High Density Lipoprotein Cholesterol Efflux Predicts Incident New Onset Diabetes After Transplantation (NODAT) in Renal Transplant Recipients Independent of High Density Lipoprotein Cholesterol Levels

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Abstract

In renal transplant recipients (RTR) new onset diabetes after transplantation (NODAT) is a frequent and serious complication limiting survival of graft and patient. However, the underlying pathophysiology remains incompletely understood. In vitro and in preclinical models, high density lipoproteins (HDL) can preserve beta cell function, largely by mediating cholesterol efflux, but this concept has not been evaluated in humans. This study investigated whether baseline cholesterol efflux capacity (CEC) in RTR is associated with incident NODAT during follow-up. This prospective longitudinal study included 405 diabetes-free RTR with a functioning graft for >1 year. During a median [interquartile range] follow-up of 9.6 [6.6-10.2] years, 57 patients (14.1%) developed NODAT. HDL CEC was quantified using incubation of human macrophage foam cells with apolipoprotein B-depleted plasma. Baseline CEC was significantly lower in patients developing NODAT during follow-up (6.84 [5.84-7.50]%) compared with the NODAT-free group (7.44 [6.46-8.60]%, p=0.001). Kaplan-Meier analysis showed a lower risk for incident NODAT with increasing gender-stratified tertiles of HDL efflux capacity (p=0.004). Linear regression analysis indicated that CEC is independently associated with incident NODAT (p=0.04). In Cox regression analyses, CEC was significantly associated with NODAT (hazard ratio [HR] 0.53; 95% CI, 0.38-0.76; p<0.001), independent of HDL cholesterol levels (p=0.015), adiposity (p=0.018), immunosuppressive medication (p=0.001) and kidney function (p=0.01). Addition of CEC significantly improved the predictive power of the Framingham Diabetes Risk Score (p=0.004). This study establishes HDL CEC as a strong predictor of NODAT in RTR, independent of several other recognized risk factors.

Introduction

Diabetes mellitus (DM) constitutes a prime risk factor for end-stage renal disease (ESRD) [1,2]. However, also ESRD patients free of DM suffer a substantial risk to develop diabetes after receiving a kidney graft [3,4]. Although the incidence of new onset diabetes after transplantation (NODAT) is estimated to be high, some studies report values up to 50%, the pathogenesis of NODAT is still incompletely understood [4,5]. Partly, similar risk factors associated with type 2 diabetes in the general population seem to play a role, partly transplantation-specific impacting factors are held responsible [3–5]. Of particular interest are conditions related to the metabolic syndrome such as obesity on the one hand and medication preventing graft rejection such as tacrolimus or corticosteroids on the other [4,5]. NODAT itself is significantly associated with decreased patient survival and increased kidney graft failure [5,6]. Still, prospective biomarkers helping to stratify patients at high or low risk for NODAT are scarce and no algorithm to predict NODAT in post-transplant RTR has been validated.

The pathophysiology of type 2 diabetes and NODAT has two components, defects in peripheral insulin sensitivity and insufficient insulin secretion by the pancreatic β-cells to meet demand [4,5]. The insulin producing pancreatic β-cell is particularly sensitive to disturbances in cholesterol homeostasis. Low density lipoprotein cholesterol (LDL-C) uptake into β-cells has been associated with cellular dysfunction, apoptosis and failure to secrete sufficient amounts of insulin [7,8]. Clinically, this might be reflected by a higher type 2 diabetes risk in subjects receiving statins that increase cellular LDLR expression [7–9]. High density lipoprotein (HDL) particles on the other hand exert protective effects on β-cells *in vitro* [10]. A key functionality of HDL in this respect is to induce cellular cholesterol efflux and thereby unload cholesterol from β-cells [7,11]. This has been e.g. illustrated by the observation that subjects lacking ABCA1, a major cellular cholesterol efflux transporter, are at an increased risk of developing

T2DM [12]. In the general population, however, an association between low HDL cholesterol (HDL-C) mass levels and diabetes risk cannot be so firmly established, as indicated by a recent Mendelian randomization study [13]. This discrepancy might also point towards an added clinical value of determining metrics of HDL function, such as cholesterol efflux. Next to cellular requirements, structure and composition of the cholesterol receiving HDL particle are key factors determining the rate of efflux [7,11]. Thus, specifically via their cholesterol efflux function, HDL particles might be able to modify the risk of developing type 2 diabetes or NODAT. However, no studies are currently available that prospectively evaluate this concept in RTR. Therefore, the present work aims to establish whether baseline cholesterol efflux in RTR is associated with incident NODAT during follow-up.

Methods

Study design and study population

For inclusion into this prospective cohort study all RTR at the UMCG (University Medical Center Groningen) with a functioning allograft for more than one year after transplantation were eligible. Patient recruitment was carried out between August 2001 and July 2003. RTR are subjected to a continuous follow-up system in the outpatient clinic with a declining frequency as outlined in the American Transplantation Society guidelines, ranging from twice a week immediately after hospital discharge to twice a year in the long-term course after transplantation [14]. Patients with known systemic illnesses such as congestive heart failure, cancer other than cured skin cancer, endocrine disorders other than diabetes or overt generalized infections were excluded. Of 847 eligible patients, 606 RTRs gave written informed consent to participate. Participants did not differ from the group that did not give consent with respect to age, gender, BMI, plasma creatinine, creatinine clearance, and proteinuria. A more comprehensive description of the study setup has been published previously[15-18]. In order to study the future development of diabetes mellitus of the 606 included RTR 105 with already existing diabetes mellitus type I and II or impaired fasting glucose or using glucose lowering drugs were excluded. Furthermore, for 92 of the remaining RTRs no baseline material was available to determine HDL cholesterol efflux. In addition, 4 RTR were not included, since they received a mixed liver-kidney transplant. Clinical information regarding donors and recipients as well as transplant characteristics were obtained from the Groningen renal transplant database. The study protocol complied with the declaration of Helsinki and was approved by the Institutional Review Board (METc 01/039).

End point of the study

The main outcome measure of this study is HDL cholesterol efflux. The primary end point is incident NODAT during follow-up.

Measurements and definitions

NODAT was defined according to the Expert Panel recommendations based on the 2003 American Diabetes Association criteria[19]. The diagnosis was based on the following criteria: classic symptoms (unexplained weight loss, polydipsia, polyuria), fasting (no caloric intake for at least 8 hours) plasma glucose concentration >126 mg/dL (7.0 mmol/L), nonfasting plasma glucose concentration of >200 mg/dL (11 mmol/L) or the use of glucose lowering medication (such medication was only prescribed to patients with manifest NODAT). Patients were categorized as having impaired plasma glucose, if fasting plasma glucose was >100 mg/dL (5.6 mmol/L) and <126 mg/dL (7.0 mmol/L). At all routine follow-up visits (see above) fasting glucose was determined using capillary glucose testing (glucose-oxidase method, YSI 2300 Stat Plus; Yellow Springs, OH). In case plasma glucose was elevated, a confirmatory laboratory test of venous plasma was done on the subsequent day or upon the next visit, after which the diagnosis of

NODAT was made. To establish/exclude diabetes at baseline the same criteria were applied as described above, thus also a confirmatory glucose measurement was required.

Waist circumference was measured on the skin midway between the iliac crest and the 10th rib. Blood pressure was measured three times in supine position after at least 6 minutes rest using an automated (Omron M4, Omron B.V., Hoofdorp, The Netherlands) device and then the average of the three measurements was taken. BMI was calculated as weight (kg) divided by height in meters squared. Insulin resistance was calculated using HOMA-IR according to the formula: HOMA-IR=glucose (mmol/l)×insulin (μU/ml)/22.5. HbA1c was assessed by high-performance liquid chromatography (VARIANTTM Hb Testing System; Bio-Rad, Hercules, CA). eGFR was calculated according to the CKD-EPI equations [20].

Blood samples were drawn in the morning after an 8–12 hour overnight fast. Total cholesterol, HDL cholesterol, triglycerides, and plasma insulin were analyzed by routine clinical chemistry methods as detailed before[15]. LDL cholesterol was calculated using the Friedewald equation, apolipoprotein A-I was determined by immunoturbidimetry (COBAS Integra System; Roche Diagnostics, Mannheim, Germany). Plasma hsCRP was measured by ELISA [15]. Plasma and urine creatinine concentrations were determined using a modified version of the Jaffé method (MEGA AU 510; Merck Diagnostica). Total urinary protein concentration was measured with the Biuret reaction (MEGA AU 510; Merck Diagnostica); proteinuria was defined as an urinary protein excretion ≥0.5 g per 24 hours.

Determination of HDL cholesterol efflux

For cholesterol efflux measurements blood samples were collected in EDTA containing tubes at time of inclusion into the study, immediately placed on ice, centrifuged at 4°C, and stored at −80°C. Efflux was determined following a previously validated protocol [14,21,22]. Briefly, HDL was isolated by precipitating apoB-containing lipoproteins with polyethylene glycol (PEG 6000, Sigma, St Louis, MO) in 10 mM HEPES (pH = 8.0) as detailed previously [15,21-24]. In order to assess HDL cholesterol efflux capacity, THP-1 human monocytes (American Type Culture Collection, Manassas, VA) were cultured in 48-well plates in RPMI 1640 Glutamax Medium (Gibco, Carlsbad, CA) containing 10% FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml) and then differentiated into macrophages by the addition of 100 nM phorbol myristate acetate (Sigma) for 24 hours. Macrophages were subsequently loaded with 50 μg/ml acetylated LDL and 1 μCi/ml ^{3}H-cholesterol (Perkin Elmer, Boston, MA) for 24 hours followed by overnight equilibration with RPMI 1640 Glutamax Medium containing 2% BSA (Sigma). After washing with PBS, efflux was determined by the addition of 2% of individual apoB-depleted plasma samples for 5h. Then medium was collected and centrifuged in a tabletop centrifuge (Hettich, Tuttlingen, Germany) for 5 minutes at 10,000 rpm to pellet cellular debris, and radioactivity was determined in an aliquot by liquid scintillation counting (Packard 1600CA Tri-Carb; Packard, Meriden, CT). To the cells, 0.1 M NaOH was added for at least 30 minutes, and then radioactivity remaining in the cells was determined. Efflux per well was calculated 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 HDL were subtracted to correct for unspecific efflux. Cholesterol efflux measurements were carried out in duplicate and in all respective patient samples at the same time to limit potential variation due to different assay conditions. To be able to correct for potential plate-to-plate variation, the same apoB-depleted control plasma was included on each plate at four different concentrations. In this assay, almost the complete biological activity (>95%) of apoB-depleted plasma for cholesterol efflux is dependent on the presence of HDL, and freezing of plasma samples has no impact on the results [22]. The intra-assay CV of this method was previously determined to be 5.4%, the interassay CV 7.9% [15,25].

Statistical Analysis

A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 24 (IBM SPSS). All variables were checked for normality. Data with skewed distribution are expressed as median [IQR] and data with normal distribution are expressed as mean ± SD. Absolute numbers (percentages) are given for categorical variables. For variables with a skewed distribution logarithmic transformation was used in order to reach normality criteria. The development of NODAT was visualized using Kaplan-Meier curves comparing all tertiles of efflux capacity; statistical significance was tested with the log rank (Mantel-Cox) test. In order to investigate parameters independently associated with NODAT, all characteristics with a P≤0.1 across gender-stratified cholesterol efflux tertiles were entered into a stepwise multivariate linear regression model with backward elimination. To determine, if cholesterol efflux was prospectively associated with the risk of NODAT, Cox proportional hazards regression analysis was performed. In order to adjust for potential confounders different models were made as indicated. Models were made based on associations (P<0.1) of respective baseline characteristics with efflux (table 1). Subsequently, the predictive capacity of cholesterol efflux was assessed using logistic regression analysis. Therefore, first the Framingham Diabetes Risk Score [26] was calculated for all participants, and in a subsequent step the performance of that diabetes prediction score was evaluated without and after the addition of cholesterol efflux.

Results

Baseline demographic characteristics

In this prospective longitudinal study HDL cholesterol efflux capacity was determined in 405 included

RTR (median [interquartile range] age 51.5 [42.2-59.4] years, 55.8% male), all free of diabetes at baseline. First, patients were divided into gender-stratified tertiles according to cholesterol efflux. The median efflux values in the respective tertiles were, first, 5.9% (5.4%–6.4%); second, 7.2% (6.8%–8.0%); and third, 9.0% (8.1%–10.0%). Baseline characteristics among the tertiles are given in Table 1. Higher efflux values were associated with higher age, lower BMI and waist circumference, lower plasma insulin levels and a lower HOMA-IR. Further, patients with better graft function, determined as serum creatinine and eGFR, had a higher efflux capacity. In addition, HDL efflux function was positively associated with plasma total cholesterol, mainly explained by the relationship of efflux with HDL-C, while LDL-C was not different among the groups. Finally, patients with higher efflux values used less antihypertensives and had a higher daily dose of prednisolone, with no significant differences being detected with respect to the use of other immunosuppressive medications or statins.

Association of cholesterol efflux with incident NODAT

During a median follow-up of 9.6 [6.6-10.2] years, a total of 57 patients (14.1%) developed NODAT. Baseline cholesterol efflux values were significantly lower in patients diagnosed with NODAT during follow-up compared with the group that remained NODAT free (6.84 [5.84-7.50]% vs. 7.44 [6.46-8.60]%, resp., $p=0.001$) already indicating a possible association between better HDL efflux capacity at baseline and less incident NODAT during follow-up. Stepwise linear regression analysis entering all patient characteristics with a p value ≤ 0.1 across the gender-stratified cholesterol efflux tertiles was carried out to identify independent determinants of NODAT (Table 2). The results indicate that with decreasing order of strength plasma glucose concentration (OR, 2.65 [1.68-4.17], $p<0.001$), plasma triglyceride concentration (OR, 1.50 [1.15-2.00], $p=0.003$), notably cholesterol efflux (OR, 0.80 [0.64-0.99], $p=0.04$) and BMI (OR, 1.10 [1.02-1.19], $p=0.01$) were independently associated with the development of NODAT in RTR, while e.g. HDL-C mass levels were not.

Next, NODAT incidence between tertiles of cholesterol efflux was compared using Kaplan-Meier analysis. Kaplan-Meier curves demonstrated a highly significant association of gender-stratified efflux percentage with the future development of NODAT (log-rank test: p=0.002, Figure 1); during follow-up 22.2% (n=30) of the patients in the lowest tertile developed NODAT, 13.2% (n=18) in the middle tertile and 6.7% (n=9) in the high efflux tertile.

Furthermore, COX proportional hazard analyses were carried out with the aim to determine the independent contribution of HDL cholesterol efflux capacity to the risk of developing NODAT (Table 3, for extended information please see supplemental table I). In univariate analysis (model 1) cholesterol efflux was significantly related to incident NODAT (HR, 0.54 [0.38-0.76], P<0.01). Further adjustment for age and gender (model 2) strengthened this association (HR, 0.53 [0.38-0.76], p<0.001). With the further addition of BMI and waist circumference to the multivariate analysis (model 3) efflux was still significantly associated with NODAT (HR, 0.65 [0.45-0.93], p=0.018). Also with adding time since renal transplantation (model 4, HR, 0.54 [0.38-0.77], p=0.01) or eGFR (model 5, HR, 0.53 [0.37-0.76], p=0.01) or plasma insulin and HOMA-IR (model 6, HR 0.56 [0.39-0.81], p=0.02) to model 2 the association between efflux and NODAT remained significant. Interestingly, model 7 demonstrates that taking account of a number of relevant lipid parameters including total cholesterol, HDL-C, apoA-I, apoB, and triglycerides also did not change the significant association of cholesterol efflux and incident NODAT (HR, 0.52 [0.31-0.88], p=0.015). Further adjustment of model 2 for a number of relevant immunosuppressive medications used by RTR did not change the conclusions reached from the other models (model 8, HR, 0.53 [0.37-0.76], p=0.001). Finally, adding statin use did not weaken the association (model 9, HR, 0.54 [0.38-0.76], p<0.001). For further adjustments for additional covariates including individual immunosuppressive medications as well as the independent determinants of NODAT identified by the linear regression analysis please see supplemental table I.

Next, logistic regression analyses were carried out to explore whether addition of cholesterol efflux would add to a classical diabetes prediction model. For this purpose we chose the Framingham Diabetes Prediction Score, which, although not fully validated, has been indicated to be of value for the prediction of NODAT in RTR [26]. As shown in table 4, also in our cohort, the Framingham Diabetes Prediction Score was useful in predicting NODAT (OR, 1.13 [1.08-1.18], $p<0.001$). Interestingly, including cholesterol efflux significantly improved the predictive capacity of the Framingham Diabetes Prediction Score, further strengthening the potential clinical utility of efflux determinations (OR, 0.74 [0.60-0.92], change from previous step, $p=0.004$).

Combined, these data demonstrate that in RTR cholesterol efflux at baseline is significantly associated with the future risk to develop NODAT, independent of a number of established and perceived risk factors. Importantly, also taking account of lipid and lipoprotein measurements determined in routine clinical chemistry evaluations such as mass HDL-C levels did not change this conclusion.

Discussion

The results of this prospective study demonstrate that the cholesterol efflux function of HDL predicts NODAT in RTR, independent of mass levels of the commonly determined biomarker HDL cholesterol as well as a number of other relevant impacting factors. Of note, cholesterol efflux represents a recognized key metric of HDL functionality with a pathophysiological link to integrity and function of pancreatic beta cells [7–9]. Thereby, these data emphasize the concept that clinically relevant information can be retrieved from HDL function studies.

With an incidence of up to 50% among RTR diabetes-free at time of transplantation, NODAT is a frequent complication after renal transplantation [3–5]. NODAT is associated with reduced graft survival as well as with an increased risk for infection and cardiovascular mortality, all contributing to decreased patient survival [4,5,27]. In the course of the rapidly increasing overall incidence of type 2 diabetes it is believed that NODAT will become an even more prominent clinical problem in the future [3–5]. In addition to the impact on health and quality of life of the individual patient, NODAT also imposes significant costs on health care systems; in the US it was estimated that within the first year after renal transplantation NODAT causes costs of $12,000/patient and more than $19,000 in the following year [28]. Although NODAT thus represents a substantial burden, surprisingly little is known about its specific pathophysiology. It is recognized, however, that general factors relevant for type 2 diabetes play a role such as age, family history of diabetes or previous glucose intolerance as well as specific factors associated with the underlying kidney disease before transplantation and e.g. the immunosuppressive medications mandatory following transplantation [3-5,27]. With respect to the glycemic effects of immunosuppressive drugs, glucose tolerance testing indicated that, next to the known negative effect of glucocorticoids[4,5], tacrolimus reduces insulin secretion by pancreatic β-cells in a dose-dependent fashion contributing to hyperglycemia in kidney transplant recipients[29]. Converting renal transplantation patients from tacrolimus to cyclosporin A on the other hand was associated with improved glucose metabolism parameters [30]. An additional improvement in glycemic control was noted when RTR were switched to a cyclosporin A sparing immunosuppression by the use of mTOR inhibitors such as everolimus [31]. Mirroring the impact of general type 2 diabetes risk on NODAT pathogenesis, risk scores for the prediction of incident type 2 diabetes have been applied with some success in an attempt to provide a better prediction for NODAT. Models using both pre-transplant data (including planned use of maintenance corticosteroids) [32] as well as early post-transplant clinical information (San Antonio Diabetes Prediction Model, Framingham Offspring Study-Diabetes Mellitus) were able to predict NODAT to a certain extent[26]. Currently, no single predictive biomarker is available to assign individual risk to patients to help identify RTR who need early therapeutic intervention. In our study the linear association of blood glucose and triglycerides levels with NODAT was stronger than the association of HDL cholesterol efflux capacity with NODAT. Nonetheless, addition of cholesterol efflux capacity to the Framingham diabetes risk score improved the prediction capacity for NODAT. So, even though the overall effect size was relatively small, these data suggest that HDL cholesterol efflux capacity provides a useful additive biomarker for NODAT that is also mechanistically linked to diabetes pathophysiology.

In the cardiovascular field studying HDL function, in particular cholesterol efflux, represents an emerging topic [11,33]. Available data indicate that low cholesterol efflux is prospectively associated with increased incident cardiovascular events [23,24] in the general population and with chronic atherosclerosis-driven graft failure in RTR [15]. However, cholesterol is not only relevant for CVD but also for both components that determine deranged glucose metabolism in type 2 diabetes/NODAT, namely increased peripheral insulin resistance and decreased functionality of pancreatic β-cells that fail to secrete sufficient amounts of insulin to meet the increased demand [4,5]. Infusion of recombinant HDL particles has e.g. been shown to improve insulin sensitivity of skeletal muscle in humans [34]. On the other hand, pancreatic beta cells maintain a tight balance of their cholesterol content pertinent to their function [7,8]. In vitro studies established that loading beta cells with cholesterol induces beta cell dysfunction and apoptosis resulting in reduced insulin secretion, factors conceivably contributing to the pathogenesis of type 2 diabetes [7,8]. On a population level these data are mirrored by the now well recognized increased risk of type 2 diabetes associated with statin therapy [9]. Statins increase the cell surface expression of LDLR and thereby stimulate LDL uptake and consequently cholesterol loading of beta cells [8,9]. On the other hand are higher HDL-C levels associated with decreased incident type 2 diabetes in the general population [35]. In vitro, HDL particles were shown to protect beta cells from ER stress and apoptosis and to preserve their functionality [36,37]. These beneficial biological effects appear intricately linked to the cholesterol efflux function of HDL particles [8,10]. In vivo support for these findings comes from studies in mice and humans demonstrating that reduced expression or lack of the major cellular cholesterol export transporter ABCA1 is associated with reduced beta cell function and an increased type 2 diabetes risk [12,38]. ABCA1 is established to interact with apoA-I, the class defining apolipoprotein of HDL, to induce cellular cholesterol unloading [11,39]. In addition, HDL also seems to have the capacity to improve peripheral insulin sensitivity as evidenced by studies using intravenous infusion of recombinant HDL particles [34]. Taken together, although these combined data indicate that HDL can conceivably protect against type 2 diabetes, literature exploring this concept in humans is scarce and especially no such results are currently available in the setting of NODAT, again emphasizing the novelty of our approach.

Several considerations with respect to potential limitations of our study should be taken into account. In general, statistical associations do not allow to draw firm conclusions on cause-effect relationships. Moreover, the interpretation of HDL function assays depends on the respective chosen assay conditions and on the HDL isolation method [11]. Currently no consensus has been reached with respect to standardization of these parameters, so that such assays are not fully comparable to e.g. values obtained by clinical chemistry determinations. In our work we employed an established assay that is using human macrophage foam cells, in which all efflux pathways are active (ABCA1, 47% contribution, determined by addition of probucol; SR-BI, 19% contribution, determined by addition of BLT-1; and ABCG1, 30% contribution, determined by the addition of probucol and BLT-1, unpublished data); this offers certain advantages over e.g. murine J774 macrophages equilibrated with cholesterol label, in which efflux mainly depends on the ABCA1 system [40]. HDL isolation was carried out with a protocol widely used in efflux studies [15,21,23,24]. In addition, to minimize experimental variation, all efflux experiments were done at the same time with identical batches of cells and reagents. Next to technical, assay-related considerations it should be pointed out that the data reported here are from a single center and that one of the inclusion criteria was to only study patients with a functioning allograft for more than one year. This was done, because we wanted to exclude the impact of acute rejections, which are most prevalent during the first year, including ample adaptations of the immunosuppressive regime and be able to evaluate the chronic long-term course after transplantation. However, that means in turn that our data do not allow to draw conclusions with respect to other factors that might play a role during the first post-transplant year. Although Transplant Lines is one of the largest prospective renal transplant cohorts, a multicenter approach appears desirable to confirm our results. Such a follow-up study would also aid to identify a simple and easy-to-measure biomarker that is reliably reflecting HDL function and thus has the potential to replace HDL cholesterol in routine clinical determinations. The final goal of such experimental efforts would be to define therapeutic interventions targeted to improve HDL function, and then test, if NODAT can either be prevented or at least substantially delayed. Metabolomics hold great potential in this respect, not only for the identification of HDL-associated biomarkers, but also in general terms for the elucidation of molecules with the capacity to serve as predictors for the development of cardiometabolic disease in patients with compromised kidney function [41].

In conclusion, the present study establishes HDL cholesterol efflux capacity as a predictor of NODAT in RTR independent of a number of other recognized risk factors. HDL function measurements thus might be promising not only for improved diagnostics but also to better characterize a possible emerging target for therapeutic intervention.

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References

1. Levin A, Tonelli M, Bonventre J, et al. Global kidney health 2017 and beyond: a roadmap for closing gaps in care, research, and policy. Lancet. 2018;390:1888-1917.
2. Webster AC, Nagler E V, Morton RL, Masson P. Chronic Kidney Disease. Lancet. 2018;389:1238-1252.
3. Montori V, Basu A, Erwin P, Velosa J, Gabriel S, Kudva Y. Posttransplantation diabetes: a systematic review of the literature. Diabetes Care. 2002;25:583–592.
4. Jenssen T, Hartmann A. Emerging treatments for post-transplantation diabetes mellitus. Nat Rev Nephrol. 2015;11:465-477.
5. Tufton N, Ahmad S, Rolfe C, Rajkariar R, Byrne C, Chowdhury TA. New-onset diabetes after renal

transplantation. Diabet Med. 2014;31:1284-1292.

6. Sharif A, Baboolal K. Complications associated with new-onset diabetes after kidney transplantation. Nat Rev Nephrol. 2011;8:34-42.

7. von Eckardstein A, Sibler R a. Possible contributions of lipoproteins and cholesterol to the pathogenesis of diabetes mellitus type 2. Curr Opin Lipidol. 2011;22:26-32.

8. Kruit JK, Brunham LR, Verchere CB, Hayden MR. HDL and LDL cholesterol significantly influence β-cell function in type 2 diabetes mellitus. Curr Opin Lipidol. 2010;21:178-185.

9. Sattar N, Preiss D, Murray HM, et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet. 2017;375:735-742.

10. von Eckardstein A, Widmann C. High-density lipoprotein, beta cells, and diabetes . Cardiovasc Res. 2014;103:384-394.

11. Triolo M, Annema W, Dullaart RPF, Tietge UJF. Assessing the functional properties of high-density lipoproteins: an emerging concept in cardiovascular research. Biomark Med. 2013;7:457-472.

12. Vergeer M, Brunham LR, Koetsveld J, et al. Carriers of loss-of-function mutations in ABCA1 display pancreatic beta-cell dysfunction. Diabetes Care. 2010;33:869-874.

13. Haase CL, Tybjærg-Hansen A, Nordestgaard BG, Frikke-Schmidt R. HDL Cholesterol and Risk of Type 2 Diabetes: A Mendelian Randomization Study. Diabetes. 2015;64:3328-3333.

14. Kasiske BL, Vazquez MA, Harmon WE, et al. Recommendations for the Outpatient Surveillance of Renal Transplant Recipients. J Am Soc Nephrol. 2000;11:S1-S86.

15. Annema W, Dikkers A, Freark de Boer J, et al. HDL Cholesterol Efflux Predicts Graft Failure in Renal Transplant Recipients. J Am Soc Nephrol. 2016;27:595-603.

16. van Ree RM, de Vries APJ, Oterdoom LH, et al. Abdominal obesity and smoking are important

determinants of C-reactive protein in renal transplant recipients. Nephrol Dial Transplant. 2005;20:2524-2531.

17. Eisenga MF, Zelle DM, Sloan JH, Gaillard CAJM, Bakker SJL, Dullaart RPF. High Serum PCSK9 Is Associated With Increased Risk of New-Onset Diabetes After Transplantation in Renal Transplant Recipients. Diabetes Care. 2017;40:894-901.
18. Zelle DM, Corpeleijn E, Deinum J, et al. Pancreatic β-Cell Dysfunction and Risk of New-Onset Diabetes After Kidney Transplantation. Diabetes Care. 2013;36:1926-1932.
19. Davidson J, Wilkinson A, Dantal J, et al. New-onset diabetes after transplantation: 2003 international consensus guidelines. Transplantation. 2003;75(10 Suppl):SS3-SS24.
20. White SL, Polkinghorne KR, Atkins RC, Chadban SJ. Comparison of the prevalence and mortality risk of CKD in Australia using the CKD Epidemiology Collaboration (CKD-EPI) and Modification of Diet in Renal Disease (MDRD) Study GFR estimating equations: the AusDiab (Australian Diabetes, Obesity and Lifestyle) Study. Am J Kidney Dis. 2010;55:660-670.
21. Kopecky C, Ebtehaj S, Genser B, et al. HDL Cholesterol Efflux Does Not Predict Cardiovascular Risk in Hemodialysis Patients. J Am Soc Nephrol . 2017;28:769-775.
22. Annema W, Willemsen HM, de Boer JF, et al. HDL function is impaired in acute myocardial infarction independent of plasma HDL cholesterol levels. J Clin Lipidol. 2016;10:1318-1328.
23. Rohatgi A, Khera A, Berry JD, et al. HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events. N Engl J Med. 2014;371:2383-2393.
24. Saleheen D, Scott R, Javad S, et al. Association of HDL cholesterol efflux capacity with incident coronary heart disease events: a prospective case-control study. Lancet Diabetes Endocrinol. 2015;3:507-513.
25. Annema W, Dikkers A, de Boer JF, et al. Impaired HDL cholesterol efflux in metabolic syndrome

is unrelated to glucose tolerance status: the CODAM study. Sci Rep. 2016;8;6:27367.

26. Rodrigo E, Santos L, Piñera C, et al. Prediction at First Year of Incident New-Onset Diabetes After Kidney Transplantation by Risk Prediction Models. Diabetes Care. 2012;35:471-473.

27. Palepu S, Prasad GVR. New-onset diabetes mellitus after kidney transplantation: Current status and future directions. World J Diabetes. 2015;6:445-455.

28. Woodward RS, Schnitzler MA, Baty J, et al. Incidence and Cost of New Onset Diabetes Mellitus Among U.S. Wait-Listed and Transplanted Renal Allograft Recipients. Am J Transplant. 2003;3:590-598.

29. Filler G, Neuschulz I, Vollmer I, Amendt P, Hocher B. Tacrolimus reversibly reduces insulin secretion in paediatric renal transplant recipients. Nephrol Dial Transplant. 2000;15:867-871.

30. Wissing KM1, Abramowicz D2, Weekers L, et al. Prospective randomized study of conversion from tacrolimus to cyclosporine A to improve glucose metabolism in patients with posttransplant diabetes mellitus after renal transplantation. Am J Transplant. 2018;18:1726-1734.

31. Kälble F, Seckinger J, Schaier M, Morath C, Schwenger V, Zeier M, Sommerer C. Switch to an everolimus-facilitated cyclosporine A sparing immunosuppression improves glycemic control in selected kidney transplant recipients. Clin Transplant. 2017;31. doi: 10.1111/ctr.13024.

32. Chakkera HA, Chang Y-H, Ayub A, Gonwa TA, Weil EJ, Knowler WC. Validation of a Pretransplant Risk Score for New-Onset Diabetes After Kidney Transplantation. Diabetes Care. 2013;36:2881-2886.

33. Rosenson RS, Brewer Jr HB, Ansell BJ, et al. Dysfunctional HDL and atherosclerotic cardiovascular disease. Nat Rev Cardiol. 2015;13:48-60.

34. Drew BG, Duffy SJ, Formosa MF, et al. High-density lipoprotein modulates glucose metabolism in patients with type 2 diabetes mellitus. Circulation. 2009;119:2103-2111.

35. Abbasi A, Corpeleijn E, Gansevoort RT, et al. Role of HDL Cholesterol and Estimates of HDL Particle Composition in Future Development of Type 2 Diabetes in the General Population: The PREVEND Study. J Clin Endocrinol Metab. 2013;98:E1352-E1359.

36. Pétremand J, Puyal J, Chatton J-Y, et al. HDLs Protect Pancreatic β-Cells Against ER Stress by Restoring Protein Folding and Trafficking. Diabetes. 2012;61:1100-1111.

37. Puyal J, Pétremand J, Dubuis G, Rummel C, Widmann C. HDLs protect the MIN6 insulinoma cell line against tunicamycin-induced apoptosis without inhibiting ER stress and without restoring ER functionality. Mol Cell Endocrinol. 2013;381:291-301.

38. Brunham LR, Kruit JK, Pape TD, et al. β-cell ABCA1 influences insulin secretion, glucose homeostasis and response to thiazolidinedione treatment. Nat Med. 2007;13:340-347.

39. Phillips MC. Is ABCA1 a Lipid Transfer Protein? J Lipid Res. 2018;59:749-763.

40. Kon V, Linton MF. HDL: Beyond Atheroprotection. J Am Soc Nephrol. 2016;27:341-344.

41. Hocher B, Adamski J. Metabolomics for clinical use and research in chronic kidney disease. Nat Rev Nephrol. 2017;13:269-284.

Table 1: Baseline patient characteristics according to gender-stratified tertiles of cholesterol efflux

n=405	Tertiles of gender-stratified HDL cholesterol efflux (%)			
variable	T1 (low tertile)	T2 (middle tertile)	T3 (high tertile)	P value for trend
HDL cholesterol efflux (%)	5.9 [5.4-6.4]	7.2 [6.8-8.0]	9.0 [8.1-10.0]	
General characteristics				
Age (years)	49.8 [41.5-58,0]	51.1 [40.4-58.9]	55.5 [45-61.5]	0.003
Male sex (%)	56.3	55.1	60.0	0.981
Smoking status				
Never smoker (%)	37	32.4	31.3	0.572
Former smoker (%)	40.7	44.9	45.5	0.693
Current smoker (%)	22.2	22.1	22.4	0.998
Alcohol consumption				0.687
None (%)	46.7	50	44	
>4 units/day (%)	0.7	0.7	2.2	
1-3 units/day (%)	11.1	14.7	13.4	
2-7 units/week (%)	23.7	22.1	25.4	
1-4 units/month (%)	17.8	11	13.4	
Body composition				
BMI (kg/m^2)	26.1 [23.5-29.2]	25.4 [23.1-27.6]	24.2 [22.6-26.6]	0.002
Waist circumference (cm)	98.6±13.7	95.6±12.6	92.4±12.4	<0.001
Transplant history				
Time since renal transplantation (years)	5.8 [2.1-9.2]	5.3 [2.4-10.6]	8.3 [4.1-13.8]	0.001
Deceased donor (%)	84.4	84.6	89.6	0.383
Donor age (years)	41 [24-52]	41 [23-52]	32 [23-50]	0.363
Dialysis duration (months)	26 [13-51]	27.5 [12.3-45]	30 [13.8-52.3]	0.363
Renal allograft function				
Serum creatinine (µmol/L)	150 [121-183]	133 [112-157]	126 [106-148]	<0.001
eGFR (mL/min/1.73 m^2)	42.3±15.3	49.4±15.8	49.9±15.5	<0.001
Urinary albumin to creatinine ratio	15.4 [5.2-50.7]	8.0 [3.4-43.2]	13.7 [3.8-68.6]	0.056
Inflammatory markers				

hsC-reactive protein	2.1 [0.9-4.6]	1.8 [0.7-4.3]	1.7 [0.7-3.5]	0.097
Blood pressure				
Diastolic blood pressure (mmHg)	90±10	89±9	90±10	0.454
Systolic blood pressure (mmHg)	149 [135-166]	147 [132-160]	152 [134-167]	0.204
Glucose homeostasis				
Fasted plasma glucose (mmol/L)	4.5 [4.1-4.9]	4.5 [4.1-4.9]	4.4 [4.0-4.8]	0.305
Plasma insulin (µmol/L)	11.5 [8.6-14.8]	10.9 [7.9-15.8]	9.0 [6.3-11.7]	<0.001
Hba1c (%)	6.4 [5.8-6.7]	6.2 [5.7-6.6]	6.1 [5.6-6.6]	0.148
Hba1c (mmol/mol)	46 [40-50]	44 [39-49]	43 [38-49]	0.148
Family history of diabetes: parent or sibling with diabetes, n (%)	13.3	8.8	9.7	0.466
Lipids and lipoproteins				
Total cholesterol (mmol/L)	5.4±0,9	5.71±1.25	5.82±0.9	0.004
LDL cholesterol (mmol/L)	3.6 [2.9-4.1]	3.5 [3.0-4.2]	3.5 [3.1-4.1]	0.824
HDL cholesterol (mmol/L)	0.86 [0.71-0.99]	1.1 [0.96-1.2]	1.4 [1.2-1.5]	<0.001
ApoA-I	1.3 [1.2-1.5]	1.6 [1.4-1.7]	1.8 [1.6-2.0]	<0.001
ApoB	1.1 ±0.2	1.1 ±0.2	1.0 ±0.2	0.006
Triglycerides (mmol/L)	2.1 [1.6-2.8]	2.0 [1.4-2.7]	1.6 [1.2-2.1]	<0.001
Medication use				
Antihypertensives (%)	92.6	80.9	82.1	<0.001
Statins (%)	48.1	48.5	51.5	0.377
Proliferation inhibitor (%)	80.7	75.7	70.1	0.129
Calcineurin inhibitor (%)	77.8	83.8	73.9	0.134
Tacrolimus (%)	15.6	14.0	8.2	0.161
Cyclosporine (%)	62.2	69.1	64.9	0.485
Prednisolone (mg/24 h)	10 [8.8-10]	10 [8.8-10]	10 [7.5-10]	0.015

Continuous data with a skewed distribution are given as median [IQR] and differences were assessed using the Kruskal-Wallis test. Normally distributed continuous data are presented as mean ± standard deviation and differences were tested using one way ANOVA. Categorical data are given as n (%), and differences were analyzed by chi-squared test.

Table 2: Variables that are determinants of NODAT

	Odds Ratio	95% CI	P value
Glucose concentration (mmol/L)	2.65	1.68-4.17	<0.001
Triglyceride concentration (mmol/L)	1.50	1.15-2.00	0.003
Cholesterol efflux (%)	0.80	0.64-0.99	0.04
BMI (kg/m^2)	1.10	1.02-1.19	0.01

Variables are listed in decreasing order of strength of association according to the odds ratio.

Table 3: Cox regression analysis to determine hazard ratios for NODAT incidence by cholesterol efflux capacity

	HR [95% CI]	P value
Model 1	0.54 [0.38-0.76]	0.01
Model 2	0.53 [0.38-0.76]	<0.001
Model 3	0.65 [0.45-0.93]	0.025
Model 4	0.54 [0.38-0.77]	0.01
Model 5	0.53 [0.37-0.75]	<0.001
Model 6	0.56 [0.39-0.81]	0.02
Model 7	0.52 [0.31-0.88]	0.015
Model 8	0.53 [0.37-0.76]	0.004
Model 9	0.54 [0.38-0.76]	<0.001

Model 1 crude analysis; model 2: adjustment for age and gender; model 3: model 2 + adjustment for BMI, waist circumference and CRP; model 4: model 2 + adjustment for time since renal transplantation; model 5: model 2 + adjustment for eGFR and urinary albumin to creatinine ratio; model 6: model 2 + adjustment for serum concentration of insulin and HOMA-IR; model 7: model 2 + adjustment for total cholesterol, HDL cholesterol, apoA-I, apoB and triglycerides; model 8: model 2 + adjustment for use of proliferation inhibitors, calcineurin inhibitors, tacrolimus, cyclosporine, anti-hypertensives and daily prednisolone dose; model 9: model 2 + adjustment for use of statins.

Table 4: Logistic regression analysis of the Framingham diabetes risk score without and with the addition of HDL cholesterol efflux measurements

	Odds Ratio [95% CI]	P value	Change from previous step	
			Model x^2	P value
Model 1:		<0.001	30.5	NA
Framingham diabetes risk score	1.13 [1.08-1.18]			
Model 2:		<0.001	8.5	0.004
Framingham diabetes risk score	1.12 [1.07-1.18]			
Cholesterol efflux (%)	0.74 [0.60-0.92]			

Figure legends

Figure 1: Kaplan-Meier analysis for cholesterol efflux and NODAT. The corresponding *P* value was obtained from log-rank tests.

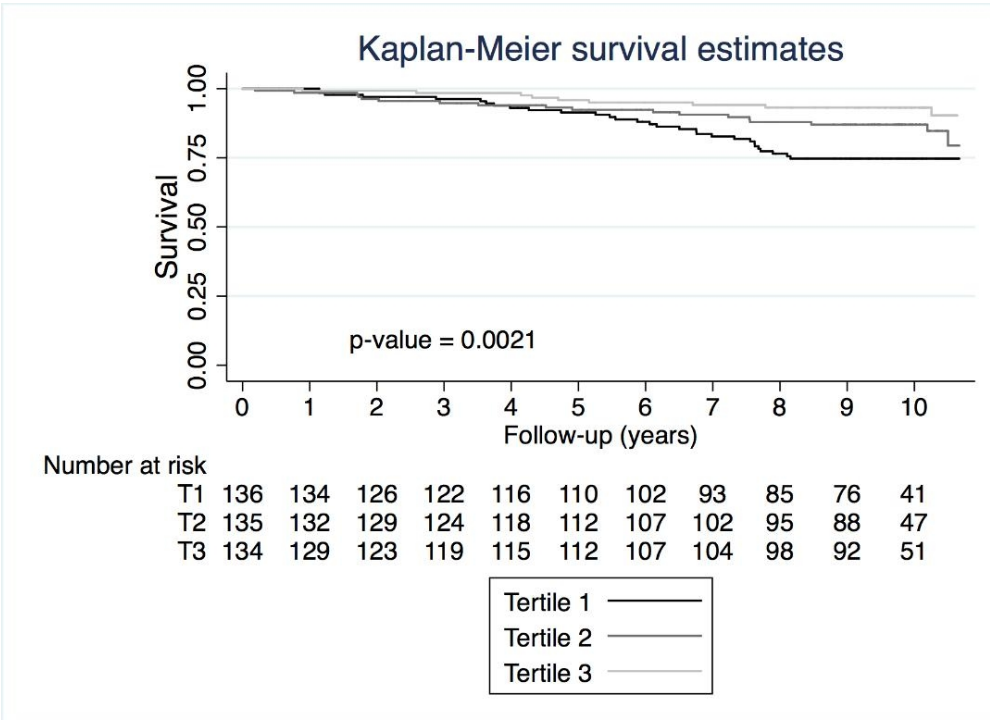

Figure 1

381x276mm (72 x 72 DPI)

Online appendix to

High Density Lipoprotein Cholesterol Efflux Predicts Incident New Onset Diabetes After Transplantation (NODAT) in Renal Transplant Recipients Independent of High Density Lipoprotein Cholesterol Levels

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Supplemental table I: Cox regression analysis to determine hazard ratios for NODAT incidence by cholesterol efflux capacity

		HR [95% CI]	B	P value
Model 1	Gender stratified cholesterol efflux	0.54 [0.38-0.76]	-0.62	0.01
Model 2	Gender stratified cholesterol efflux	0.53 [0.38-0.76]	-0.63	<0.001
	Age	1.01 [0.98-1.03]	0.01	0.579
	Sex	1.16 [0.69-1.98]	0.15	0.576
Model 3	Gender stratified cholesterol efflux	0.66 [0.46-0.95]	-0.42	0.025
	BMI	1.10 [0.97-1.24]	0.10	0.127
	Waist circumference	1.01 [0.97-1.05]	0.01	0.667
	Concentration CRP	1.03 [0.97-1.10]	0.03	0.386
Model 4	Gender stratified cholesterol efflux	0.54 [0.38-0.77]	-0.61	0.01
	Time since renal transplantation	0.97 [0.93-1.02]	-0.03	0.257
Model 5	Gender stratified cholesterol efflux	0.53 [0.37-0.75]	-0.64	<0.001
	eGFR	1.00 [0.98-1.02]	0.00	0.967
	Albumin to creatinine ratio	1.02 [0.73-1.42]	0.00	0.996
Model 6	Gender stratified cholesterol efflux	0.56 [0.39-0.81]	-0.54	0.02
	Concentration insulin	0.76 [0.65-0.88]	-0.28	<0.001
	HOMA-IR	4.39 [2.41-8.00]	1.48	<0.001
Model 7	Gender stratified cholesterol efflux	0.52 [0.31-0.88]	-0.66	0.015
	Total cholesterol	0.92 [0.63-1.36]	-0.08	0.678
	HDL cholesterol	1.41 [0.32-6.14]	0.34	0.650

	apoA-1	1.39 [0.22-8.91]	0.33	0.730
	apoB	0.79 [0.13-4.96]	-0.23	0.802
	Triglycerides	1.48 [1.29-1.70]	0.39	<0.001
Model 8	Gender stratified cholesterol efflux	0.59 [0.41-0.85]	-0.53	0.004
	Proliferation inhibitors	0.73 [0.38-1.39]	0.32	0.338
	Cakcineurin inhibitors	9.24 [1.09-78.36]	2.22	0.42
	Tacrolimus	0.29 [0.04-2.41]	-1.22	0.254
	Cyclosporine	0.11 [0.01-0.88]	-2.21	0.038
	Concentration prednisolone	1.02 [0.82-1.26]	0.02	0.888
	Antihypertensives	1.27 [1.02-1.59]	0.24	0.032
Model 9	Gender stratified cholesterol efflux	0.54 [0.38-0.76]	-0.62	<0.001
	Statin use	2.15 [1.23-3.77]	0.77	0.007
Model 10	Gender stratified cholesterol efflux	0.53 [0.37-0.75]	-0.64	<0.001
	Proliferation inhibitors	0.77 [0.43-1.41]	-0.26	0.400
Model 11	Gender stratified cholesterol efflux	0.53 [0.38-0.76]	-0.63	<0.001
	Calcineurin inhibitors	1.48 [0.72-3.03]	0.39	0.290
Model 12	Gender stratified cholesterol efflux	0.55 [0.39-0.78]	-0.60	0.001
	Tacrolimus	2.37 [1.27-4.42]	0.86	0.006
Model 13	Gender stratified cholesterol efflux	0.54 [0.38-0.76]	-0.62	<0.001
	Cyclosporine	0.72 [0.43-1.22]	-0.33	0.222
Model 14	Gender stratified cholesterol efflux	0.54 [0.38-0.77]	-0.62	0.001
	Dose prednisolone	1.07 [0.86-1.32]	0.07	0.546

Model 15	Gender stratified cholesterol efflux	0.53 [0.37-0.76]	-0.63	0.001
	Proliferation inhibitors	0.69 [0.36-1.31]	-0.38	0.257
	Calcineurin inhibitors	6.32 [0.77-51.84]	1.84	0.086
	Tacrolimus	0.43 [0.05-3.33]	-0.86	0.413
	Cyclosporine	0.17 [0.02-1.30]	-1.77	0.88
	Dose prednisolone	1.04 [0.84-1.28]	0.04	0.724
Model 16	Gender stratified cholesterol efflux	0.52 [0.36-0.76]	-0.65	0.001
	Glucose concentration	4.02 [2.72-5.95]	1.39	<0.001
Model 17	Gender stratified cholesterol efflux	0.59 [0.42-0.85]	-0.52	0.004
	Triglyceride concentration	1.44 [1.28-1.62]	0.36	<0.001
Model 18	Gender stratified cholesterol efflux	0.64 [0.45-0.92]	-0.44	0.016
	BMI	1.13 [1.07-1.19]	0.12	<0.001
Model 19	Gender stratified cholesterol efflux	0.63 [0.43-0.92]	-0.47	0.018
	Glucose concentration	3.49 [2.32-5.26]	1.25	<0.001
	Triglycerides concentration	1.20 [1.04-1.38]	0.18	0.11
	BMI	1.07 [1.02-1.13]	0.07	0.013

Model 1 crude analysis; model 2: adjustment for age and gender; model 3: model 2 + adjustment for BMI, waist circumference and concentration of CRP; model 4: model 2 + adjustment for time since renal transplantation; model 5: model 2 + adjustment for eGFR and urinary albumin to creatinine ratio; model 6: model 2 + adjustment for serum concentration insulin and HOMA-IR; model 7: model 2 + adjustment for total cholesterol, HDL cholesterol, apoA, apoB and triglycerides; model 8: model 2 + adjustment for use of proliferation inhibitors, calcineurin inhibitors, tacrolimus, cyclosporine, use of antihypertensive medication and daily prednisolone dose; model 9: model 2 + adjustment for use of statins.

Model 10: model 2 + adjustment for use of proliferation inhibitors; model 11: model 2 + adjustment for the use of calcineurin inhibitor; model 12: model 2 + adjustment for the use of tacrolimus; model 13: model 2 + adjustment for the use of cyclosporine; model 14: model 2 + adjustment for daily dose prednisolone; model 15: model 2 + adjustment for the use of proliferation inhibitors, calcineurin inhibitors, tacrolimus, cyclosporine and daily dose prednisolone; model 16: model 2 + adjusting for glucose concentration; model 17: model 2 + adjusting for triglyceride concentration; model 18: model 2 + adjusting for BMI; model 19: model + adjusting for glucose concentration, triglycerides concentration and BMI.