Understanding HDL function

Annema, Wijtske

CHAPTER 8

HDL cholesterol efflux predicts graft failure but not cardiovascular and overall mortality in renal transplant recipients

Wijtske Annema[1,2]
Arne Dikkers[1]
Jan Freark de Boer[1]
Robin P.F. Dullaart[3]
Jan-Stephan F. Sanders[4]
Stephan J.L. Bakker[4]
Uwe J.F. Tietge[1,2]

[1]Department of Pediatrics, Center for Liver, Digestive, and Metabolic Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.
[2]Top Institute Food and Nutrition, Wageningen, The Netherlands.
[3]Department of Endocrinology, University of Groningen, University Medical Center Groningen, The Netherlands.
[4]Department of Internal Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.

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Abstract

Background. A key function of high-density lipoprotein (HDL) particles in cardiovascular protection is cholesterol efflux, the removal of cholesterol from macrophage foam cells and first step in reverse cholesterol transport. This study prospectively investigated whether HDL cholesterol efflux capacity is associated with cardiovascular mortality, all-cause mortality, and graft failure in renal transplant recipients, patients with accelerated atherosclerosis formation.

Methods and Results. In renal transplant recipients (n = 495, median follow-up 7.0 years) cholesterol efflux capacity at baseline was quantified using incubation of human macrophage foam cells with apolipoprotein B-depleted plasma. Baseline efflux capacity was not different in deceased patients compared to survivors (P = 0.60 or P = 0.50 for cardiovascular or all-cause mortality, respectively), whereas renal transplant recipients developing graft failure had lower efflux capacity than those with functioning grafts (P < 0.001). Kaplan-Meier analysis demonstrated a lower risk for graft failure (P = 0.004), but not cardiovascular (P = 0.30) or all-cause mortality (P = 0.31) with increasing gender-stratified tertiles of efflux capacity. Cox regression analyses adjusted for age and gender showed that efflux capacity was not associated with cardiovascular mortality (hazard ratio [HR] = 0.891 [0.668-1.188], P = 0.43); the association between efflux capacity and all-cause mortality (HR = 0.786 [0.631-0.978], P = 0.031) disappeared after further adjustment for potential confounders. However, efflux capacity at baseline significantly predicted graft failure (HR = 0.433 [0.291-0.644], P < 0.001), independent of apolipoprotein A-I, HDL cholesterol, or creatinine clearance.

Conclusions. This prospective study demonstrates that cholesterol efflux capacity from macrophage foam cells is not associated with cardiovascular or all-cause mortality, but is a strong predictor of graft failure independent of plasma HDL cholesterol levels in renal transplant recipients.

8.1 Introduction

Over the last decades large population-based studies established low levels of high-density lipoprotein (HDL) cholesterol as an important independent risk factor for atherosclerotic cardiovascular disease (CVD).[1, 2] However, several recent observations shifted the focus of cardiovascular research to the concept of HDL functionality, i.e. the functional quality of HDL particles being at least equally important as HDL cholesterol mass levels. Firstly, on the individual level there is substantial variation in the relationship between CVD and plasma HDL cholesterol.[3, 4] Further support for the concept of HDL functionality came from pharmacological intervention studies designed to raise plasma HDL cholesterol levels, which failed to show a clinical benefit.[5-7] Although many different functions of HDL have been described thus far, cholesterol efflux, which is the capacity of HDL to remove cholesterol from macrophage foam cells, is one of the best established beneficial properties of HDL. Specifically, cholesterol efflux is the first step in reverse cholesterol transport, the major HDL-mediated atheroprotective pathway for eliminating excess cholesterol from the body via liver and bile.[8-10] A recent cross-sectional study illustrated the clinical potential of HDL function measurements by showing that a decreased cholesterol efflux capacity from macrophages was associated with increased subclinical atherosclerosis and a higher prevalence of coronary artery disease.[11] However, prospective data on the association between HDL cholesterol efflux potential and long-term CVD outcomes are lacking.

Renal transplant recipients (RTRs) have accelerated atherosclerosis resulting in a four to six times increased incidence of CVD compared to the general population,[12] and CVD is the leading cause of mortality in RTRs.[13] Interestingly, there is also evidence that intragraft atherosclerosis plays a role in the pathogenesis of chronic renal transplant dysfunction [14] and thereby contributes to graft failure. Graft failure represents another important clinical problem in RTRs, and despite progressive improvements in one-year graft survival rates, specifically graft failure after the first year has not been reduced substantially over the last decades.[15]

Therefore, the aim of this study was to use RTRs as a model of clinically relevant accelerated atherosclerosis formation to prospectively determine whether cholesterol efflux capacity at baseline is associated with future cardiovascular mortality, all-cause mortality, and graft failure.

8.2 Materials and Methods

8.2.1 Study design and patients

In this study, all adult RTRs who visited the outpatients clinic at the University Medical Center Groningen between August 2001 and July 2003 and who survived with a functioning graft for at least 1 year (1 year post-transplant was considered baseline) were invited to participate at their next visit to the outpatient clinic. The outpatient follow-up constitutes a continuous surveillance system in which patients visit the outpatient clinic with declining frequency, in accordance with the American Transplantation Society guidelines, that is, ranging from twice a week immediately after hospital discharge to twice a year in the long-term course after transplantation.[16] Patients with overt congestive heart failure and patients diagnosed with cancer other than cured skin cancer were not considered eligible

for the study. In patients with fever or other signs of infection (e.g. complaints of upper respiratory tract infection or urinary tract infection), baseline visits were postponed until symptoms had resolved. From the 847 eligible RTRs, 606 gave signed written informed consent (72% consent rate) and were included in the study. The group that decided not to participate was comparable with the group that consented with respect to age, gender, body mass index, plasma creatinine, creatinine clearance, and proteinuria.
Cholesterol efflux was determined in 517 RTRs. Of this group, 22 patients were excluded from analyses because of evidence of acute inflammation (high sensitivity C-reactive protein [hsCRP] values > 20 mg/l), leaving a total of 495 recipients for analyses. A more complete description of the overall study design has been published previously.[17] The Institutional Review Board approved the study protocol (METc2001/039), which complied with the Declaration of Helsinki.

8.2.2 End points of the study

The primary end points of this study were recipient mortality and death-censored graft failure. Death-censored graft failure was defined as return to dialysis therapy or retransplantion. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. 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. Causes of death were coded according to the International Classification of Diseases, 9th revision (ICD-9).[18] Cardiovascular mortality was defined as deaths in which the principal cause of death was cardiovascular in nature, using ICD-9 codes 410 to 447. Graft failure and mortality were recorded until May 2009. There was no loss during follow-up.

8.2.3 Renal transplant characteristics

Relevant transplant characteristics, such as age, gender, and date of transplantation, were extracted from the Groningen Renal Transplant Database. This database contains information on all renal transplantations that have been performed at the University Medical Center Groningen since 1968, including dialysis history. Details of the standard immunosuppressive treatment were described previously.[19] Current medication was extracted from the medical record. Smoking status and CVD history were obtained using a self-report questionnaire at inclusion. CVD history was considered positive if participants had a previous myocardial infarction, transient ischemic attack, or cerebrovascular accident.

8.2.4 Measurements and definitions

For metabolic syndrome the definition of the National Cholesterol Education Program Expert Panel was used.[20] In 2008, the American Diabetes Association (ADA) lowered the cut-off point for impaired fasting glucose to ≥ 5.6 mmol/l.[21] For our analysis of the prevalence of the metabolic syndrome, we used this ADA cut-off point. Diabetes mellitus was defined according to the guidelines of the ADA as a fasting plasma glucose ≥ 7.0 mmol/l or the use of anti-diabetic medication.[22]
Body mass index was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured on bare skin midway between the iliac crest

and the 10th rib using a plastic tape measure. Blood pressure was measured using an automated device (Omron M4; Omron Europe B.V., The Netherlands) in supine position after a 6-minute rest as the average of three measurements at 1-minute intervals.
Blood was drawn after a 8 to 12 hour overnight fasting period. 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.[23] HDL cholesterol was measured with the cholesterol oxidase-phenol aminophenazone method on a Technikon RA-1000 (Bayer Diagnostics, Mijdrecht, The Netherlands). Apolipoprotein A-I was determined by immunoturbidimetry (COBAS Integra System; Roche Diagnostics, Mannheim, Germany). Plasma triglycerides were determined with the glycerol-3-phosphate oxidase-phenol aminophenazone method (Roche Diagnostics). The glucose-oxidase method (YSI 2300 Stat Plus; Yellow Springs, OH) was used to determine plasma glucose levels. Plasma insulin was measured using an AxSym autoanalyzer (Abbott Diagnostics, Abbott Park, IL). HbA1c was assessed by high performance liquid chromatography (VARIANTTM Hb Testing System; Bio-Rad, Hercules, CA). Insulin resistance was calculated using Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) as follows: HOMA-IR = glucose (mmol/l) x insulin (μU/ml)/22.5.[24] Plasma hsCRP was assessed by ELISA as described before.[25] Plasma and urine creatinine concentrations were determined using a modified version of the Jaffé method (MEGA AU 510; Merck Diagnostica). Creatinine clearance was calculated from 24-hour urinary creatinine excretion and plasma creatinine. Total urinary protein concentration was analyzed using the Biuret reaction (MEGA AU 510; Merck Diagnostica), and proteinuria was defined as urinary protein excretion ≥ 0.5 g per 24 hours.

8.2.5 Assessment of cholesterol efflux

Blood samples from RTRs were collected after a 8 to 12 hour overnight fasting period in plasma separator tubes containing EDTA, placed on ice, centrifuged at 4°C, and immediately stored at -80°C until further analysis. To isolate total HDL, apolipoprotein B (apoB)-containing lipoproteins were precipitated from EDTA plasma using polyethylene glycol (PEG 6000, Sigma, St. Louis, MO) in 10 mM HEPES (pH = 8.0) as described previously.[11, 26-29] After 30 minutes centrifugation at 2200 *g*, the HDL-containing supernatant was collected, kept on ice, and used directly for cholesterol efflux measurement.
To assess cholesterol efflux, THP-1 human monocytes (ATCC via LGC Promochem, Teddington, UK) were differentiated into macrophages by the addition of 100 nM phorbol myristate acetate.[30] Differentiated THP-1 macrophages were then loaded with 50 μg/ml acetylated LDL and 1 μCi/ml ^{3}H-cholesterol (Perkin Elmer, Boston, MA) for 24 hours followed by equilibration for 24 hours in RPMI 1640 medium containing 2% bovine serum albumin.[30] Thereafter, 2% apoB-depleted plasma was added to the macrophage foam cells. After 6 hours an aliquot of medium was counted to quantitate the effluxed cholesterol label following table-top centrifugation to pellet cellular debris. Meanwhile the cells were incubated for at least 30 minutes with 0.1 M NaOH at room temperature, whereupon the radioactivity remaining within the cells was determined by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT). Efflux per well is expressed as the percentage of counts released into the medium related to the total dose of radioactivity initially present (counts recovered within the medium added to the counts recovered from the cells). Values obtained from control cells without added apoB-

depleted patient plasma were subtracted to correct for unspecific efflux.
Cholesterol efflux measurements were carried out in all respective patient samples at the same time to limit potential variation due to different assay conditions. All measurements were performed in duplicate. To correct for potential plate-to-plate variation, apoB-depleted control plasma was included on each plate at four different concentrations. Additional validation experiments showed that almost 90% of the cholesterol efflux capacity of apoB-depleted plasma was explained by the presence of HDL (Supplemental figure 8.I).

8.2.6 Statistical analysis

Normally distributed continuous variables are presented as mean ± standard deviation, whereas continuous variables with a skewed distribution are given as median [25^{th}-75^{th} percentile]. Categorical variables were summarized by absolute numbers (percentages). Logarithmic transformation was used for variables with a skewed distribution in order to reach normality criteria. Hazard ratios (HRs) are reported with 95% confidence intervals (CIs).
Recipient baseline characteristics were analyzed separately for gender-stratified tertiles of cholesterol efflux. Differences among tertiles were tested with one-way analysis of variance followed by Bonferroni post hoc test for normally distributed variables and with Kruskal-Wallis test followed by Mann-Whitney *U* test for variables with a skewed distribution. Chi-square test was used to compare categorical data. Subsequently, all characteristics with a $P \leq 0.1$ across gender-stratified tertiles of cholesterol efflux were entered into a stepwise multivariate linear regression model with backward elimination ($P \leq 0.05$) in order to identify variables independently associated with cholesterol efflux.
Graft failure, all-cause mortality, and cardiovascular mortality rates in gender-stratified tertiles of cholesterol efflux were compared using the Kaplan-Meier method and tested for significant differences by log-rank test. Receiver operating characteristic (ROC) curves were generated to evaluate the predictive capability of cholesterol efflux capacity at baseline for cardiovascular mortality, all-cause mortality, and graft failure, and the area under the ROC curve and the 95% CIs were computed. Additionally, univariate and multivariate Cox proportional hazard regression analysis models were used to estimate hazard ratios and 95% CIs for mortality from all-causes or CVD and for graft failure. In the multivariate analyses, the associations of cholesterol efflux with both graft failure and mortality were adjusted for recipient age and gender (model 2) and further adjusted for apolipoprotein A-I (model 3), for HDL cholesterol (model 4), and for creatinine clearance (model 5). Power calculations showed that the minimum detectable hazard ratio based on an assumption of 90% power and two-sided alpha significance of 0.05 was 0.769 for CVD mortality, 0.827 for overall mortality, and 0.748 for graft failure.
A two-sided *P* value of < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using the Statistical Package for the Social Sciences version 20 (SPSS, Chicago, IL) and GraphPad Prism version 5.00 (GraphPad software, San Diego, CA).

Table 8.1. Baseline characteristics according to gender-stratified tertiles of cholesterol efflux.

Characteristics	Gender-stratified tertiles of cholesterol efflux			*P* value
	First (n = 164)	Second (n = 166)	Third (n = 165)	
Cholesterol efflux, %	5.8 [5.3-6.4]	7.3 [6.8-7.9]‡	9.0 [8.2-9.8]‡,#	<0.001
Recipient demographics				
Age, years	50.6 ± 11.8	50.7 ± 12.7	53.6 ± 11.1	0.03
Male gender, n (%)	89 (54)	90 (54)	90 (55)	1.00
Current smoking, n (%)	36 (22)	34 (21)	35 (21)	0.95
Previous smoking, n (%)	65 (40)	74 (45)	74 (45)	0.56
Metabolic syndrome, n (%)	126 (77)	104 (63)*	52 (32) ‡,#	<0.001
Body composition				
BMI, kg/m^2	26.9 ± 4.6	25.9 ± 3.9	25.0 ± 3.9‡	<0.001
Waist circumference men, cm	103.4 ± 12.7	101.3 ± 10.8	94.4 ± 11.7‡,#	<0.001
Waist circumference women, cm	96.5 ± 14.6	92.1 ± 14.9	91.4 ± 13.8	0.07
Lipids				
Total cholesterol, mmol/L	5.4 ± 1.0	5.7 ± 1.2	5.8 ± 1.0†	0.008
LDL cholesterol, mmol/L	3.4 ± 1.0	3.6 ± 1.1	3.6 ± 0.9	0.20
HDL cholesterol, mmol/L	0.9 ± 0.2	1.1 ± 0.2‡	1.4 ± 0.3‡,#	<0.001
Apolipoprotein A-I, g/L	1.3 ± 0.2	1.6 ± 0.2‡	1.8 ± 0.3‡,#	<0.001
Triglycerides, mmol/L	2.2 [1.6-3.0]	2.0 [1.4-2.6]*	1.6 [1.2-2.2]‡,#	<0.001
Use of statins, n (%)	76 (46)	85 (51)	91 (55)	0.28
Cardiovascular disease history				
Myocardial infarction, n (%)	15 (9)	10 (6)	17 (10)	0.36
TIA/CVA, n (%)	7 (4)	9 (5)	9 (6)	0.85
Blood pressure				
Systolic blood pressure, mmHg	154 ± 24	151 ± 22	153 ± 23	0.35
Diastolic blood pressure, mmHg	90 ± 10	89 ± 9	90 ± 10	0.65
Use of ACE inhibitors, n (%)	73 (45)	53 (32)*	46 (28)†	0.004
Use of β-blockers, n (%)	120 (73)	97 (58)†	85 (52)‡	<0.001
Use of diuretics, n (%)	92 (56)	65 (39)†	57 (35)‡	<0.001
Number of antihypertensive drugs, n	2 [1-3]	2 [1-3]‡	2 [1-2]‡	<0.001
Glucose homeostasis				
Glucose, mmol/L	4.7 [4.1-5.2]	4.6 [4.1-5.1]	4.4 [4.0-4.9]†	0.03
Insulin, µmol/L	12.3 [8.7-17.1]	11.4 [8.4-16.9]	9.4 [6.9-12.1]‡,#	<0.001
HbA1c, %	6.5 [6.0-7.0]	6.4 [5.8-6.9]	6.2 [5.7-6.9]	0.06
HOMA-IR	2.5 [1.7-3.9]	2.3 [1.6-3.9]	1.9 [1.3-2.6]‡,#	<0.001
Post-Tx diabetes mellitus, n (%)	37 (23)	26 (16)	26 (16)	0.18

Use of anti-diabetic drugs, n (%)	31 (19)	18 (11)	19 (12)	0.06
Use of insulin, n (%)	13 (8)	10 (6)	9 (6)	0.63
Inflammation				
hsCRP, mg/L	2.6 [1.1-5.6]	1.9 [0.8-4.7]	1.7 [0.7-3.2]†	0.007
Donor demographics				
Age, years	38.2 ± 15.6	37.4 ± 15.4	35.6 ± 15.7	0.29
Male gender, n (%)	90 (55)	91 (55)	93 (56)	0.96
Living kidney donor, n (%)	23 (14)	23 (14)	17 (10)	0.52
Postmortem donor, n (%)	141 (86)	143 (86)	148 (90)	
(Pre)transplant history				
Dialysis time, months	25.5 [13.0-48.0]	28.5 [14.0-45.0]	29.0 [13.0-51.0]	0.73
Time between Tx and inclusion, years	5.8 [2.3-10.0]	5.4 [2.5-10.2]	8.3 [4.0-13.9]‡,#	<0.001
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]	0.20
Calcineurin inhibitors, n (%)	128 (78)	140 (84)	123 (75)	0.09
Proliferation inhibitors, n (%)	128 (78)	125 (75)	114 (69)	0.16
Renal allograft function				
Creatinine clearance, mL/min	58.4 ± 23.1	64.2 ± 20.6	63.8 ± 22.7	0.03
Urinary protein excretion, g/24h	0.3 [0.1-0.5]	0.2 [0.0-0.5]	0.2 [0.0-0.5]	0.11
Proteinuria ≥ 0.5 g/24h, n (%)	46 (28)	43 (26)	48 (29)	0.80

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-75th percentile], and differences were tested by Kruskal-Wallis test followed by Mann Whitney *U* test. Categorical data are summarized by n (%), and differences were tested by χ^2 test. ACE, angiotensin-converting enzyme; BMI, body mass index; CVA, cerebrovascular event; HDL, high-density lipoprotein; HOMA, homeostatic model assessment; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; TIA, transient ischemic attack; Tx, transplantation. *Tertile significantly different from the first tertile, $P < 0.05$; †Tertile significantly different from the first tertile, $P < 0.01$; ‡Tertile significantly different from the first tertile, $P < 0.001$; §Tertile significantly different from the second tertile, $P < 0.05$; ||Tertile significantly different from the second tertile, $P < 0.01$; #Tertile significantly different from the second tertile, $P < 0.001$.

8.3 Results

In this prospective longitudinal study, cholesterol efflux capacity was measured in a total of 495 RTRs (mean age 51.6 ± 12.0; 54% men). Patients were divided in gender-stratified tertiles based on baseline cholesterol efflux capacity with the following median values: first tertile, 5.8% [5.3-6.4%]; second tertile, 7.3% [6.8-7.9%]; and third tertile, 9.0% [8.2-9.8%]. Baseline patient characteristics according to gender-stratified tertiles of cholesterol efflux are summarized in Table 8.1. The prevalence of the metabolic syndrome decreased significantly with increasing tertiles of cholesterol efflux. Additionally, there was an inverse association between cholesterol efflux and plasma triglycerides, whereas cholesterol efflux was positively associated with HDL cholesterol and apolipoprotein A-I levels. Patients in the highest tertile of cholesterol efflux had a lower body mass index,

a smaller waist circumference, higher plasma total cholesterol levels, lower plasma glucose, lower plasma insulin, a lower HOMA-IR, lower hsCRP values, and a longer time between kidney transplantation and inclusion. Mean blood pressure was similar over the cholesterol efflux tertiles, although patients in the lowest tertile more frequently used anti-hypertensive drugs.

Table 8.2. Variables that have independent associations with or are determinants of cholesterol efflux capacity.

	β	95% CI	Standardized beta	*P* value
Apolipoprotein A-I	2.805	2.368; 3.241	0.493	<0.001
HDL cholesterol	1.913	1.509; 2.317	0.367	<0.001
Time between Tx and inclusion	0.026	0.012; 0.040	0.099	<0.001
Use of calcineurin inhibitors	0.380	0.158; 0.601	0.093	0.001
Recipient age	0.011	0.004; 0.018	0.079	0.003
Waist circumference	-0.009	-0.015; -0.003	-0.073	0.006
HbA1c	-0.113	-0.196; -0.031	-0.068	0.007
$R^2 = 0.75$				

Variables are listed in decreasing order of strength of association according to the absolute value of the standardized beta. HDL, high-density lipoprotein; Tx, transplantation.

Subsequently, backward multiple linear regression analysis was used to assess which variables are independently associated with and are determinants of cholesterol efflux capacity in RTRs (Table 8.2). Cholesterol efflux capacity was found to have a strong, independent relationship with plasma apolipoprotein A-I and HDL cholesterol mass. Furthermore, cholesterol efflux capacity was independently and positively associated with time between kidney transplantation and inclusion, use of calcineurin inhibitors, and recipient age. On the other hand, cholesterol efflux capacity independently and inversely correlated with both waist circumference and HbA1c. R^2 of the final model was 0.75.

During a median follow-up of 7.0 years [6.3-7.5 years], a total of 102 (21%) patients died, including 54 (11%) from confirmed cardiovascular causes. In addition, 46 (9%) RTRs experienced graft failure during the follow-up period. Baseline cholesterol efflux from macrophage foam cells was not statistically different between the patients that survived during follow-up and the patients that died. This holds true for both cardiovascular mortality (7.3% [6.4-8.4%] vs. 7.6% [6.3-8.7%], $P = 0.60$) and all-cause mortality (7.3% [6.4-8.4%] vs. 7.2% [6.2-8.6%], $P = 0.50$). However, cholesterol efflux capacity at baseline was significantly lower in RTRs with graft failure compared to RTRs whose graft survived (6.5% [5.2-7.4] vs. 7.4% [6.4-8.6%], $P < 0.001$).

Next, mortality rates and graft failure among tertiles of cholesterol efflux were compared using Kaplan-Meier analysis. There was no relationship between cholesterol efflux tertiles and cardiovascular mortality (log-rank test: $P = 0.30$; Figure 8.1A). During follow-up, the corresponding numbers of death from apparent cardiovascular origin were 16 (10%) in the first tertile, 15 (9%) in the second tertile, and 23 (14%) in the third tertile. Likewise, Kaplan-Meier curves did not reveal an association of cholesterol efflux with all-cause mortality (log-rank test: $P = 0.31$; Figure 8.1B). The incidence of death from all-causes was 37 (23%) in the first tertile, 28 (17%) in the second tertile, and 37 (22%) in the third tertile.

However, the cumulative incidence of graft failure significantly decreased in a step-wise fashion with increasing tertiles of cholesterol efflux (log-rank test: *P* = 0.004; Figure 8.1C), with respective numbers of 23 (14%) in the lowest tertile, 17 (10%) in the middle tertile, and 6 (4%) in the highest tertile.

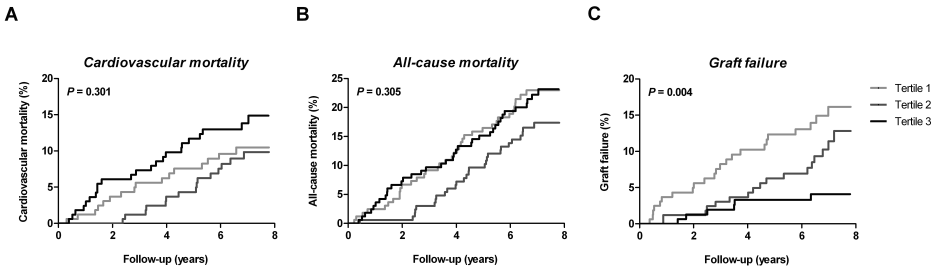

Figure 8.1. Kaplan-Meier curves of (A) cardiovascular mortality, (B) all-cause mortality, and (C) graft failure according to gender-stratified tertiles of cholesterol efflux. The corresponding *P* value was obtained from the log-rank test.

ROC curves were plotted to assess the prognostic value of cholesterol efflux capacity for cardiovascular mortality, all-cause mortality, and graft failure in RTRs within the median follow-up time of 7.0 years. The area under the ROC curve for prediction of cardiovascular mortality was 0.48 (95% CI: 0.39-0.56, *P* = 0.60; Figure 8.2A) and for prediction of all-cause mortality 0.52 (95% CI: 0.46-0.59, *P* = 0.50; Figure 8.2B). On the other hand, the area under the ROC curve showed that baseline cholesterol efflux capacity is a predictor of graft failure (0.69 [95% CI: 0.62-0.77], *P* < 0.001; Figure 8.2C).

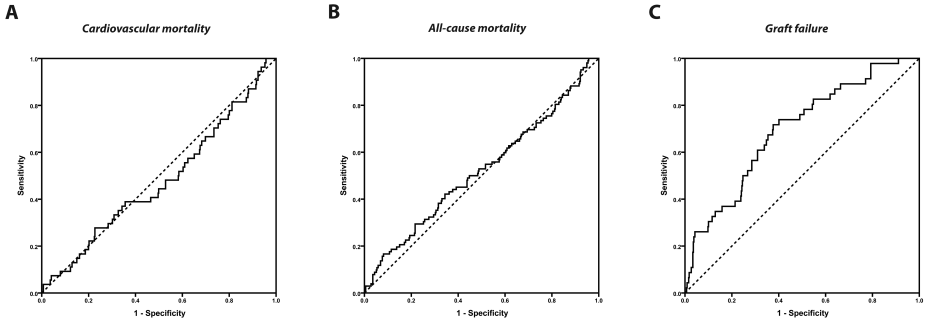

Figure 8.2. Receiver operating characteristic (ROC) curves of cholesterol efflux capacity for (A) cardiovascular mortality, (B) all-cause mortality, and (C) graft failure. The dashed line represents the reference line**.**

Finally, Cox proportional hazard analyses were performed to evaluate the independent contribution of cholesterol efflux capacity to the risk for patient mortality and graft failure (Table 8.3). Cholesterol efflux capacity was not associated with future cardiovascular mortality in both univariate (HR = 1.014 [0.777-1.323], *P* = 0.92; Table 8.3, model 1) and multivariate analyses (Table 8.3, model 2-5 and Supplemental table 8.I). Similar results were obtained for the association of HDL cholesterol levels and apolipoprotein A-I levels with CVD mortality (Supplemental table 8.II and 8.III).

Table 8.3. Hazard ratios for cardiovascular mortality, all-cause mortality, and graft failure by cholesterol efflux capacity.

	Cardiovascular mortality (54 events)		**All-cause mortality (102 events)**		**Graft failure (46 events)**	
	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	1.014 [0.777-1.323]	0.92	0.908 [0.741-1.112]	0.35	0.428 [0.293-0.625]	<0.001
Model 2	0.891 [0.668-1.188]	0.43	0.786 [0.631-0.978]	0.031	0.433 [0.291-0.644]	<0.001
Model 3	1.050 [0.683-1.615]	0.83	0.841 [0.594-1.191]	0.33	0.417 [0.226-0.769]	0.005
Model 4	1.255 [0.833-1.891]	0.28	0.918 [0.659-1.280]	0.62	0.556 [0.313-0.987]	0.045
Model 5	0.955 [0.718-1.269]	0.75	0.839 [0.677-1.040]	0.11	0.524 [0.363-0.758]	0.001

Model 1: crude; model 2: model 1 + adjustment for recipient age and gender; model 3: model 2 + adjustment for apolipoprotein A-I; model 4: model 2 + adjustment for HDL cholesterol; model 5: model 2 + adjustment for creatinine clearance. HR, hazard ratio; CI, confidence interval.

While cholesterol efflux capacity was not related to all-cause mortality in an univariate model (HR = 0.908 [0.741-1.112], *P* = 0.35; Table 8.3, model 1), this association became significant after adjustment for recipient age and gender (HR = 0.786 [0.631-0.978], *P* = 0.031; Table 8.3, model 2). Following additional adjustments for apolipoprotein A-I (HR = 0.841 [0.594-1.191], *P* = 0.33; Table 8.3, model 3), HDL cholesterol (HR = 0.918 [0.659-1.280], *P* = 0.62; Table 8.3, model 4), and creatinine clearance (HR = 0.839 [0.677-1.040], *P* = 0.11; Table 8.3, model 5), cholesterol efflux capacity was no longer associated with all-cause mortality. The age- and gender-specific association between cholesterol efflux and all-cause mortality was also not independent of several other known mortality risk factors (Supplemental table 8.I). Comparably, also low plasma levels of HDL cholesterol or apolipoprotein A-I did not independently associate with a higher risk for all-cause mortality (Supplemental table 8.II and 8.III). When analyses were repeated for development of graft failure, in an univariate Cox regression model cholesterol efflux capacity was found to predict graft failure with a HR 0.428 ([0.293-0.625], *P* < 0.001; Table 8.3, model 1). Adjustment for recipient age and gender did not appreciably change this association (HR = 0.433 [0.291-0.644], *P* < 0.001; Table 8.3, model 2). Importantly, cholesterol efflux capacity at baseline remained a significant predictor of graft failure, even after further controlling for apolipoprotein A-I (HR = 0.417 [0.226-0.769], *P* = 0.005; Table 8.3, model 3) and for HDL cholesterol mass levels (HR = 0.556 [0.313-0.987], *P* = 0.045; Table 8.3, model 4). Although taking into account renal allograft function, estimated by creatinine clearance, attenuated the independent predictive power of graft failure by cholesterol efflux in RTRs (HR = 0.524 [0.363-0.758], *P* = 0.01; Table 8.3, model 5), it still remained significant. On the other hand, cholesterol efflux predicted renal graft outcome independent of various other potential confounders (Supplemental table 8.I). Although the association of HDL cholesterol mass levels as well as apolipoprotein A-I levels with graft failure was significant, absolute hazard ratios per 1-SD increase showed that cholesterol efflux capacity was the most powerful predictor for graft failure (Supplemental table

8.II and 8.III). Additionally, plasma levels of HDL cholesterol and apolipoprotein A-I no longer predicted graft failure after adjusting for cholesterol efflux capacity (Supplemental table 8.II and 8.III), suggesting that HDL cholesterol and apolipoprotein A-I levels are not independent risk markers but that the association with graft failure may be explained by cholesterol efflux capacity. These combined data demonstrate that HDL cholesterol efflux function is a strong independent predictor of graft failure in RTRs.

8.4 Discussion

This prospective study is to our knowledge the first to examine the predictive value of cholesterol efflux capacity as a measure of HDL functionality for relevant long-term clinical outcomes. Our data indicate that cholesterol efflux capacity from macrophage foam cells did not independently predict risk for cardiovascular and all-cause mortality after kidney transplantation. Interestingly, however, a higher cholesterol efflux capacity in RTRs at baseline was associated with significant protection against the future development of graft failure, a condition previously linked to accelerated atherosclerosis formation.[14] Importantly, this clinical association of cholesterol efflux capacity, as a mechanistically relevant surrogate of HDL function, was independent of plasma HDL cholesterol as well as apolipoprotein A-I mass levels. Thereby, our data lend strong support to the emerging concept that important additional clinical information can be derived from the assessment of HDL function as compared to HDL cholesterol mass measurements.

In recent years, a growing amount of literature has been published on HDL functionality. These studies demonstrated that significant differences in the functional properties of HDL may exist between patients and healthy control subjects (reviewed by [31, 32]). In addition, recent work demonstrated that HDL function, as measured by the cholesterol efflux capacity from macrophage foam cells, inversely related to subclinical atherosclerosis and coronary artery disease.[11] However, in contrast to our present study a major disadvantage of current published research on HDL function is that the cross-sectional nature of these studies does not allow to draw any definite conclusion about whether dysfunctional HDL is cause or consequence of a specific clinical condition.

An important result of the current study is that cholesterol efflux capacity is not an independent predictor of mortality in RTRs. HDL function was only linked to all-cause mortality when patient age and gender were taken into account, and this relationship could be largely explained by a variety of other conventional risk factors. Furthermore, no evidence was found for an association between HDL function and specific cardiovascular mortality. However, the nature of cardiovascular disease in RTRs is not well-defined and might differ from the general population.[33] Such a concept is supported by traditional risk factors not consistently being the major determinants of cardiovascular events in RTRs.[34] Although myocardial infarction due to obstructive coronary artery disease, the principal type of CVD in the general population, is not uncommon in RTRs, increased cardiovascular mortality among RTRs might be largely attributable to an excess prevalence of sudden cardiac death and heart failure.[33] Moreover, as their kidney function declines RTRs may develop uremia, which in turn can cause deleterious changes in the structure and function of the heart, a condition termed uremic cardiomyopathy.[35] Therefore, our results might not be readily translated to other groups of patients or the general population. Further research is warranted to address this issue.

The most interesting finding of our study was that cholesterol efflux capacity independently identified subjects at risk for graft failure. There are several potential explanations for the association between HDL function and graft failure after kidney transplantation. First, progressive atherosclerosis in the vasculature of the transplanted kidney is a major pathological manifestation of chronic renal transplant dysfunction, one of the leading causes of graft failure in RTRs after the first year following transplantation.[14] A better functionality of HDL in removing cholesterol from macrophage foam cells in the vascular wall is conceivably expected to contribute to prevent or reverse intragraft atherosclerosis, and thereby slow the decline in kidney function. It is also plausible that an increased cellular cholesterol efflux capacity, as one key metric of HDL function, reflects an overall improvement in the functionality of HDL particles. One could hypothesize that in this line other functions of HDL, such as endothelial protection, might also contribute to improved graft survival. One of the hallmarks of chronic allograft dysfunction in renal transplantation is an enhanced endothelial expression of adhesion molecules.[36] HDL has the ability to inhibit adhesion molecule expression on endothelial cells,[37] which in turn may help restrain the recruitment of potentially harmful proinflammatory mononuclear cells into the kidney graft.

With regard to potential clinical implications, our findings suggest that HDL function might be an attractive novel treatment target for the prevention of graft failure in RTRs. Several new therapeutic strategies to potentially increase the functionality of HDL are currently under investigation. These include apolipoprotein A-I/reconstituted HDL infusions, apolipoprotein A-I mimetic peptides, niacin, and maybe still cholesteryl ester transfer protein inhibitors.[38, 39] According to our data additional research is clearly required to address the effectiveness of such interventions for kidney graft preservation.

In conclusion, our results indicate that baseline cholesterol efflux capacity is not a significant risk factor for cardiovascular mortality and all-cause mortality, at least not in this specific patient population of RTRs. However, higher cholesterol efflux capacity was independently associated with an increased long-term graft survival after kidney transplantation. The findings of this study thereby provide the basis for an important new understanding of the prognostic value of HDL functionality independent of HDL cholesterol mass levels.

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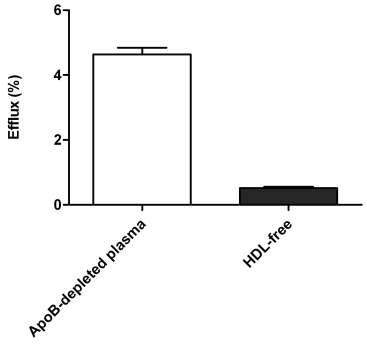
SUPPLEMENTAL INFORMATION CHAPTER 8



Supplemental figure 8.I. Cholesterol efflux capacity measurement. Cholesterol efflux capacity of apoB-depleted plasma and apoB-depleted plasma devoid of HDL (HDL-free). Plasma was depleted from apoB-containing lipoproteins by PEG precipitation as described in Materials and Methods, and subsequently HDL was removed from the apoB-depleted plasma using ultracentrifugation (*d* = 1.25 g/ml) to obtain the HDL-free fraction. Cholesterol efflux capacity was determined as described in Material and Methods.

Supplemental table 8.I. Hazard ratios for cardiovascular mortality, all-cause mortality, and graft failure by cholesterol efflux capacity.

	Cardiovascular mortality (54 events)		**All-cause mortality (102 events)**		**Graft failure (46 events)**	
	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	0.901 [0.668-1.216]	0.50	0.814 [0.650-1.021]	0.074	0.438 [0.291-0.661]	<0.001
Model 2	0.846 [0.629-1.138]	0.27	0.743 [0.593-0.931]	0.010	0.414 [0.275-0.623]	<0.001
Model 3	1.102 [0.781-1.554]	0.58	0.923 [0.709-1.203]	0.55	0.377 [0.242-0.587]	<0.001
Model 4	0.957 [0.709-1.292]	0.77	0.832 [0.662-1.045]	0.11	0.505 [0.342-0.744]	0.001
Model 5	0.914 [0.676-1.235]	0.56	0.806 [0.642-1.012]	0.063	0.453 [0.309-0.662]	<0.001
Model 6	0.919 [0.687-1.230]	0.57	0.805 [0.645-1.003]	0.054	0.439 [0.294-0.657]	<0.001
Model 7	0.882 [0.658-1.182]	0.40	0.775 [0.620-0.968]	0.025	0.425 [0.285-0.634]	<0.001
Model 8	0.891 [0.668-1.189]	0.43	0.786 [0.632-0.978]	0.031	0.425 [0.286-0.634]	<0.001

All models were adjusted for recipient age and gender. Model 1: adjustment for LDL cholesterol and triglycerides; model 2: adjustment for current and past smoking; model 3: adjustment for presence of the metabolic syndrome, BMI, and waist circumference; model 4: adjustment for systolic blood pressure, use of ACE inhibitors, use of β-blockers, use of diuretics, and number of anti-hypertensive drugs; model 5: adjustment for presence of diabetes, glucose, fasting insulin, HbA1c, HOMA-IR, and use of anti-diabetic drugs; model 6: adjustment for hsCRP; model 7: adjustment for dialysis time and time between kidney transplantation and inclusion; model 8: adjustment use of calcineurin inhibitors. HR, hazard ratio; CI, confidence interval.

Supplemental table 8.II. Hazard ratios for cardiovascular mortality, all-cause mortality, and graft failure by HDL cholesterol levels.

	Cardiovascular mortality (54 events)		**All-cause mortality (102 events)**		**Graft failure (46 events)**	
	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	0.838 [0.627-1.120]	0.23	0.858 [0.696-1.058]	0.15	0.450 [0.307-0.660]	<0.001
Model 2	0.745 [0.550-1.009]	0.057	0.763 [0.613-0.950]	0.016	0.458 [0.307-0.684]	<0.001
Model 3	0.664 [0.397-1.112]	0.12	0.779 [0.546-1.111]	0.17	0.481 [0.256-0.906]	0.023
Model 4	0.625 [0.402-0.913]	0.037	0.814 [0.584-1.135]	0.23	0.705 [0.400-1.242]	0.23
Model 5	0.791 [0.586-1.066]	0.12	0.807 [0.650-1.001]	0.051	0.526 [0.359-0.772]	0.001

Model 1: crude; model 2: model 1 + adjustment for recipient age and gender; model 3: model 2 + adjustment for apolipoprotein A-I; model 4: model 2 + adjustment for efflux capacity; model 5: model 2 + adjustment for creatinine clearance. HR, hazard ratio; CI, confidence interval.

Supplemental table 8.III. Hazard ratios for cardiovascular mortality, all-cause mortality, and graft failure by apolipoprotein A-I levels.

	Cardiovascular mortality (54 events)		**All-cause mortality (102 events)**		**Graft failure (46 events)**	
	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	0.963 [0.735-1.263]	0.79	0.927 [0.759-1.132]	0.46	0.537 [0.380-0.759]	<0.001
Model 2	0.839 [0.630-1.118]	0.23	0.806 [0.653-0.995]	0.044	0.555 [0.386-0.799]	0.002
Model 3	0.809 [0.523-1.250]	0.34	0.920 [0.655-1.291]	0.63	1.047 [0.589-1.858]	0.88
Model 4	1.147 [0.706-1.865]	0.58	0.975 [0.691-1.376]	0.89	0.943 [0.529-1.683]	0.84
Model 5	0.887 [0.664-1.185]	0.42	0.849 [0.687-1.050]	0.13	0.591 [0.407-0.857]	0.006

Model 1: crude; model 2: model 1 + adjustment for recipient age and gender; model 3: model 2 + adjustment for cholesterol efflux capacity; model 4: model 2 + adjustment for HDL cholesterol; model 5: model 2 + adjustment for creatinine clearance. HR, hazard ratio; CI, confidence interval.