HDL Cholesterol Efflux Predicts Graft Failure in Renal Transplant Recipients

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ABSTRACT

High-density lipoprotein (HDL) particles are involved in the protection against cardiovascular disease by promoting cholesterol efflux, in which accumulated cholesterol is removed from macrophage foam cells. We investigated whether HDL cholesterol efflux capacity is associated with cardiovascular mortality, all-cause mortality, and graft failure in a cohort of 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 and survivors (P=0.60 or P=0.50 for cardiovascular or all-cause mortality, respectively), whereas 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], 0.89; 95% confidence interval [95% CI], 0.67 to 1.19; P=0.43). Furthermore, the association between efflux capacity and all-cause mortality (HR, 0.79; 95% CI, 0.63 to 0.98; P=0.031) disappeared after further adjustment for potential confounders. However, efflux capacity at baseline significantly predicted graft failure (HR, 0.43; 95% CI, 0.29 to 0.64; P<0.001) independent of apolipoprotein A-I, HDL cholesterol, or creatinine clearance. In conclusion, this prospective study shows 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.


Over recent decades large population-based studies established low levels of HDL cholesterol as an important independent risk factor for coronary heart disease.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. Indeed, on the individual level there is substantial variation in the relationship between coronary heart disease and plasma HDL cholesterol.3,4 Further support for the concept of HDL functionality came from pharmacologic intervention studies designed to raise plasma HDL cholesterol levels, which failed to show a clinical benefit.5–7 Although several functions of HDL have been described thus far, cholesterol efflux, the capacity of
HDL to remove cholesterol from macrophage foam cells and the first step in reverse cholesterol transport, is one of the best established beneficial properties of HDL.6-10

Patients with renal failure as well as ESRD are commonly encountered in clinical practice, and renal transplantation represents a frequently performed therapeutic option.11 There is an exceptionally high burden of cardiovascular disease (CVD) in patients with advanced kidney disease and ESRD, with an up to 30-fold age-adjusted increase in mortality, thereby accounting for about half of the total deaths.12 Successful kidney transplantation dramatically reduces cardiovascular mortality rates in patients with ESRD compared with hemodialysis treatment.13 However, renal transplant recipients (RTRs) still have a four to six times higher incidence of CVD than the general population.14 Graft failure represents another important clinical problem in RTRs, and despite progressive improvements in one-year graft survival rates, specifically chronic graft failure after the first year has not been reduced substantially over recent decades.15 In this situation, transplant vasculopathy plays a major role16,17 and pathophysiologic links between chronic humoral rejection and transplant vasculopathy are increasingly recognized.18 In particular, the binding of circulating donor-specific antibodies to mismatched human leukocyte antigen molecules expressed by the graft microvasculature may lead to chronic inflammation and progressive tissue destruction.19 Classic immunosuppressive therapy does not substantially influence chronic humoral rejection and subsequent transplant vasculopathy.16,20 About 50% of transplant recipients develop transplant vasculopathy after 5 years and 90% after 10 years following transplantation, underlining the clinical importance of the problem.16 Interestingly, transplant vasculopathy is a rather diffuse process affecting the whole vascular bed of the transplanted organ and closely resembles classic atherosclerotic lesions.16,21 The vascular alterations are thought to lead to progressive fibrosis and structural loss of organ function.17,20 Importantly, a role for macrophages as well as endothelial inflammation has been indicated for chronic transplant vasculopathy.16,20,21 All being processes on which HDL function conceivably has an impact.6,22

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

RESULTS

This prospective longitudinal study measured cholesterol efflux capacity in a total of 495 RTRs (mean age 51.6±12.0; 54% men). Patients were divided into 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 1. The prevalence of metabolic syndrome decreased significantly with increasing tertiles of cholesterol efflux. Additionally, cholesterol efflux was inversely associated with plasma triglycerides and positively with HDL cholesterol and apolipoprotein A-I levels. Patients in the highest tertile of cholesterol efflux had a lower body mass index (BMI), smaller waist circumference, higher plasma total cholesterol levels, lower plasma insulin, a lower Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), lower high sensitive C-reactive protein (hsCRP), 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 antihypertensive drugs.

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 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. R² of the final model was 0.75. While hsCRP showed an inverse relationship to cholesterol efflux capacity in univariable linear regression (β = -0.101, P = 0.025), this association disappeared when adjusted for confounders (data not shown).

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 patients that survived during follow-up and patients that died. This holds true for both cardiovascular mortality (7.3% [6.4%-8.4%] versus 7.6% [6.3%-8.7%]; P = 0.60) and all-cause mortality (7.3% [6.4%-8.4%] versus 7.2% [6.2%-8.6%]; P = 0.50). However, cholesterol efflux capacity at baseline was significantly lower in RTRs with graft failure compared with RTRs whose graft survived (6.5% [5.2-7.4] versus 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 1A). During follow-up, the corresponding numbers of deaths 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 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. Of note, the cumulative incidence of graft failure significantly decreased in a step-wise fashion with increasing
Table 1. Baseline characteristics according to gender-stratified tertiles of cholesterol efflux

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Gender-stratified Tertiles of Cholesterol Efflux</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First (n=164)</td>
<td>Second (n=166)</td>
</tr>
<tr>
<td>Cholesterol efflux (%)</td>
<td>5.8 [5.3–6.4]</td>
<td>7.3 [6.8–7.9]</td>
</tr>
<tr>
<td>Body composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.9 ± 4.6</td>
<td>25.9 ± 3.9</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>103.4 ± 12.7</td>
<td>101.3 ± 10.8</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>154 ± 24</td>
<td>151 ± 22</td>
</tr>
<tr>
<td>Glucose homeostasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.7 [4.1–5.2]</td>
<td>4.6 [4.1–5.1]</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.4 ± 1.0</td>
<td>5.7 ± 1.2</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2c</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>2.2 [1.6–3.0]</td>
<td>2.0 [1.4–2.6]</td>
</tr>
<tr>
<td>Cardiovascular disease history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction, n (%)</td>
<td>15 (9)</td>
<td>10 (6)</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
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<tr>
<td>Myocardial infarction, n (%)</td>
<td>15 (9)</td>
<td>10 (6)</td>
</tr>
<tr>
<td>TIA/CVA, n (%)</td>
<td>7 (4)</td>
<td>9 (5)</td>
</tr>
<tr>
<td>Donor demographics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>38.2 ± 15.6</td>
<td>37.4 ± 15.4</td>
</tr>
<tr>
<td>Living kidney donor, n (%)</td>
<td>23 (14)</td>
<td>23 (14)</td>
</tr>
<tr>
<td>Renal allograft function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>25.5 [13.0–48.0]</td>
<td>28.5 [14.0–45.0]</td>
</tr>
</tbody>
</table>
| 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 by n (%), and differences were tested by chi-squared test. TIA, transient ischemic attack; CVA, cerebrovascular event; ACE, angiotensin-converting enzyme; Tx, transplantation.

aTertile significantly different from the first tertile, P<0.05.
bTertile significantly different from the first tertile, P<0.01.
cTertile significantly different from the first tertile, P<0.001.
dTertile significantly different from the second tertile, P<0.001.
tertiles of cholesterol efflux (log-rank test: $P=0.004$; Figure 1C), with respective numbers of 23 (14%) in the lowest tertile, 17 (10%) in the middle tertile, and six (4%) in the highest tertile.

Receiver operating characteristic (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 of cholesterol efflux capacity for prediction of cardiovascular mortality was 0.48 (95% confidence interval [95% CI], 0.39 to 0.56, $P=0.60$; Figure 2A) and that for prediction of all-cause mortality was 0.52 (95% CI, 0.46 to 0.59, $P=0.50$; Figure 2B). On the other hand, the area under the ROC curve showed that baseline cholesterol efflux capacity predicted graft failure (0.69 [95% CI, 0.62 to 0.77], $P<0.001$; Figure 2C). Furthermore, the area under the ROC curve concerning prediction of graft failure was slightly higher for cholesterol efflux capacity than for HDL cholesterol mass levels or apolipoprotein A-I levels (Supplemental Figure 1).

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 3). Cholesterol efflux capacity was not associated with future cardiovascular mortality in both univariate (hazard ratio [HR], 1.014 [95% CI, 0.777–1.323], $P=0.92$; Table 3, model 1) and multivariate analyses (Table 3, models 2–5, Supplemental Table 1). Similar results were obtained for the association of HDL cholesterol levels and apolipoprotein A-I levels with CVD mortality (Supplemental Tables 2 and 3). While cholesterol efflux capacity was not related to all-cause mortality in a univariate model (HR, 0.908 [95% CI, 0.741–1.112], $P=0.35$; Table 3, model 1), this association became significant after adjustment for recipient age and gender (HR, 0.786 [95% CI, 0.631–0.978], $P=0.031$; Table 3, model 2). Following additional adjustments for apolipoprotein A-I (HR, 0.841 [95% CI, 0.594–1.911], $P=0.33$; Table 3, model 3), HDL cholesterol (HR, 0.918 [95% CI, 0.659–1.280], $P=0.62$; Table 3, model 4), and creatinine clearance (HR, 0.839 [95% CI, 0.677–1.040], $P=0.11$; Table 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 1). Comparably, low plasma levels of HDL cholesterol or apolipoprotein A-I also did not independently associate with a higher risk for all-cause mortality (Supplemental Tables 2 and 3). Repeating analyses for development of graft failure, in a univariate Cox regression model cholesterol efflux capacity was found to predict graft failure with a HR of 0.428 (95% CI, [0.293–0.625], $P<0.001$; Table 3, model 1). Adjustment for recipient age and gender did not appreciably change this association (HR, 0.433 [95% CI, 0.291–0.644], $P<0.001$; Table 3, model 2).

Prospective study demonstrates 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 future development of graft failure, a condition-

### Table 2. Variables that have independent associations with or are determinants of cholesterol efflux capacity

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>95% CI</th>
<th>Standardized $\beta$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A-I</td>
<td>2.805</td>
<td>2.37 to 3.24</td>
<td>0.493</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.913</td>
<td>1.51 to 2.32</td>
<td>0.367</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Time between Tx and inclusion</td>
<td>0.026</td>
<td>0.01 to 0.04</td>
<td>0.099</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Use of calcineurin inhibitors</td>
<td>0.380</td>
<td>0.16 to 0.60</td>
<td>0.093</td>
<td>$0.001$</td>
</tr>
<tr>
<td>Recipient age</td>
<td>0.011</td>
<td>0.004 to 0.02</td>
<td>0.079</td>
<td>$0.003$</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>$-0.009$</td>
<td>$-0.015$ to $-0.003$</td>
<td>$-0.073$</td>
<td>$&lt;0.01$</td>
</tr>
<tr>
<td>HbA1c</td>
<td>$-0.113$</td>
<td>$-0.20$ to $-0.03$</td>
<td>$-0.068$</td>
<td>$&lt;0.01$</td>
</tr>
</tbody>
</table>

Variables are listed in decreasing order of strength of association according to the absolute value of the standardized beta. Tx, transplantation.
previously linked to accelerated atherosclerosis formation.\textsuperscript{16,21} 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 with 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 Triolo et al.,\textsuperscript{22} Ansell et al.,\textsuperscript{23} and Sviridov et al.\textsuperscript{24}). Importantly, cholesterol efflux capacity from macrophages was inversely related to subclinical atherosclerosis and coronary artery disease in a cross-sectional study.\textsuperscript{25} While in the two published prospective studies one reported an inverse relationship between efflux function of HDL and future events\textsuperscript{26} and the other a protective impact of efflux at baseline with respect to future cardiovascular events.\textsuperscript{27} Previously, the cholesterol acceptor capacity of HDL was indicated to be reduced in RTRs.\textsuperscript{28} Consistently, also with our efflux assay conditions, we observed a significant decrease in the cholesterol acceptor capacities of HDL from RTRs when compared with controls matched for age, sex, and BMI (Supplemental Figure 3). Whether this reduction is a reflection of the reduced kidney function in RTRs or is caused by the immunosuppressive medication or represents a feature of the preexisting kidney disease that transplantation fails to correct remains to be determined in future studies.

An important result of the current study is that cholesterol efflux capacity is not an independent predictor of overall or cardiovascular mortality in RTRs. However, the nature of CVD in RTRs is not well defined and might differ from the general population.\textsuperscript{29} Such a concept is supported by traditional risk factors not consistently being the major determinants of cardiovascular events in RTRs.\textsuperscript{30} 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 also attributable to an excess prevalence of sudden cardiac death and heart failure.\textsuperscript{29} Moreover, as kidney function declines, RTRs may develop uremia, which can cause uremic cardiomyopathy.\textsuperscript{31} 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, known as transplant vasculopathy, is a major pathogenetic factor of chronic renal transplant dysfunction, one of the leading causes of graft failure in RTRs after the first year following transplantation.\textsuperscript{16,21} Importantly, in this process macrophages as well as endothelial activation and inflammation play major roles.\textsuperscript{16} Given the biologic activities of HDL these could conceivably all be beneficially impacted by well functioning HDL.\textsuperscript{4,22} Specifically, a better functionality of HDL in removing cholesterol from macrophage foam cells in the vascular wall would be 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. Thereby, 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 enhanced endothelial expression of adhesion molecules.32 HDL has the ability to inhibit adhesion molecule expression on endothelial cells,33 which may help restrain the recruitment of potentially harmful proinflammatory mononuclear cells into the graft.

Some general methodologic considerations need to be taken into account that apply to all HDL function studies published to date. There is, in contrast to standard clinical chemistry methods, currently no gold standard available regarding use of cell lines and assay conditions used.22 Thus, results of such determinations need to be interpreted in the context of the assay conditions applied. In the present study we (1) aimed to minimize experimental variation by analyzing efflux in all samples at the same time with the same batch of cells and reagents; (2) chose, with the rationale of analyzing patient material, a human macrophage cell line, THP-1, that expresses all relevant efflux transporters (ATP-binding cassette transporter A1 [ABCA1], ABCG1 and scavenger receptor BI), which is in contrast to other work employing mouse macrophage cell lines such as J774 and RAW 264.7,25–27 which require additional treatments with, for example, cyclic AMP to induce ABCA1, lack apoE expression and are poorly responsive to liver X receptor agonists, because liver X receptor α/β expression is almost absent.8,22 However, because a consensus and respective comparative studies are lacking, we cannot formally exclude that the choice of cell line and assay conditions might have an impact on the results.

In conclusion, our results indicate that baseline cholesterol efflux capacity is not a significant risk factor for cardiovascular and all-cause mortality, at least not in RTRs. However, higher cholesterol efflux capacity was independently associated with an increased long-term graft survival after kidney transplantation, which as a potential clinical implication suggests that increasing HDL function might be an attractive novel treatment target for the prevention of graft failure in RTRs.

Table 3. HRs for cardiovascular mortality, all-cause mortality, and graft failure by cholesterol efflux capacity

<table>
<thead>
<tr>
<th>Model</th>
<th>HR [95% CI] per 1-SD increase</th>
<th>P value</th>
<th>HR [95% CI] per 1-SD increase</th>
<th>P value</th>
<th>HR [95% CI] per 1-SD increase</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>1.01 [0.78 to 1.32]</td>
<td>0.92</td>
<td>0.91 [0.74 to 1.11]</td>
<td>0.35</td>
<td>0.43 [0.29 to 0.63]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.89 [0.67 to 1.19]</td>
<td>0.43</td>
<td>0.79 [0.63 to 0.98]</td>
<td>0.031</td>
<td>0.43 [0.29 to 0.64]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.05 [0.68 to 1.62]</td>
<td>0.83</td>
<td>0.84 [0.59 to 1.19]</td>
<td>0.33</td>
<td>0.42 [0.23 to 0.77]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Model 4</td>
<td>1.26 [0.83 to 1.89]</td>
<td>0.28</td>
<td>0.92 [0.66 to 1.28]</td>
<td>0.62</td>
<td>0.56 [0.31 to 0.99]</td>
<td>0.045</td>
</tr>
<tr>
<td>Model 5</td>
<td>0.96 [0.72 to 1.27]</td>
<td>0.75</td>
<td>0.84 [0.68 to 1.04]</td>
<td>0.11</td>
<td>0.52 [0.36 to 0.76]</td>
<td>0.001</td>
</tr>
</tbody>
</table>

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.

CONCISE METHODS

Study Design and Patients
In this study, all adult RTRs who visited the outpatient 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.34 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, BMI, 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 (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.35
Board approved the study protocol (METc2001/039), which complied with the Declaration of Helsinki.

**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 retransplantation. This occurred for the following reasons: recurrence of original disease 4.3%, chronic allograft dysfunction 93.5%, thrombosis/vascular reasons 2.2%. 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 cases where 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). Cardiovascular mortality was defined as deaths in which the principal cause of death was cardiovascular in nature, using ICD-9 codes 410–447. Graft failure and mortality were recorded until May 2009. There was no loss during follow-up.

**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. 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.

**Measurements and Definitions**
For metabolic syndrome the definition of the National Cholesterol Education Program Expert Panel was used. In 2008, the American Diabetes Association (ADA) lowered the cut-off point for impaired fasting glucose to ≥5.6 mmol/l. 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 antidiabetic medication. BMI 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 BV, The Netherlands) in supine position after a 6-minute rest as the average of three measurements at 1-minute intervals.

Blood samples were drawn after an 8–12 hour overnight fasting period. Total cholesterol was determined using 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 (VARIANT TSM Hb Testing System; Bio-Rad, Hercules, CA). Insulin resistance was calculated using HOMA-IR as follows: HOMA-IR=glucose (mmol/l)×insulin (µU/ml)/22.5. Plasma hsCRP was assessed by ELISA as described before.

**Assessment of Cholesterol Efflux**
THP-1 human monocytes (ATTCC via LCG Promochem, Teddington, UK) were seeded in 48-well plates at a concentration of 187,500 cells/well in RPMI 1640 Glutamax medium containing 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 µg/ml) and proteinuria was determined as urinary protein excretion ≥0.5 g per 24 hours.

Blood samples from RTRs for cholesterol efflux measurements, collected at time of inclusion into the study, were placed on ice, centrifuged at 4°C, and immediately stored at −80°C for a period of 11–13 years until 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. After 30 minutes centrifugation at 2200 g, the HDL-containing supernatant was collected, kept on ice, and used directly for cholesterol efflux measurement.

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quantitate the effluxed cholesterol label. 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 (on average 1.55%).

Cholesterol efflux measurements were carried out in all respective patient samples in duplicate at the same time to limit potential variation due to different assay conditions. 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 2).

Statistical Analysis

Normally distributed continuous variables are presented as mean (SD), whereas continuous variables with a skewed distribution are given as median (25th to 75th 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. HRs are reported with 95% CIs.

Recipient baseline characteristics were analyzed separately for gender-stratified tertiles of cholesterol efflux. Subsequently, all characteristics with a P≤0.10 across gender-stratified tertiles of cholesterol efflux were entered into a step-wise multivariate linear regression model with backward elimination (P≤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. 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 HRs 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 HR based on an assumption of 90% power and two-sided α 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 (IBM SPSS, Chicago, IL) and GraphPad Prism version 5.00 (GraphPad Software Inc., San Diego, CA).

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DISCLOSURES

None.

REFERENCES


34. van Ree RM, de Vries AP, Oterdoom LH, The TH, Gansevoort RT, Homan van der Heide JJ, van Son WJ, Bloeg RJ, de Jong PE, Gans RO, Bakker SJ: Abdominal obesity and smoking are important determinants of C-reactive protein in renal transplant recipients. Nephrol Dial Transplant 20: 2524–2531, 2005


