m^2), UAE (<12.2 versus ≥ 12.2 mg/24 h), total cholesterol (<229 and ≥ 229 mg/dL), triglyceride (<121 and ≥ 121 mg/dL) and time between blood sampling and CVD event (<8.6 and ≥ 8.6 years). To assess the functional relationship of anti-inflammatory capacity with the probability of CVD events we used restricted cubic spline analysis with four knots placed at recommended percentiles according to Harrell[23]. A logistic regression with the spline term was performed, with adjustment for BMI, diabetes mellitus, LDL cholesterol, triglyceride levels, hypertension and hsCRP.

To further illustrate the relationship of HDL cholesterol levels and the metric of HDL functionality, anti-inflammatory capacity, we investigated the association of both variables with CVD in a mutually adjusted analysis. In this analysis cases and controls were used that were matched for age, sex and smoking status, however not for HDL cholesterol levels. Analyses were adjusted for CVD risk factors, namely BMI, diabetes mellitus, LDL cholesterol, triglyceride levels, hypertension and hsCRP.

Furthermore, the contribution of the two HDL function metrics anti-inflammatory capacity and cholesterol efflux capacity to disease prediction was assessed. Due to the nested nature of the analysis, the addition of anti-inflammatory capacity, cholesterol efflux capacity and both these function metrics together to the Framingham risk score was assessed using likelihood ratio statistics[24]. Additionally, we used Akaike's information criterion (AIC) and Bayesian information criterion (BIC)[25] to estimate whether substitution of HDL cholesterol level with the HDL function metric anti-inflammatory capacity in the Framingham risk score improved the predictive value of the risk equation.

Two-sided p-values <0.05 were considered statistically significant. Statistical analysis was performed using STATA version 15.0 (StataCorp, College Station, TX: StataCorp LP).

RESULTS

The median follow-up time was not different in cases (10.5 [9.9–10.8] years) and in controls (10.4 [9.9–10.8] years). As shown in table 1, cases had a significantly higher prevalence of hypertension and diabetes, used more lipid lowering drugs as well as having higher hsCRP, total cholesterol, LDL cholesterol, triglyceride and apoB levels. Cases had a significantly lower cholesterol efflux capacity. HDL cholesterol and apoA1 concentrations were similar between groups as a consequence of the matching procedure. Notably, the HDL anti-inflammatory capacity was significantly lower in cases, despite virtually no difference in HDL cholesterol levels (31.6% (15.7, 44.2) vs. 27.0% (7.4, 36.1), p<0.001). In univariate, as well as age, and age- and HDL cholesterol-adjusted analyses no clinical or laboratory value was significantly correlated with the HDL anti-inflammatory capacity (supplemental table 1).

Table 1. Characteristics of the 680 study participants according to case-control status at end of follow-up

	Controls	**Cases**	***p*-value**
Number	340	340	
Male gender (%)	239 (70%)	239 (70%)	1.00
Age, yr	59.0 ±10.8	59.2 ±10.9	0.88
BMI, kg/m^2	26.9 ±4.3	27.5 ±4.1	0.084
Smoking (%)			0.50
Current	71 (20.9%)	60 (17.6%)	
Former	125 (36.8%)	136 (40.0%)	
Never	144 (42.4%)	144 (42.4%)	
Alcohol intake (%)			0.60
<10g/d	247 (72.9%)	253 (74.6%)	
≥10g/d	92 (27.1%)	86 (25.4%)	
Hypertension (%)	164 (48.2%)	206 (60.6%)	0.001
Diabetes (%)	12 (3.5%)	24 (7.1%)	0.040
Lipid lowering drug use (%)	9 (2.6%)	20 (5.9%)	0.037
Anti-hypertensive medication use (%)	69 (20.3%)	97 (28.5%)	0.012
Systolic blood pressure, mmHg	136.7 ±20.4)	142.6 ±22.4)	<0.001
Diastolic blood pressure, mmHg	77.7 ±9.3	80.0 ±9.8	0.002
Glucose lowering drug use (%)	4 (1.2%)	13 (3.8%)	0.027
Fasting glucose, mg/dL	91.4 ±18.8	93.1 ±26.9	0.33
hsCRP, mg/L	1.6 (0.7, 3.4)	2.1 (0.9, 4.5)	0.004
eGFR, ml/min/1.73 m^2	89.3 ±15.6	87.9 ±15.9	0.26
UAE, mg/24 h	11.5 (7.0, 24.5)	13.0 (7.7, 28.4)	0.078
Total cholesterol, mg/dL	226.1 ±43.8	241.3 ±44.5	<0.001
LDL cholesterol, mg/dL	153.2 ±41.7	166.4 ±40.1	<0.001
HDL cholesterol, mg/dL	45.6 ±13.6	45.1 ±13.2	0.59
Triglycerides, mg/dL	115.9 (85.0, 160.6)	125.7 (88.5, 181.0)	0.044
ApoA1, mg/dL	1.3 ±0.3	1.3 ±0.3	0.11
ApoB, mg/dL	1.1 ±0.3	1.2 ±0.3	0.006
Cholesterol efflux capacity (AU)	1.0±0.2	0.9±0.3	0.002
Anti-inflammatory capacity, %	31.6 (15.7, 44.2)	27.0 (7.4, 36.1)	<0.001

Normally distributed continuous variables are presented as mean±SD. Continuous variables with a skewed distribution are presented as median (25th, 75th percentile). Categorical data are summarized by n (%). BMI, body mass index; hsCRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; UAE, urinary albumin excretion; LDL, low density lipoproteins; HDL, high density lipoproteins; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B.

In a univariate conditional logistic regression analysis, the HDL anti-inflammatory capacity showed an inverse association with incident CVD events (OR per 1SD =0.77, 95%CI= 0.66-0.91, p=0.002, table 2). When adjusting for BMI, alcohol intake, diabetes status, hypertension and use of lipid lowering drugs this association remained essentially unaltered (model 1, OR per 1SD =0.78, 95%CI= 0.66-0.93, p=0.005). After further adjustment for total cholesterol, apoA1 and triglyceride levels (model 2, OR per 1SD = 0.75, 95%CI=0.63-0.90, p=0.002) and hsCRP, UAE and eGFR (model 3, OR per 1SD = 0.74, 95%CI= 0.61-0.90, p=0.002) the inverse association of the anti-inflammatory capacity with incident CVD also did not materially change.

Table 2. Association of the HDL anti-inflammatory capacity with incident cardiovascular events in 340 control participants and 340 matched case participants expressed per 1 SD increase in anti-inflammatory capacity.

	OR per SD increase (1 SD= 22.2%)	95% confidence interval	P-value
Crude	0.77	0.66-0.91	0.002
Model 1	0.78	0.66-0.93	0.005
Model 2	0.75	0.63-0.90	0.002
Model 3	0.74	0.61-0.90	0.002

Data are ORs (95% CI) for incident cardiovascular disease events obtained with multivariable conditional logistic regression models. Triglycerides, urinary albumin excretion and hsCRP values were $\log_e$ transformed. Abbreviations: OR, odds ratio; SD, standard deviation. The use of glucose lowering drugs and antihypertensive medication is included in the definition of diabetes and hypertension, respectively.

Model 1: Crude + body mass index, alcohol intake (<10g per day or ≥10g per day), diabetes status, hypertension and use of lipid lowering drugs

Model 2: Model 1 + total cholesterol, apolipoprotein A1 and triglycerides

Model 3: Model 2 + high sensitivity C-reactive protein, urinary albumin excretion and estimated glomerular filtration rate

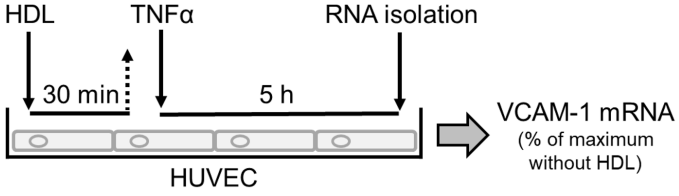

Figure 1. Schematic representation of the HDL anti-inflammatory assay

HUVECs were incubated with HDL for 30 minutes, whereafter TNFα was added for 5 hours. Then RNA was isolated and VCAM-1 mRNA expression determined. Abbreviations: HDL, high density lipoprotein; TNFα, tumor necrosis factor α; RNA, ribonucleic acid; HUVEC, human umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule-1.

In sensitivity analyses adjustment was performed for individual CVD risk factors (supplemental table 2), which did not significantly alter the association. Analysis were also repeated for individual components of the combined CVD endpoint. In crude analyses the HDL anti-inflammatory capacity was suggested to be inversely associated with incident CVD death (n=17, OR per 1SD =0.61, 95%CI=0.25-1.49, p=0.28), ischemic heart disease (n=92,

OR per 1SD =0.75, 95%CI=0.55-1.00, p=0.06), hospitalization for MI (n=139, OR per 1SD =0.85, 95%CI=0.65-1.10, p=0.22), PTCA (n=54, OR per 1SD =0.74, 95%CI=0.48-1.16, p=0.19) and CABG (n=38, OR per 1SD =0.72, 95%CI=0.44-1.18, p=0.19). Furthermore, in sensitivity analyses the association between cholesterol efflux capacity and incident CVD events was assessed as a comparison exposure (table 3). When combining the cholesterol efflux capacity and the anti-inflammatory capacity in one model, both these HDL function metrics were significantly and independently associated with incident CVD events in a crude (OR per 1SD =0.74, p=0.002 and OR per 1SD =0.69 p<0.001, respectively, table 3), as well as fully adjusted model (OR per 1SD =0.74, p=0.002 and OR per 1SD =0.66 p<0.001, respectively, table 3).

As shown in figure 2, the association of the HDL anti-inflammatory capacity with CVD was different for males and females (p for interaction =0.008), participants with high versus low BMI (p for interaction = 0.003) and high versus low triglyceride levels (p for interaction <0.001, figure 2). These interactions were also present with BMI (p for interaction = 0.003) and triglycerides (p for interaction <0.001) as continuous variables.

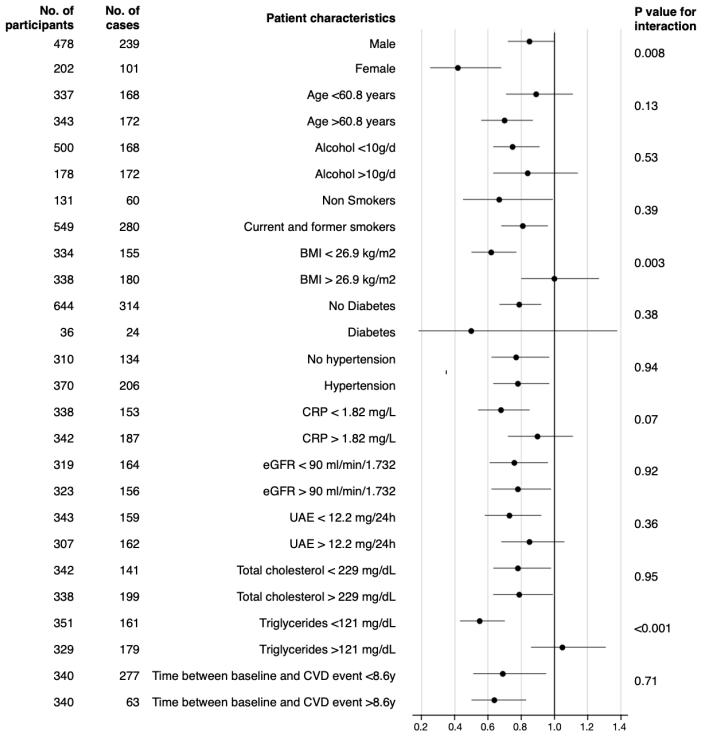

Figure 2. Odds ratios for incident cardiovascular events per 1 SD increase in the HDL anti-inflammatory capacity, by several participant level characteristics

Data are odds ratios (95% CI) for incident cardiovascular disease events obtained with logistic regression models. Triglycerides, urinary albumin excretion and hsCRP values were $\log_e$ transformed. Abbreviations: SD, standard deviation; BMI, body mass index; hsCRP, high sensitive- C-reactive protein; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; UAE, urinary albumin excretion.

Table 3. Comparison of the association of different HDL function metrics with incident CVD in 340 matched control participants and 340 case participants

	Anti-inflammatory capacity			Cholesterol efflux capacity			Combined model anti-inflammatory capacity and cholesterol efflux			
	OR per 1 SD increase	95% CI	P-value	OR per 1 SD increase	95% CI	P-value		OR per 1 SD increase	95% CI	P-value
Crude	0.77	0.66-0.91	0.002	0.70	0.59-0.83	<0.001	Anti-inflammatory capacity	0.74	0.62-0.87	<0.001
							Cholesterol efflux capacity	0.69	0.57-0.82	<0.001
Model 1	0.78	0.66-0.93	0.005	0.70	0.59-0.84	<0.001	Anti-inflammatory capacity	0.75	0.63-0.90	0.001
							Cholesterol efflux capacity	0.69	0.58-0.83	<0.001
Model 2	0.75	0.63-0.90	0.002	0.70	0.58-0.85	<0.001	Anti-inflammatory capacity	0.72	0.61-0.87	0.001
							Cholesterol efflux capacity	0.69	0.57-0.84	<0.001
Model 3	0.74	0.61-0.90	0.002	0.67	0.55-0.82	<0.001	Anti-inflammatory capacity	0.74	0.61-0.90	0.002
							Cholesterol efflux capacity	0.66	0.54-0.81	<0.001

Data are ORs (95% CI) for incident cardiovascular disease events obtained with multivariable conditional logistic regression models. Triglycerides, urinary albumin excretion and hsCRP values were $\log_e$ transformed. Abbreviations: OR, odds ratio; SD, standard deviation; CEC, cholesterol efflux capacity. The use of glucose lowering drugs and antihypertensive medication is included in the definition of diabetes and hypertension, respectively.

Model 1: Crude + body mass index, alcohol intake (<10g per day or ≥10g per day), diabetes status, hypertension and use of lipid lowering drugs

Model 2: Model 1 + total cholesterol, apolipoprotein A1 and triglycerides

Model 3: Model 4b + high sensitivity C-reactive protein, urinary albumin excretion and estimated glomerular filtration rate

Restricted cubic spline analysis showed that the probability of a CVD event is approximately constant for values of HDL anti-inflammatory capacity ranging from -40% to 10%, while at higher values of the HDL anti-inflammatory capacity an increase of anti-inflammatory capacity is directly proportional to a decrease in risk (figure 3).

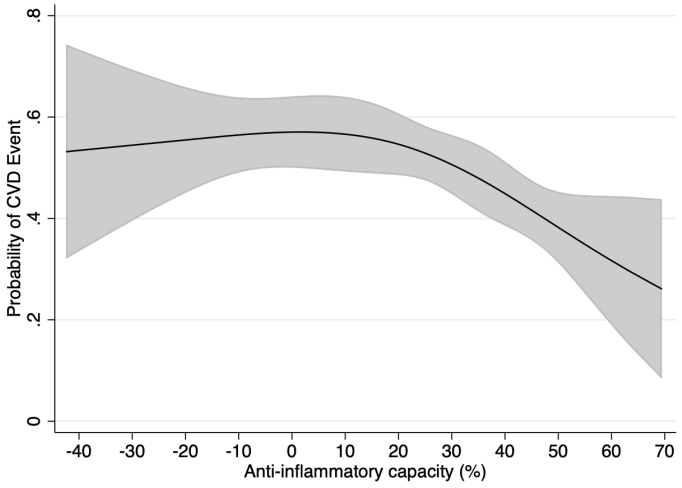

Figure 3. Probability of CVD events according to HDL anti-inflammatory capacity
Probabilities were obtained by multivariate conditional logistic regression using restricted cubic splines with four knots, adjusted for body mass index, diabetes mellitus, low density lipoprotein cholesterol, triglyceride levels, hypertension and high sensitivity C-reactive protein.

When adding anti-inflammatory capacity to the well-established Framingham risk score, the model likelihood ratio statistic increases from 10.50 to 20.40. A significant likelihood-ratio test (p=0.002) indicated a statistically significant greater predictive power. With further addition of the cholesterol efflux capacity the model likelihood ratio statistic again increased to 32.84, with a significant likelihood ratio test (p=0.0005). When substituting HDL cholesterol with anti-inflammatory capacity in the FRS equation the AIC decreases from 936 to 542 and the BIC from 945 to 550, again suggesting an increase in model fit.

The HDL anti-inflammatory capacity was inversely associated with risk of CVD events in a CVD risk-adjusted model (OR per 1SD =0.74, 95% CIs=0.60-0.92, p=0.007), which remained unchanged after further adjustment for HDL cholesterol (OR per 1SD =0.74, 95% CIs=0.60-0.92, p=0.008) (figure 4). On the other hand, plasma HDL cholesterol was not significantly associated with a lower risk of CVD events in a model adjusted for CVD risk factors (OR per 1SD =1.19, 95% CIs=0.61-2.33, p=0.60). Further adjustment for anti-inflammatory capacity changed the OR slightly (OR per 1SD =1.10, 95% CIs=0.55-2.14, p=0.81, figure 4).

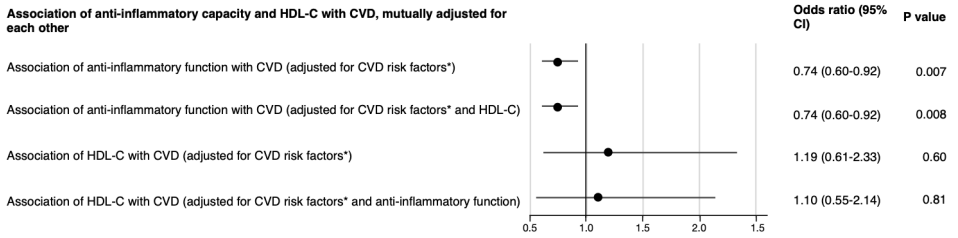

Figure 4. Association of the HDL anti-inflammatory capacity and HDL cholesterol levels with CVD risk mutually adjusted for each other.
Data are odds ratios (95% CI) for incident cardiovascular disease events obtained with multivariable logistic regression models. Abbreviations: HDL-C, high density lipoprotein cholesterol; CVD, cardiovascular disease.
* Diabetes, hypertension, body mass index, plasma low density lipoprotein cholesterol, triglycerides and high sensitivity C-reactive protein.

DISCUSSION

The results of this prospective individually matched nested case-control study demonstrate that the anti-inflammatory capacity of HDL is associated with incident CVD events in a general population cohort independent of HDL cholesterol and apoA1 levels. Since only very limited associations with established CVD biomarkers were observed, notably including HDL cholesterol, hsCRP and HDL cholesterol efflux capacity, we surmise that determining the HDL anti-inflammatory capacity has the potential to provide independent clinical information on risk assessment for patients initially free of clinically manifest CVD. In support of this point, we found that either adding HDL anti-inflammatory capacity to the Framingham risk score or substituting HDL cholesterol for this functional metric in the Framingham risk score significantly improved risk prediction. Notably, the association of the HDL anti-inflammatory capacity with incident CVD was also independent of HDL cholesterol efflux capacity.

Atherosclerosis has a strong inflammatory component. Endothelial inflammation, specifically as indicated by increased expression of adhesion molecules such as VCAM-1, is observed in animal models of atherosclerosis as well as in human atherosclerotic plaques[15]. The importance of inflammation is further evidenced by the usefulness of hsCRP or adhesion molecules as CVD biomarkers [26,27] and by the recent results of the CANTOS trial showing that treatment with Canakinumab, which targets the interleukin-1β innate immunity pathway, improves CVD outcomes [28]. In addition, decreasing functional VCAM-1 expression either by the use of an antibody[29] or genetically[30] resulted in decreased atherosclerosis development in mouse models. HDL is regarded as a component of the innate immune system and has been shown in *in vitro* as well as *in vivo* studies to decrease VCAM-1 expression in vascular tissue, next to other markers of inflammation[31–34], neutralize bacterial lipopolysaccharide [35]

and protect against bacterial infections [14]. Therefore, the anti-inflammatory activity of HDL, as measured in our current study, represents conceivably a reflection of a pathophysiologically relevant process with respect to atherosclerotic cardiovascular disease. Since the cholesterol content of HDL has not been mechanistically implicated in the anti-inflammatory or anti-infectious properties of these particles, it is biologically plausible that we did not observe a correlation between HDL cholesterol and anti-inflammatory capacity. On the other hand, also no correlation of the HDL anti-inflammatory capacity with hsCRP was detected. These results could potentially indicate that the processes causing systemic inflammation with the secretion of acute phase proteins by hepatocytes are distinct from the ones impacted by the action of HDL in the vessel wall. Of note, the association of the HDL anti-inflammatory capacity was less strong in participants with higher BMI and higher triglycerides, suggesting that maybe a higher endogenous inflammatory load, commonly associated with these conditions, attenuates the effect. Conversely, it appears that the HDL anti-inflammatory activity could be of greater predictive utility in subgroups with lower BMI or triglycerides. However, more work and data would be required before such a general conclusion could be formally drawn.

It is currently incompletely understood which components of the complex HDL particles, that carry a large amount of protein and lipid cargo, govern the anti-inflammatory activity towards endothelial or vascular smooth muscle cells. It has been previously shown that the acute phase protein serum amyloid A (SAA) can replace apoA1 on HDL particles and that enrichment of HDL with SAA can give rise to a pro-inflammatory HDL particle in end-stage renal disease patients; in addition, SAA can signal via the formyl-peptide receptor 2 [31]. However, such a mechanism does not appear highly likely in our study from the general population investigating participants with mostly normal renal function and an overall low inflammatory load. Changes in the composition of phospholipid species have also been reported to impact the anti-inflammatory activity of HDL; specifically, an increased phosphatidyl-serine content was associated with a better anti-inflammatory capacity [36]. Directly [33], but also in a more indirect fashion via induction of endothelial nitric oxide synthase and the subsequent production of nitric oxide[37], sphingosine-1-phosphate within HDL can act to decrease inflammation. More mechanistic studies seem warranted to explore HDL components and underlying molecular pathways, since such an improved understanding could result in the identification of a simple biomarker reflecting HDL (dys)function that would be more suitable for clinical routine than a rather complex assay based on primary cells. In addition, such mechanistic insights would also provide novel intervention targets aimed at reducing CVD risk.

Several methodological aspects and limitations need to be considered. Our study included participants of Caucasian ethnicity with a relatively narrow genetic background. Replication is needed before generalizing our findings. In addition, stroke was not included as an endpoint.

The case-control design of this study limits the ability to investigate the association of the HDL anti-inflammatory capacity with other known CVD risk factors. Further, HDL function assays are not yet standardized, thus allowing interpretations only in context of the assay conditions applied. A similar reasoning holds true for the isolation of HDL. It is noteworthy that although precipitation of apoB-containing lipoproteins has the advantage to preserve all HDL subfractions, still up to approximately 17% of the anti-inflammatory biological activity captured by the assay is contained within the fraction that is by ultracentrifugation rendered devoid of more mature HDL, but conceivably still contains biological activity from preβ-HDL particles. Since these data were generated in subjects from the general population participating in the current study, it is currently unclear if such a reasoning would also apply to patients with a high inflammatory load. In contrast to precipitation of apoB-containing lipoproteins, during isolation by ultracentrifugation, although cleaner in terms of eliminating plasma components, high centrifugal forces and ionic strengths are applied resulting in the loss of numerous HDL-associated proteins and a relative depletion in preβ-HDL [9]. Further, for large cohorts such as the present one, ultracentrifugation is technically not feasible, if re-freezing of the isolates is to be avoided. For this reason, apoB-depletion has been largely applied in other cohort studies dealing with HDL function [11–13].

In summary, the current work identified an impaired HDL anti-inflammatory capacity as a functional metric prospectively associated with increased cardiovascular risk in the general population. The results of the HDL anti-inflammatory assay cover a considerable dynamic range from largely suppressing endothelial cell inflammation (positive values) towards capturing pro-inflammatory HDL particles (negative values). Notably, the HDL anti-inflammatory capacity was independent of a large number of established cardiovascular biomarkers including hsCRP. In contrast to the cholesterol efflux function of HDL that tracks moderately with HDL cholesterol levels in case of the radiolabel assay (in this cohort, $r=0.439$, $p<0.001$,[10,12,13] and much less in case of the BODIPY-cholesterol efflux assay[11], the anti-inflammatory capacity did not show any significant correlation with HDL cholesterol or apoA-I. Combined our results place clinical meaning to the potential of testing for anti-inflammatory properties of HDL to achieve improved cardiovascular risk prediction.

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THE TRIGLYCERIDE TO HDL CHOLESTEROL RATIO IS ASSOCIATED WITH NEW-ONSET CHRONIC KIDNEY DISEASE IN THE GENERAL POPULATION

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ABSTRACT

BACKGROUND

Chronic kidney disease (CKD) represents a global burden with high morbidity and mortality. Due to the largely asymptomatic course of CKD, disease prediction in the general population is an unmet clinical need. We investigated whether dyslipidemia reflected by the triglyceride (TG)/high density lipoprotein cholesterol (HDL-C) ratio is associated with incident CKD in the general population.

METHODS

The current prospective observational study in the Prevention of REnal and Vascular End stage Disease (PREVEND) cohort (8,592 subjects, median follow-up 11.7 [11.3-12.6] years) included 6,629 participants after exclusion due to pre-existent CKD or suspected acute infection. The primary end point was incident CKD during follow-up, defined as eGFR <60 ml/min/1.73 m2 or urinary albumin excretion (UAE) >30 mg/24h.

RESULTS

383 subjects reached the endpoint. Cox regression showed a significant association of the TG/HDL-C ratio with incident CKD in a fully adjusted model independent of the metabolic syndrome (HR= 1.06, 95% CI=1.01-1.11, p=0.013), that was more pronounced in the 2,453 participants with UAE ≥10 mg/L (HR=1.13, 95% CI=1.02-1.26, p=0.025) than in 4,176 subjects with UAE <10 mg/L (HR=1.08, 95% CI=0.98-1.18, p=0.11). This association stayed significant after sensitivity analysis with exclusion of participants with metabolic syndrome (HR= 1.06, 95% CI =1.00-1.11, p=0.039). The TG/HDL-C ratio improved risk prediction by the validated kidney failure risk equation with a significant model likelihood-ratio statistic increase (p<0.001).

CONCLUSION

The TG/HDL-C ratio serves as a predictive biomarker for incident CKD. These data stress the need for thorough lipid management in the general population to prevent new-onset CKD.

INTRODUCTION

Chronic kidney disease (CKD) is a growing global health burden, with 10% of the adult population in developed countries being affected, ranking it the 12th leading cause of death worldwide.[1] Patients with CKD suffer an incremental increase in cardiovascular risk and a substantial proportion will eventually progress to end-stage renal disease requiring renal replacement therapy.[2,3] CKD is defined as kidney damage for ≥ 3 months, confirmed by biopsy or markers of kidney damage or a GFR < 60 ml/min/1.73 m^2 or an urinary albumin excretion (UAE) > 30 mg/24h.[4] Optimal treatment is challenging, as CKD often goes undiagnosed until later stages due to being essentially asymptomatic in nature.[5] Identification of causative risk factors enabling early detection is therefore of great relevance, in order to limit disease progression as early as possible. This point is clinically even more important, since no dedicated medicines for primary prevention of CKD are currently available and therapeutic intervention is thus limited to the modulation of risk factors.

Diabetes is a well-known risk factor for incident CKD.[6] For subjects without diabetes the largest risk prediction effort for primary CKD prevention so far, carried out by the CKD Prognosis Consortium, identified age, sex, race/ethnicity, eGFR, history of cardiovascular disease, smoking, hypertension, BMI, and urinary albumin concentration as additional risk factors.[7] Lipid parameters were not included in this study.

A potential link between kidney and lipid metabolism, however, has been suggested. Dyslipidemia is frequent in CKD patients, particularly reflected in increased triglyceride and decreased HDL cholesterol levels.[8,9] Dyslipidemia has also been linked to the progression of pre-existing CKD.[10,11] On the other hand, only limited information is available how dyslipidemia impacts incident CKD in the general population. Three studies identified low HDL cholesterol as a risk factor for new-onset CKD,[12–14] but two of these were conducted only in males [12,13] and all lacked triglyceride values. Another work, including a more comprehensive lipid panel,[15] concluded that high triglycerides and low HDL cholesterol associate with risk for an increase in serum creatinine, but the number of cases was low and no GFR measurements were available. Further, dyslipidemia associated with the metabolic syndrome was reported to confer an increased incident CKD risk, however, eGFR was calculated based on the Modification of Diet in Renal Disease (MDRD) formula that uses serum creatinine.[16] Of note, none of the above-mentioned studies reported urinary albumin excretion, an essential parameter to establish a CKD diagnosis.[17]

Therefore, the current work aimed to assess whether dyslipidemia as an easy-to-determine, modifiable risk factor increases the risk of incident CKD in the general population using both accepted clinical measures for CKD as outcome definition, eGFR and urinary albumin excretion. Since it has been shown that capturing dyslipidemia in the form of the triglyceride/

high density lipoprotein cholesterol (TG/HDL-C) ratio has a greater predictive value for CVD than single measures of lipid abnormalities and that this ratio predicts disease progression in patients with established kidney disease, we decided to also use this parameter in the evaluation of new-onset CKD.[18]

METHODS

STUDY POPULATION

We performed a prospective observational study in the Prevention of REnal and Vascular End stage Disease (PREVEND) cohort. PREVEND was initiated to investigate the association of renal damage with CVD in a large cohort from inhabitants in the city of Groningen in the North of the Netherlands. Details of the study have been described elsewhere.[19,20] In the period between 1997-98, all inhabitants of Groningen aged 28-75 years (a total of 85,421 participants) were sent a postal questionnaire and a vial to collect an early morning urine sample. In total, 40,856 subjects (47.8%) responded. The questionnaire collected information about the presence of risk factors of CVD and CVD morbidity. Diabetic subjects using insulin and pregnant women were excluded. The study population comprised 8,592 subjects who completed the total screening program, until end of follow-up on 01-01-2009.

STUDY DESIGN

Participants with a suspected active infection at the time of blood sampling, as defined by a hs-CRP>20mg/L, were excluded from the present study (n=528), since inflammation has a known impact on lipid metabolism resulting in higher triglyceride and lower HDL-C levels.[21]

Furthermore, participants who had CKD at baseline, defined as eGFR < 60 ml/min/1.73 m^2 or urinary albumin excretion (UAE) > 30 mg/24h were excluded (n= 1,306).
The association of the TG/HDL-C ratio with deterioration of kidney function was assessed both in individuals with a beginning kidney dysfunction, defined as UAE above 10mg/L (n= 2,453)[4,17] and those without discernible impairment, defined as UAE below 10mg/L (n= 4,176).

Furthermore, as dyslipidemia is closely associated with the metabolic impairments summarized under the term “metabolic syndrome”, sensitivity analysis was performed after exclusion of participants with an established diagnosis of the metabolic syndrome. The study was approved by the medical ethics committee of the University Medical Center Groningen. All participants gave written informed consent.

OUTCOME MEASURES

The main predictor in this study was the TG/HDL-C ratio, which was computed by dividing the triglyceride concentration by the HDL-C concentration (both in mmol/l). The primary

end point of this study was CKD, defined as an eGFR < 60 ml/min/1.73 m^2 or urinary albumin excretion (UAE) > 30 mg/24h, in accordance with KDIGO guidelines.[4,17]

MEASUREMENTS AND DEFINITIONS

For a detailed description of all parameters collected for the PREVEND participants please see.[22] Briefly, body mass index (BMI) was calculated as the ratio between weight and height squared (in kg/m^2). Blood pressure was measured using an automatic Dinamap XL model 9300 series device (Johnson-Johnson Medical, Tampa, FL, USA). Hypertension was defined as systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg or the use of anti-hypertensive drugs. Type 2 diabetes mellitus (T2DM) was defined as a fasting glucose ≥ 7.0 mmol/L, a random glucose ≥ 11.1 mmol/L, self-report of a physician diagnosis or the use of glucose lowering drugs. Alcohol consumption was recorded assuming one drink to contain 10 grams of alcohol. Smoking was categorized into current, former and never. Estimated glomerular filtration rate (eGFR) was calculated applying the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation.[23] Metabolic syndrome was ascertained based on the criteria of the National Cholesterol Education Program Expert Panel.[24] UAE was measured by nephelometry with a threshold of 2.3 mg/L (Dade Behring Diagnostic, Marburg, Germany) and was multiplied by urine volume to obtain a value of UAE in mg/24 h. Two 24-h urine samples were obtained and the values averaged. HDL cholesterol was measured with a homogeneous method (direct HDL, AEROSETTM System, Abbott Laboratories, Abbott Park, USA). Low density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula if triglycerides were ≤ 399 mg/dL (4.5 mmol/l). Triglycerides were measured enzymatically. Information on medication use was combined with information from a pharmacy-dispensing registry, which has complete information on drug use of > 95% of subjects in the PREVEND study.

STATISTICAL ANALYSES

Sex-stratified quartiles of the TG/HDL-C ratio were computed and differences in baseline characteristics were tested between quartiles. Categorical variables are expressed as total numbers (%) and differences between groups were tested with the chi^2 test. Normally distributed continuous variables were expressed as mean ± SD and differences tested using one-way ANOVA; skewed continuous variables were presented as median [25th, 75th quartile] and differences were assessed using Kruskal-Wallis test.

Cox proportional hazard regression was used to assess the association of the TG/HDL-C ratio with the endpoint CKD, with results expressed as hazard ratios (HRs) with 95% confidence intervals (CI). Multivariable models for Cox regression were adjusted for known predictors of kidney function and dyslipidemia, namely sex, age, BMI, smoking, alcohol consumption, diabetes, history of CVD, use of antihypertensive drugs, systolic blood pressure, use of lipid lowering drugs, total cholesterol, hsCRP and metabolic syndrome. Schoenfeld residuals

test was used to test the proportional hazard assumption and was found not to be violated. Cox proportional hazard regression was performed separately for individuals with a UAE >10mg/L and UAE <10mg/L at baseline. Sensitivity analysis was performed after exclusion of participants with a confirmed diagnosis of the metabolic syndrome.

Furthermore, subgroup analysis using interaction tests were performed in which HR were determined across categories of baseline characteristics. For continuous variables the median value was used as cutoff. The subject characteristics were sex (male versus female), age (<46.8 versus ≥46.8 years), alcohol consumption (<10 or ≥10 g/d), current or former smoking (yes versus no), BMI (<25.3 versus ≥25.3 kg/m^2), diabetes mellitus (yes versus no), hypertension (yes versus no), hsCRP <1.12 and ≥1.12 mg/L), total cholesterol (<5.5 and ≥5.5 mmol/L), eGFR (<99.2 and ≥99.2 mL/min per 1.73 m^2), UAE (<8.1versus ≥8.1 mg/24 h), and triglycerides (<1.4 and ≥1.4mmol/L).

The contribution of the TG/HDL-C ratio to disease prediction was also evaluated. We assessed the addition of the TG/HDL-C ratio to the kidney failure risk equation (KFRE), a validated and well implemented predictor of kidney function decline.[25] Due to the nested nature of the analysis, the addition of the TG/HDL-C ratio to the KFRE was assessed using likelihood ratio statistics.[26] Two-sided P values <0.05 were considered statistically significant. Statistical analysis was performed using STATA version 15.0 (StataCorp, College Station, TX: StataCorp LP).

RESULTS

BASELINE DEMOGRAPHIC CHARACTERISTICS

After exclusion due to suspected acute infection, as determined by a hsCRP >20 mg/l, and exclusion of participants with pre-existing CKD, 6629 subjects were eligible for inclusion in the study. Subjects were followed for a median of 11.7 (11.3-12.6) years. As shown in table 1, subjects in the higher quartiles of the TG/HDL-C ratio were significantly older, were less likely to be smokers, had a lower alcohol intake, a higher prevalence of hypertension, diabetes, history of CVD, and used more lipid lowering, anti-hypertensive as well as anti-diabetic drugs. Subjects in increasing quartiles had significantly higher systolic and diastolic blood pressure, BMI, fasting glucose, hsCRP, eGFR, UAE, total cholesterol, LDL-cholesterol, and apoB. As expected, a higher quartile of the TG/HDL-C ratio coincided with lower HDL-C and apoA1 levels as well as higher triglycerides.

Table 1. Characteristics of the 6,629 study participants according to quartiles of the triglyceride/HDL-C ratio

	First quartile	Second quartile	Third Quartile	Fourth Quartile	*P*-value
Number	1,660	1,655	1,658	1,656	
TG/HDL-C	0.4 (0.3, 0.5)	0.7 (0.6, 0.9)	1.1 (0.8, 1.4)	2.1 (1.5, 2.9)	<0.001
Male gender	874 (52.7%)	874 (52.8%)	874 (52.7%)	873 (52.7%)	1.00
Age, years	45.5 ±11.8	47.3 ±12.1	49.2 ±12.2	51.1 ±11.7	<0.001
BMI, kg/m^2	23.8 ±3.1	25.0 ±3.6	26.1 ±3.9	27.9 ±4.1	<0.001
Smoking					<0.001
Current	603 (36.3%)	536 (32.4%)	487 (29.4%)	442 (26.7%)	
Former	656 (39.5%)	578 (34.9%)	555 (33.5%)	540 (32.6%)	
Never	392 (23.6%)	536 (32.4%)	611 (36.9%)	666 (40.2%)	
Alcohol intake					<0.001
<10g/d	1172 (70.9%)	1225 (74.4%)	1263 (76.6%)	1281 (77.8%)	
≥10g/d	480 (29.1%)	422 (25.6%)	385 (23.4%)	366 (22.2%)	
Hypertension	265 (16.0%)	372 (22.5%)	500 (30.2%)	682 (41.2%)	<0.001
Diabetes	8 (0.5%)	21 (1.3%)	34 (2.1%)	90 (5.4%)	<0.001
History of CVD	32 (1.9%)	61 (3.6%)	60 (3.6%)	96 (5.8%)	<0.001
Lipid lowering drug use	30 (1.8%)	64 (3.9%)	102 (6.2%)	147 (8.9%)	<0.001
Anti-hypertensive medication use	95 (5.7%)	154 (9.3%)	211 (12.7%)	345 (20.8%)	<0.001
Systolic blood pressure, mmHg	121.3 ±17.0	124.0 ±17.5	127.3 ±18.3	132.1 ±18.8	<0.001
Diastolic blood pressure, mmHg	70.5 ±9.0	71.8 ±8.9	73.5 ±9.2	75.7 ±8.8	<0.001
Glucose lowering drug use	5 (0.3%)	15 (0.9%)	19 (1.1%)	46 (2.8%)	<0.001
Fasting glucose, mmol/L	4.5 ±0.6	4.7 ±0.7	4.8 ±0.8	5.1 ±1.3	<0.001
hsCRP, mg/L	0.6 (0.3, 1.4)	0.9 (0.4, 2.1)	1.5 (0.6, 3.0)	1.8 (0.9, 4.0)	<0.001
eGFR, ml/min/1.73 m^2	100.6 ±13.8	99.2 ±13.8	97.4 ±14.1	95.8 ±14.2	<0.001
UAE, mg/24 h	7.7 (5.7, 11.4)	7.8 (5.8, 11.6)	8.3 (5.7, 12.4)	8.8 (6.0, 14.1)	<0.001
Total cholesterol, mmol/L	5.1 ±1.0	5.4 ±1.0	5.7 ±1.1	6.1 ±1.2	<0.001
LDL cholesterol, mmol/L	3.1 ±0.9	3.5 ±0.9	3.9 ±1.0	4.0 ±1.1	<0.001
HDL cholesterol, mmol/L	1.7 ±0.4	1.4 ±0.3	1.2 ±0.3	1.0 ±0.2	<0.001
ApoA1, g/L	1.5 ±0.3	1.4 ±0.3	1.4 ±0.3	1.3 ±0.2	<0.001
ApoB, g/L	0.8 ±0.2	0.9 ±0.2	1.1 ±0.3	1.2 ±0.3	<0.001
Triglycerides, mmol/L	0.7 (0.6, 0.8)	1.0 (0.8, 1.1)	1.3 (1.1, 1.5)	2.1 (1.7, 2.7)	<0.001

Normally distributed continuous variables are presented as mean±SD. Continuous variables with a skewed distribution are presented as median (25th, 75th percentile). Categorical data are summarized by n (%). Abbreviations: TG, triglyceride; HDL-C, high density lipoprotein cholesterol; BMI, body mass index; hsCRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; UAE, urinary albumin excretion; LDL, low density lipoproteins; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B.

TIME TO EVENT ANALYSIS

The CKD endpoint was reached in 383 subjects (5.7%). Of the individuals with a UAE < 10 mg/L at inclusion 117 (2.8%) reached the endpoint, whereas 266 (10.7%) individuals with a baseline UAE > 10mg/L reached the endpoint. Cox regression showed a significant association of the TG/HDL-C ratio with incident CKD (HR= 1.06, 95%CI 1.03-1.09, p<0.001, table 2) in an age- and sex-adjusted model. This association remained largely unchanged after adjusting for potential confounders, including a fully adjusted model (HR= 1.06, CI 95% 1.01-1.11, p=0.013, table 2). These results indicate that a higher TG/HDL-C ratio is prospectively associated with an increased risk of CKD independent of other known risk factors and importantly also independent of the metabolic syndrome.

Table 2. The association of the triglyceride/HDL-C ratio with chronic kidney disease

	Hazard ratio	95% CI	*p*
Model 1	1.06	1.03-1.09	<0.001
Model 2	1.05	1.01-1.10	0.008
Model 3	1.05	1.01-1.10	0.016
Model 4	1.06	1.01-1.11	0.011
Model 5	1.06	1.01-1.11	0.011
Model 6	1.06	1.01-1.11	0.013

Data are hazard ratios (95% CI) for incident chronic kidney disease, obtained with multivariable cox regression models.
Abbreviations: HDL-C, high density lipoprotein cholesterol; CI, confidence interval.
Model 1: Crude + age and sex
Model 2: Model 1 + body mass index, alcohol intake (<10g per day or ≥10g per day), diabetes status and smoking status (never or former/current)
Model 3: Model 2 + history of cardiovascular disease, use of anti-hypertensives and systolic blood pressure
Model 4: Model 3 + total cholesterol and use of lipid lowering drugs
Model 5: Model 4 + high sensitivity C-reactive protein
Model 6: Model 5 + metabolic syndrome

Since a higher percentage of participants with increased UAE at baseline reached the CKD end point during follow-up, we next stratified the cohort by UAE. In patients with low UAE (<10mg/L) the TG/HDL-C ratio was not significantly associated with CKD in a crude (HR= 1.06, 95%CI 1.00-1.13, p=0.06, table 3) or fully adjusted model (HR= 1.08, 95%CI 0.98-1.18, p=0.11, table 3). The TG/HDL-C was, however, significantly associated with CKD in patients with a high UAE (>10mg/L) in an age- and sex-adjusted (HR= 1.14, 95%CI 1.05-1.24, p=0.003, table 3) as well as a fully adjusted model (HR= 1.13, 95%CI 1.02-1.26, p=0.025, table 3).

Table 3. The association of the triglyceride/HDL-C ratio with chronic kidney disease according to UAE

	UAE < 10mg/L (n= 4,176)			UAE ≥ 10mg/L (n= 2,453)		
	Hazard ratio	**95% CI**	***p***	**Hazard ratio**	**95% CI**	***p***
Model 1	1.06	1.00-1.13	0.06	1.14	1.05-1.24	0.003
Model 2	1.05	0.99-1.12	0.10	1.13	1.03-1.23	0.007
Model 3	1.05	0.99-1.12	0.10	1.13	1.04-1.23	0.006
Model 4	1.07	0.10-1.15	0.07	1.13	1.04-1.24	0.006
Model 5	1.07	0.99-1.15	0.07	1.13	1.03-1.24	0.008
Model 6	1.08	0.98-1.18	0.11	1.13	1.02-1.26	0.025

Data are hazard ratios (95% CI) for incident chronic kidney disease, obtained with multivariable cox regression models.
Abbreviations: HDL-C, high density lipoprotein cholesterol; CI, confidence interval.
Model 1: Crude + age and sex
Model 2: Model 1 + alcohol intake (<10g per day or ≥10g per day) and smoking status (never or former/current)
Model 3: Model 2 + history of cardiovascular disease,
Model 4: Model 3 + total cholesterol and use of lipid lowering drugs
Model 5: Model 4 + high sensitivity C-reactive protein
Model 6: Model 5 + metabolic syndrome

To further confirm that an increased TG/HDL-C ratio confers risk of CKD outside of the metabolic syndrome, sensitivity analysis was performed after exclusion of participants with a diagnosis of the metabolic syndrome. Still the TG/HDL-C ratio was significantly associated

with CKD in an age- and sex-adjusted model (HR= 1.05, 95%CI 1.00-1.10, p=0.038, table 4), as well as in a fully adjusted model (HR= 1.06, 95%CI 1.00-1.10, p=0.039, table 4).

As shown in figure 1, the association of the TG/HDL-C ratio with CVD was different for males versus females (p for interaction = 0.02), subjects with lower versus higher age (p for interaction = 0.02), high versus low alcohol consumption (p for interaction <0.001), high versus low BMI (p for interaction = 0.02), high versus low CRP (p for interaction = 0.003), high versus low total cholesterol (p for interaction <0.001), high versus low eGFR (p for interaction = 0.008) and high versus low UAE (p for interaction = 0.001).

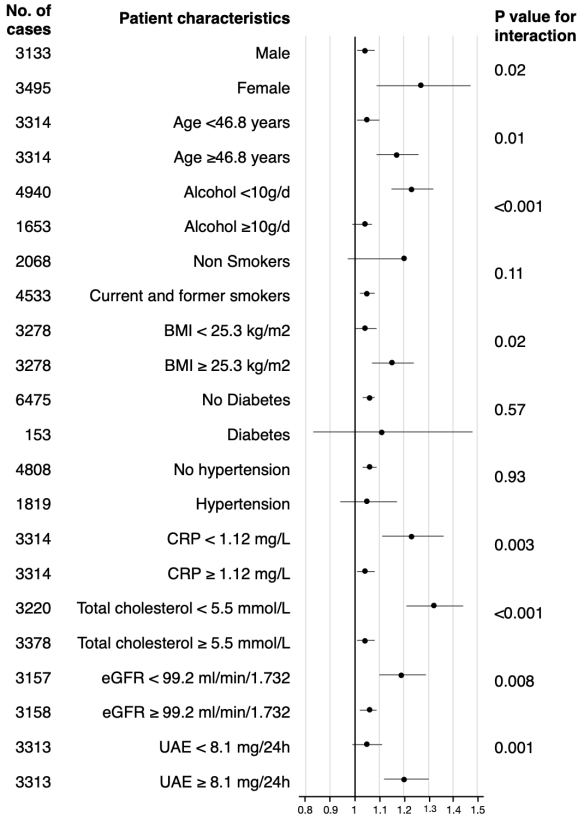

Figure 1. Hazard ratios for the association of the TG/HDL-C ratio with incident chronic kidney disease, by several participant level characteristics

Data are hazard ratios (95% CI) for incident chronic kidney disease obtained with Cox regression models. Abbreviations: BMI, body mass index; CRP, high sensitive- C-reactive protein; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; UAE, urinary albumin excretion.

Table 4. Sensitivity analysis of the association of the triglyceride/HDL-C ratio with chronic kidney disease in participants without metabolic syndrome (n= 5,601)

	Hazard ratio	95% CI	*p*
Model 1	1.05	1.00-1.10	0.038
Model 2	1.05	1.00-1.10	0.038
Model 3	1.05	1.00-1.10	0.037
Model 4	1.05	1.00-1.11	0.043
Model 5	1.06	1.00-1.11	0.039

Data are hazard ratios (95% CI) for incident chronic kidney disease, obtained with multivariable cox regression models.
Abbreviations: HDL-C, high density lipoprotein cholesterol; CI, confidence interval.
Model 1: Crude + age and sex
Model 2: Model 1 + body mass index, alcohol intake (<10g per day or ≥10g per day), diabetes status and smoking status (never or former/current)
Model 3: Model 2 + history of cardiovascular disease, use of anti-hypertensives and systolic blood pressure
Model 4: Model 3 + total cholesterol and use of lipid lowering drugs
Model 5: Model 4 + high sensitivity C-reactive protein

DISEASE PREDICTION

When adding the TG/HDL-C ratio to the KFRE the model likelihood ratio statistic increases from 208 to 225. A likelihood ratio test gives a p value of <0.001, demonstrating that adding the TG/HDL-C ratio to the KFRE results in a significantly improved model fit.

DISCUSSION

The results of this study demonstrate that in the general population the TG/HDL-C ratio is significantly associated with incident CKD, defined as an eGFR < 60 ml/min/1.73 m2 or UAE > 30 mg/24h. Importantly, the increase in risk conferred by the higher TG/HDL-C ratio occurred outside of the metabolic syndrome, but showed an interaction with albuminuria. Hereby, our results stress the importance of good lipid control in clinical practice, particularly of triglycerides.

Precise risk prediction is an important, thus far not fully met, clinical need for CKD patients. The early stages of the disease are largely asymptomatic. Identification of at-risk members of the general population is therefore vital in order to initiate adequate treatment to prevent CKD development or limit disease progression. Treatment includes elimination of reversible causes of kidney failure. However, for a large number of patients no direct cause can be identified. Research into the etiology of CKD, with the aim of identifying modifiable risk factors is therefore not only important for disease prediction, but also for finding new treatment modalities to slow or ideally halt disease progression.

We identified few previous general population studies addressing a potential prospective relationship between dyslipidemia and CKD.[12–16] Of note, albuminuria was determined in none of these and not included as CKD end point, further stressing the novelty of the current

approach. Two of these studies included only male participants and in both triglycerides were also not determined.[12,13] Using serum creatinine as proxy for CKD, one concluded that the LDL-C/HDL-C ratio increases CKD risk,[12] while in the other low HDL-C associated with an increase in serum creatinine during follow-up, but not with a decline in eGFR.[13] In the Framingham Heart Study low HDL-C increased CKD risk as judged by a decline in eGFR, but also in this study triglycerides were not mentioned .[14] From ARIC two publications are available. The first did not determine eGFR, included few cases and handled a relatively high cut-off with respect to serum creatinine as measure of pre-existing renal disease; however, both high triglycerides and low HDL-C associated prospectively with an increase in serum creatinine.[15] The second analysis from ARIC used eGFR values and reported that in the context of the metabolic syndrome increased triglycerides confer an increased CKD risk.[16]

In the cardiovascular field triglycerides represent an emerging risk factor reflecting incompletely lipolyzed so-called remnant lipoproteins.[27] Several Mendelian randomization studies suggested that triglycerides are a causal risk factor for atherosclerotic CVD,[28,29] while HDL-C is increasingly regarded as a sort of long-term integrator of circulating triglycerides comparable to HbA1c reflecting longer term alterations in blood glucose.[30] One way to characterize dyslipidemia that takes this concept into account is via the TG/HDL-C ratio. It was e.g. shown that the combination of these two risk factors has a higher predictive value for CVD events, compared to single measures of dyslipidemia.[18,31] Consequently, also a number of novel therapeutic strategies focus on lowering raised circulating triglyceride levels. Among these are, next to lifestyle interventions and fibrates, omega-3-fatty acids, as exemplified by the recent icosapent ethyl outcomes trial.[32] Further, anti-sense oligonucleotide strategies are in development aiming to decrease the expression of endogenous inhibitors of lipoprotein lipase such as apoC3 or ANGPTL3.[33,34] Our data suggest that extrapolation of such treatments to the kidney field would be worthwhile. Of note, statins have almost no impact on circulating triglycerides.[35]

With respect to the interaction between triglycerides and albuminuria on CKD risk that we observed it is currently unclear which component represents the cause and which the consequence. More studies are required to clarify this pathophysiologically important point. However, a potential detrimental role of dyslipidemia for the kidney has been suggested by the lipid nephrotoxicity hypothesis indicating that concomitant hyperlipidemia and proteinuria causes self-perpetuating renal disease even once the initial insult was no longer present.[36] Preclinical models lend further evidence to the lipid nephrotoxicity hypothesis. Several studies indicated that feeding mice a high fat diet caused severe glomerular disease and increased the severity of proteinuria.[37–39] Lipid deposition in the kidney could be caused by a certain spill-over of lipids into non-adipose tissue once energy intake gradually exceeds the body's ability to store fat in adipose tissue.[40] Such 'ectopic lipid

accumulation' can also be seen in the kidneys, where lipids can sediment in virtually all cell types including podocytes and proximal tubules.[41]

Specific strengths of our current work should be pointed out and certain limitations considered. Strengths include a large number of participants that were thoroughly prospectively followed in a general population cohort specifically designed to address the CKD end point. This contrasts to the other available general population studies investigating CKD as an end point, which constitute secondary analyses in cohorts primarily set up to investigate CVD outcomes.[12–16] This point is illustrated by our study being the only one published thus far that includes both definitions of CKD according to current guidelines, namely increased UAE and decreased eGFR. Further strengths are that our data are of relevance for primary prevention of CKD instead of secondary prevention, and that we investigated the consequences of dyslipidemia outside the context of the metabolic syndrome. A limitation of our work is that PREVEND consists of White participants with a relatively narrow genetic background. Therefore, validation in other cohorts including different ethnicities and ideally also different social and economic settings is desirable. Further, only a single measurement of plasma lipids at time of inclusion was performed leaving a certain possibility of day-to-day variation in triglyceride levels. However, since HDL-C values are more stable,[42] we believe that by using the TG/HDL-C ratio a substantial amount of a potential daily variation in triglycerides is normalized for. If anything, we expect circulating triglycerides to be underestimated in our cohort, since participants were informed beforehand of the date of blood draw, which usually triggers a healthier lifestyle behavior.

In conclusion, our results show that the TG/HDL-C ratio is a risk factor for CKD in the general population, independent of the metabolic syndrome but interacting with albuminuria. Our results underline the importance of good lipid management in the general population, especially in a context of developing albuminuria. Due to the low costs and broad availability of the measurements, using the TG/HDL-C ratio in daily clinical practice is realistic and potentially very valuable.

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DISCUSSION AND FUTURE PERSPECTIVES

The importance of studying lipids in renal transplant recipients is twofold: firstly, although short term outcomes such as acute graft failure have improved substantially in recent decades, long term outcomes of RTR are still disappointing, with a high rate of CVD, relatively short life expectancy and a substantial threat of chronic graft failure. The potential contribution of lipid parameters to these outcomes has largely been overlooked, despite the fact that modifiable lipid risk factors potentially have a substantial effect on patient outcomes.

Secondly, as RTR have a vastly increased risk of CVD when compared to the general population they are an ideal model population. CVD processes can be studied in RTR and findings can potentially be extrapolated to the general population. Two types of atherogenic processes can be observed in RTR, (i) preexisting, mostly complex atherosclerotic lesions, which are the underlying pathology for CVD, and (ii) *de novo* atherosclerotic lesion formation in the graft, known as transplant vasculopathy (TV), a major contributing cause for chronic graft failure.

The aim of this thesis were to identify novel lipid biomarkers in i. patients with advanced stages of kidney disease and ii. members of the general population. With the goal of deepening the understanding about disease etiology, it is possible to identify predictive biomarkers and ideally to identify potential targets for future intervention.

Atherosclerosis is a complex and protracted pathophysiological process, which often begins in adolescence[1]; however clinical consequences are only mainly observed in later life. The process of atherosclerosis is initiated by retention of oxidized macrophages, which bind to proteoglycans in the vessel wall.[2] These macrophages take up lipids, turning them into foam cells. Progressive buildup of plaques leads to narrowing of the vessel lumen, with possible occlusion or rupture. LDL and HDL play a central role in the pathogenesis of atherosclerosis. HDL, the traditional 'good' cholesterol, can not only stop the buildup of atherosclerotic plaques, but indeed also contribute the reduction of plaques.[3] Reverse cholesterol transport (RCT) describes the take up of lipids into HDL from plaques, and transportation and excretion in the liver.[3] However, the beneficial functions of HDL are more extensive than only RCT, with increasing numbers of anti-oxidant and anti-inflammatory functions of HDL being discovered.

PART I:
ADVANCED STAGES OF KIDNEY DISEASE

The main anti-atherogenic effect of HDL is arguably its ability to promote RCT, the transport of cholesterol out from foam cells contained in atherogenic plaques.[3] The ability of HDL to carry out this process is therefore a vital protective effect for CVD. HDL is prone to oxidative alterations, which impacts its ability to carry out various functions.[4,5] **Chapter 2** demonstrates that HDL from ESRD is dysfunctional in mediating reverse cholesterol transport. As ESRD patients face a large oxidative stress load, it is conceivable that the oxidative stress affects the apolipoproteins associated with HDL, resulting in a lack of function. In order to assess this hypothesis, TBAR levels within HDL were measured, as a marker of oxidative modification. ESRD HDL had significantly higher levels of TBARS, which negatively correlated with two functional parameters of HDL, namely cholesterol efflux from macrophage foam cells and cholesterol uptake in hepatocytes. Furthermore, we were able to replicate the functional deficits of ESRD-HDL through in vitro oxidation. Our findings therefore lend evidence to the notion that ESRD-HDL is modified by oxidative stress, resulting in a loss of function which could impact the risk of CVD.

The main structural protein of HDL is apo-A1, which drives many of the beneficial anti-atherogenic functions of HDL.[6] The occurrence of auto-antibodies to apoA1 has been described in the literature in various high-risk populations as well as in the general population.[7–10] In **chapter 3** we found that 11.5% of RTR had high levels of anti-apoA1 IgG. These auto-antibodies were associated with CVD mortality, but not all-cause mortality or graft failure. Again, the occurrence of anti-apoA1 IgG may be linked to oxidative stress. Due to oxidative stress the apoA1 molecules are altered and not recognized as 'self' by the immune system, therefore aiding the development of auto-antibodies. The exact mechanism by which anti-apoA-1 leads to increased CVD risk is unknown. Anti-apoA1 potentially disrupts the functionality of apoA1, and can therefore have a deleterious effect on the athero-protective functions of this important protein.

The initiation of atherosclerotic plaque formation is the binding of LDL to the vessel wall, described in the widely accepted response-to-retention hypothesis.[2] This process is enabled by proteoglycans on the vessel wall, that allow LDL particles to bind.[2] The affinity with which LDL and proteoglycans are bound is therefore an important factor in the extent to which an individual might be susceptible to atherosclerotic disease. It has previously been shown that oxidized LDL has a higher affinity for proteoglycans.[11,12] In **chapter 7** we showed that binding of LDL from RTR to proteoglycans is significantly associated with graft failure, but not CVD. Interestingly, measures of LDL-C were not associated with either endpoint, despite clear recommendations for LDL-C lowering in RTR.[13] Our results suggest that focus should be shifted from static measures of LDL-C, to functional measures of the LDL particle.

Additional clinical information could be obtained from function measures, with relevance to the atherogenic process.

The notion that non-traditional CVD risk factors impact chronic graft failure is strengthened in **chapter 4**, where we show that the TG/HDL-C ratio is associated with chronic graft failure and premature mortality. The TG/HDL-C ratio is a reliable reflection of dyslipidemia, with a higher predictive value than the individual measures of TG and HDL-C.[14,15] Furthermore, TG levels fluctuate heavily, whereas the TG/HDL-C ratio is a relatively stable marker.[16,17] Unlike measures of TG, the TG/HDL-C does not have to be assessed in a fasting state, therefore increasing its applicability in clinical practice. As of yet there are no guidelines recommending a cut-off value for the TG/HDL-C ratio in RTR. As a first step to lowering TG, lifestyle changes should be implemented. Fibrates have shown to successfully and safely lower TG in the general population, but concerns exist regarding application in RTR.[18,19] Omega 3 fatty acids could offer an alternative, as they have been shown to be safe and effective in RTR.[20,21]

Clinical treatment guidelines recommend statin therapy in all RTR, irrespective of age and LDL-C levels.[22] Use of statins lowers LDL-C cholesterol levels, which translates to a reduced CVD risk in the general population. However, as we have demonstrated in chapter 6, LDL-C levels are not associated with CVD death in RTR in observational studies. The evidence to support the recommendation of statin therapy in all RTR is sparse, as only one trial with statin therapy in RTR has been carried out. The ALERT trial showed that the use of fluvastatin did not lower CVD risk in the main analysis, but a significant association was found in post-hoc analyses.[23] Furthermore, concern has been raised about the simultaneous use of the immunosuppressant cyclosporine and statins. *In vitro* and *in vivo* studies have shown raised cyclosporine and statin levels with concomitant administration of both drugs, however the clinical consequences of this pharmacological interaction have thus far not been explored in detail.[24–27] **Chapter 6** shows that use of statins was not associated with CVD outcomes in a propensity matched cohort of RTR. Subsequent sensitivity analyses in those RTR that received cyclosporine treatment showed that statin use was even associated with an increased risk of CVD. Different statins vary in their metabolism, bioavailability and excretion, however, a number of them (simvastatin, lovastatin and atorvastatin) are metabolized by cytochrome P450 3A4.[25] Cyclosporine is also metabolized by cytochrome P450 3A5,[26] making it plausible that a saturation of this enzyme is reached, with subsequent rises of both cyclosporine and statins. Cyclosporine has a wide variety of dose dependent adverse effects and therefore requires very precise dosages with frequent evaluation of titers. Adverse effects include nephrotoxicity[28], induction of the hemolytic-uremic syndrome[28], an elevated risk of infection,[29] raised blood pressure,[30] increased circulation LDL particle numbers and increased oxidisability of LDL[28,31] and unfavorable effects of the fibrinolytic system[32]. A raise of serum cyclosporine levels can therefore contribute to a vastly increased CVD risk.

Due to the pathophysiological similarities between TV and classic atherosclerosis it is plausible that the same risk factors apply to both. In **chapter 5,** we found that the Framingham risk score (FRS) is associated with graft failure. The FRS is to date the best researched CVD risk prediction model and is used world-wide.[33] It includes the risk factors gender, age, smoking, systolic blood pressure, total cholesterol, and HDL-C, all key metrics thought to pathophysiologically contribute to the formation of atherosclerotic plaques.[33] Chapter 5 therefore strengthens the notion that TV and classic atherosclerosis share a similar aetiology, with possible implications for risk stratification and management techniques.

PART II: GENERAL POPULATION

A growing body of evidence is showing that inflammation is a fundamental part of atherogenesis. Biomarkers for inflammation predict risk of CVD independently of other risk factors[34,35] and trials involving interleukin antagonists have been shown to successfully reduce CVD risk.[36]

Chapter 8 successfully demonstrated that the HDL anti-inflammatory capacity is associated with future CVD events, independently of HDL-C levels in the general population. The significance of our results is two-fold. Firstly, our results allow for further delineation and deepening of our understanding of the HDL functional properties; Secondly, they portray an interesting potential target for pharmacological intervention. Although HDL is regarded a component of the innate immune system[37], the exact mechanisms with which HDL contributes to immunology are still being unraveled. Chapter 8 successfully quantifies the anti-inflammatory capacity of HDL and provides proof of concept for the association of the HDL anti-inflammatory capacity with CVD events.

The early stages of CKD are largely asymptomatic, therefore at the time of diagnosis patients often have advanced disease, accompanied by irreversible damage.[38] Early identification of at-risk patients is therefore an important clinical goal, in order to allow for preventative actions, as well as timely treatment. The TG/HDL-C has the potential to act as a predictive biomarker to identify patients at risk for CKD, as **Chapter 9** shows that the TG/HDL-C ratio is associated with CKD in the general population. The results of chapter 9 draw attention to the need for good lipid control, in order to slow disease progression in the general population.

FUTURE PERSPECTIVES

Studies from this thesis show that in the framework of atherogenesis the functionality of lipoproteins might be a more important factor with regards to outcomes, than static measures of lipid concentration. However, these findings have thus far not been reflected in clinical treatment guidelines, which largely focus on concentrations of LDL-C and disregard other lipid parameters. The translation of recent findings into clinical practice are an important future perspective in the field of lipidology.

A number of potential modifiable risk factors have been described in this thesis. Lowering of triglycerides could potentially result in a substantial benefit with regards to long term outcomes in both RTR as well as the general population. Randomized clinical trials (RCT) therefore form a vital and immensely interesting next step in unraveling the possibilities that close lipid control has. Furthermore, our results highlight a number of important functional aspects of lipoproteins in disease prediction. In order to utilize these assays in daily clinical practice standardized protocols have to be developed, with methodological techniques that allow for well-standardized, higher throughput measures. Our assays are very informative, however also time consuming and costly; developing assays that can be performed on a large scale or alternatively allow for identification of surrogate biomarkers will therefore be of interest for new opportunities in disease prediction.

We have demonstrated that functionality of lipoproteins is an important risk factor. However, the factors that influence loss of function in both HDL and LDL particles remain to be further delineated. We have shown that oxidative changes are associated with loss of function of HDL in ESRD. Furthermore, it remains plausible that oxidative stress is also an important modifiable factor in RTR. A period of dialysis prior to transplantation results in oxidative stress with consequent changes in lipoproteins, which can plausibly be associated with outcomes post transplantation. Furthermore, oxidative stress caused by the transplantation and the lowered kidney function of RTR can further contribute to this process. The exact mechanism that leads to lipoprotein dysfunction remains to be unraveled, with the ultimate goal of implementing strategies to minimize these factors.

CONCLUSION

Lipoproteins and lipids are overlooked modifiable risk factors in the general population and in populations with decreased renal function. There is a need for closer attention to lipids in clinical guidelines.

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NEDERLANDSE SAMENVATTING
LIST OF PUBLICATIONS
ABOUT THE AUTHOR
ACKNOWLEDGEMENTS

NEDERLANDSE SAMENVATTING

Deel 1: De rol van functionaliteit van lipoproteïnen bij niertransplantatiepatiënten.
In **deel 1** van dit proefschrift is de rol van verschillende lipiden parameters bij patiënten met eindstadium nierfalen en bij niertransplantatiepatiënten onderzocht.

In **hoofdstuk 2** wordt de mogelijkheid van patiënten met eindstadium nierfalen om reversed *cholesterol transport* uit te voeren onderzocht. Daarnaast werd onderzocht of een verminderde functie hiervan toegeschreven kan worden aan oxidatieve modificatie, veroorzaakt door hemodialyse. Onze data demonstreert dat HDL van patiënten met eindstadium nierfalen uitgebreid oxidatief gemodificeerd is en verminderde effectiviteit laat zien in essentiële beschermende functies gerelateerd aan cardiovasculaire ziekte, zoals het stimuleren van cellulair cholesterol efflux en SR-BI-gemedieerde cholesterol afgifte. Deze resultaten hebben mogelijk grote impact bij de verklaring van het zeer verhoogde risico op cardiovasculaire ziekte bij uremische patiënten.

Apolipoproteïne A-1 (apoA-1) is het belangrijkste eiwit van het high density lipoprotein (HDL) partikel. Recentelijk is de bepaling opkomend van de rol van auto-antilichamen tegen apoA-1 voor het voorspellen van cardiovasculaire ziekte, in zowel de algehele populatie als bij hoog-risico patiënten. In **hoofdstuk 3** wordt de associatie tussen anti-apoA-1 IgG en cardiovasculaire ziekte gerelateerde specifieke mortaliteit, algehele mortaliteit en chronisch transplantaatfalen bestudeerd. Verder werd getracht de relatie tussen anti-apoA-1 IgG spiegels en HDL functionaliteit te beschrijven. De resultaten laten zien dat anti-apoA-1 IgG onafhankelijk geassocieerd is met cardiovasculaire ziekte. Het ontstaan van deze antilichamen is mogelijk gerelateerd aan oxidatieve stress: door oxidatieve verandering wordt het apoA-1 eiwit niet meer als eigen herkend waardoor er antilichamen gevormd worden.

Het is bekend dat verhoogde triglyceride (TG) spiegels bijdragen aan het proces van atherosclerose, en hiermee een groot risico vormen voor cardiovasculaire ziekte. Niertransplantatiepatiënten hebben uitgesproken dyslipidemie, met verhoogde TG spiegels en verminderde concentraties van HDL. Het is denkbaar dat deze afwijkingen bijdragen aan de vorming van transplantaat vasculopathie, de novo atherosclerose in het nier transplantaat, welke kan leiden tot chronisch transplantaatfalen. Echter, in tegenstelling tot HDL-C, variëren TG spiegels veel, gerelateerd aan voedingsstatus. Om deze reden zijn TG spiegels een slechte biomarker, ondanks de grote klinische implicaties. Daarentegen is een TG/HDL-C ratio een stabiele biomarker en een betere representatie van dyslipidemie dan individuele markers. **Hoofstuk 4** laat zien dat het TG/HDL-C ratio geassocieerd is met chronisch transplantaatfalen en mortaliteit. Gezien de lage kosten en algeheel brede beschikbaarheid van de TG en HDL-C meting, zou het een realistische optie zijn om het gebruikt van de TG/ HDL-C ratio te implementeren in de dagelijkse klinische praktijk.

Chronisch transplantaatfalen gaat vaak gepaard met nieuw ontstaan atherogenese in het nier transplantaat, de transplantaat vasculopathie. Hierom werd de hypothese gesteld dat dezelfde risicofactoren die gelden voor cardiovasculaire ziekte, ook toepasbaar zijn op chronisch transplantaatfalen. In **hoofdstuk 5** wordt onderzocht of de Framingham risk score (FRS), een veelgebruikte cardiovasculaire risicoscore, ook kan dienen als marker om het risico op het ontwikkelen van chronisch transplantaatfalen in kaart te brengen bij niertransplantatiepatiënten. Onze resultaten laten zien dat de 10-jaars cardiovasculaire FRS significant is geassocieerd met chronisch transplantaatfalen. De FRS heeft het potentieel een klinische marker en voorspeller te zijn om het risico op chronisch transplantaatfalen te berekenen. Effectieve risico-analyse gecombineerd met adequate therapeutische interventie kunnen een belangrijke bijdrage leveren om de overleving van niertransplantaties te verlengen.

Statines hebben een low-density lipoproteïne (LDL) verlagend effect in de algemene populatie, welke zich vertaald in een verminderde incidentie van cardiovasculaire events en mortaliteit. Richtlijnen voor de behandeling van niertransplantatiepatiënten omvatten het geven van statines, echter is er onvoldoende bewijs dat deze leiden tot een verminderd aantal cardiovasculaire events. Daarnaast zijn er zorgen over het tegelijkertijd toedienen van het immunosuppressivum cyclosporine, in combinatie met een statine, dit vanwege de gemeenschappelijke metabole pathway. **Hoofdstuk 6** onderzoekt hierom of statines een effect hebben op cardiovasculaire ziekte en sterfte bij niertransplantatie patiënten, met hierin subgroep analyse bij patienten die cyclosporine gebruiken. Uit de resultaten is gebleken dat er geen beschermend effect is van statines met betrekking tot het verlagen van cardiovasculaire events en mortaliteit bij niertransplantatiepatiënten. Integendeel, het gebruik van statines bij niertransplantatiepatiënten die ook cyclosporine gebruiken is potentieel schadelijk. De pathofysiologische mechanismen die hieraan ten grondslag liggen zijn nog niet geheel duidelijk, maar het is plausibel om aan te nemen dat een interactie tussen cyclosporine en statine leidt tot een verhoogde biobeschikbaarheid van beide medicijnen, en zo een keten in gang zet van negatieve bijwerkingen. Gebaseerd op deze data is er een klinische noodzaak voor prospectief gerandomiseerd onderzoek om de impact van verschillende LDL-C verlagende medicijnen op cardiovasculaire ziekte bij niertransplantatiepatiënten te onderzoeken.

De relatie tussen lipide spiegels uitgezet tegen lipoproteïne functie met betrekking tot uitkomstmaten bij niertransplantatiepatiënten is verder onderzocht in **hoofdstuk 7**. De binding van LDL aan proteoglycanen in de vaatwand is een vroeg essentieel moment bij de vorming van atherosclerotische plaques. De gevoeligheid waarmee LDL partikels binden aan proteoglycanen varieert sterk. Deze gevoeligheid kan mogelijk beïnvloedt worden door chemische modificatie van het LDL partikel. Het is aan te nemen dat dit proces van chemische modificatie gebeurd bij niertransplantatiepatiënten, welke normaal gesproken

een periode van hemodialyse hebben ondergaan. Gedurende deze hemodialyse zijn zij in een uremische staat en worden hierbij blootgesteld een verhoogde oxidatieve stress. Hierom is onderzocht of lipoproteïne-proteogclyaan binding gevoeligheid van LDL is geassocieerd met twee atherosclerotische uitkomstmaten bij niertransplantatiepatiënten, namelijk cardiovasculaire ziekte en chronisch transplantaatfalen. De studie laat zien dat lipoproteïne-proteogclyaan binding gevoeligheid, als een dynamische test voor de individuele pro-atherogeneticiteit van LDL-partikels, is geassocieerd met chronisch transplantaatfalen.

Deel 2: De rol van lipoproteïne functionaliteit in de algemene populatie.
In het tweede deel van dit proefschrift is de rol van lipiden in de algemene populatie onderzocht.

Hoofdstuk 8 heeft het doel te bepalen of de anti-inflammatoire capaciteit van HDL is geassocieerd met cardiovasculaire events in de algemene populatie. Uit de resultaten is gebleken dat de anti-inflammatoire capaciteit van HDL zorgt voor vasculaire bescherming (in de keten van atherogenese) en zo omgekeerd evenredig is geassocieerd met cardiovasculaire events, ongeacht van HDL-cholesterol concentratie en HDL efflux capaciteit.

Atherosclerotische ziekte kan zich op verschillende manieren manifesteren. Het valt aan te nemen dat cardiovasculaire ziekte en chronische nierziekte uitingen zijn van het zelfde ziekteproces, gebaseerd op atherosclerotische plaquevorming in bloedvaten, leidend tot micro-infarcten in de nier, ischemie en verlies van functie. De nauwe correlatie tussen cardiovasculaire ziekte en chronische nierziekte suggereren dat dezelfde risicofactoren relevant zijn. **Hoofdstuk 9** laat zien dat de TG/HDL-C ratio (als marker van dyslipidemie) een risicofactor is voor chronische nierziekte in de algemene populatie. Dit onderstreept het belang van adequaat lipide management in de algemene populatie, zeker in de context van een aanwezige albuminurie. Vanwege de lage kosten en brede beschikbaarheid van deze metingen, zou het gebruik van de TG/HDL-C ratio in de dagelijkse klinische praktijk realistisch en potentieel zeer waardevol kunnen zijn.

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Josephine L.C. Anderson was born on the 19th of June 1993 in Hamburg, Germany to a German mother and English father. She grew up in the town of Wedel with her parents and brother Sean. After completing the International Baccalaureate at the International School of Hamburg she moved to Groningen, the Netherlands in 2012 to study medicine at the Rijksuniversiteit Groningen. During the bachelor she spent three months in Yogyakata, Indonesia where she completed a clinical internship. She developed an avid interest in research during her master thesis project at the Laboratory of Pediatrics under supervision of Prof. Dr. U.J.F. Tietge. She decided to extend the project and successfully applied for an MD/PhD grant from the Junior Scientific Masterclass from the Rijksuniversiteit Groningen. She performed both laboratory and clinical research on the topic of lipid biomarkers in kidney transplant recipients under the supervision of Prof. Dr. U.J.F. Tietge and Prof Dr. S.J.L.Bakker. During this program she combined research with clinical internships at the University Medical Centre Groningen and the Nij Smellinghe Ziekenhuis in Drachten. She completed her medical studies at the ward of plastic surgery at the Martini Ziekenhuis in Groningen.

After obtaining her qualifications as a medical doctor she started her clinical career at the department of surgery at the Ziekenhuis Gelderse Vallei.

ACKNOWLEDGEMENTS

Je moet het zelf doen maar niet alleen.

Allereerst, dankjewel aan alle **patiënten** die onderdeel uit hebben gemaakt van mijn onderzoek: Jullie zijn het fundament van dit project en de reden waarom dit onderzoek zo veel voor mij betekent.

Prof. Dr. S.J.L. Bakker, beste Stephan, mijn rots in de branding gedurende dit project. Met alle ups en downs kon ik op je vertrouwen. Ik ken jou niet anders dan met een brede glimlach op je gezicht en zo heb je mij ook altijd weten te overtuigen dat alles goed komt. Bedankt voor jouw steun, jouw enthousiasme en alles wat je mij hebt geleerd.

Prof. Dr U.J.F. Tietge, lieber Uwe. Von Anfang an hast du an mich geglaubt, mir manchmal mehr zugetraut als ich mir selbst. Wir ergänzen uns super, deswegen ist unsere Zusammenarbeit auch so ein Erfolg. Dank dir war meine Promotion nicht nur erfolgreich, sondern hat mir auch besonders viel Freude bereitet. Deine Leidenschaft für die Wissenschaft, dein unermüdlicher Enthusiasmus und dein grenzenloses Wissen sind für mich eine große Inspiration. Für die Zukunft freue ich mich auf eine Fortsetzung unseres regen Austausches, natürlich bei Kaffee.

Prof dr. F. Kuipers, beste Folkert, dankjewel voor je begeleiding en supervisie, voor je wijze woorden en je adviezen. **Dr. R.P.F. Dullaart**, beste Robin, dankjewel voor de belangrijke bijdrage die je hebt geleverd aan dit proefschrift.

Dear **co-authors**: thank you for your valuable input from all over the world. Research is teamwork and I had a great team.
Hartelijk dank aan alle **analisten** en **laboratoriummedewerkers** die dit onderzoek mogelijk hebben gemaakt.

Dear members of the assessment committee, **Prof. Dr. G.J. Navis, Prof. Dr. R.A. de Boer** en **Prof. Dr. J.W.J. Beulens**. Thank you for the time you took to evaluate my work. I am looking forward to being challenged on the details of my research by you.

Dear **Sandra**, you've been by my side for this entire journey. Thank you for all your support and the many good times. **Roos**, I am so glad we got to work together. We had some crazy times, ranging from office yoga, to visits to the emergency room, to putting my western block on fire. **Congzhuo**, thank you for being a great colleague. It's been a pleasure working with you!

Aan mijn collega's van de nefrologie. Wat hebben we een geweldige tijd samen gehad. Dankjewel aan de ASN 2018 gang **Joelle, Camillo, Maryse, Quinquin, Stanley** en **Antonio** voor een onvergetelijke tijd in San Diego (en Tijuana). Mijn zusterhuisgenootjes, **Suzanne**,

Amber, **Anne-Sophie**, **Lianne**, **Rianne**, **Yusof**, **Daan**, (mede) dankzij jullie ging ik met een brede glimlach naar mijn werk. Dankjewel voor alle gezelligheid.

En mijn **collegas uit het ZGV** mogen ook zeker niet ontbreken: samen maken wij er iedere dag opnieuw een feestje van. Jullie staan altijd klaar voor een lekkere kop koffie of een koud biertje. Op naar de volgende borrel, feestje en avontuur!

Beste **chirurgen uit het Ziekenhuis Gelders Vallei**, onder jullie hoede heb ik enorm veel geleerd (en gelachen). Dank voor jullie vertrouwen.

Lieve **Els** en **Paul**, **Guido** en **Amanda**, dankjewel dat jullie mij met zo veel liefde hebben opgenomen in de familie.

Esther en Nynke: Laiverds, wat ontzettend fijn dat ik jullie aan ons Fryslân avontuur heb overgehouden. Samen zijn wij dokter geworden. Bij jullie in het 'dagboek' kan ik alles kwijt, zonder enige gêne. Dankjewel voor alle ondersteuning de afgelopen jaren.

Mein Happy Trio, **Annika** und **Annette**, mit euch am Küchentisch zu sitzen, über das Leben zu philosophieren und nahezu obsessiv Karten zu spielen tut mir so gut. Danke für eure Freundschaft.

Aan de Italië gang: **Martijn, Emma, Rens, Juul**. Wat een prachtig avontuur hebben we gehad! Ik kijk uit naar de volgende uitdaging die wij samen aangaan, met uiteraard een gezonde dosis weizen en monopoly deal.

Hannah, kaum jemand versteht mich so gut wie du, vielleicht nicht mal ich selbst. Danke, dass du mein Kompass bist in den größeren (und kleineren) Fragen im Leben.

Kim, je houdt me met beide benen op de grond en bied me tegelijkertijd onvoorwaardelijke steun. Als ik het even niet meer weet kijk ik naar jou. Dankje, dat ik altijd op je kan vertrouwen, dat wij kunnen lachen tot wij buikpijn krijgen en dankje dat je mijn paranimf bent!

Margot, not only best friend but also roommate, travel buddy, colleague, collaborator and now paranimf. Your contribution to this PhD was endless: in the form of encouragement (at times brutal), proof reading of countless documents and undying support. I think it's safe to say this wouldn't have been possible without you. Thank you for being my rock.

Thomas, ik had de pieken en dalen van de afgelopen jaren met niemand anders willen doorstaan. Door jou zijn alle pieken net wat hoger en alle dalen te overzien. Thank you for being at my side, thank you for reminding me of the important things in life. Thank you for

making me happy.

Sean, dass du stolz auf mich bist ist das wichtigste Kompliment auf der Welt.
Mama und Papa, euer uneingeschränktes Vertrauen hat mir immer die Gewissheit gegeben, dass nichts unmöglich ist. Dank euch traue ich mich groß zu träumen.