Sphingomyelin is associated with kidney disease in type 1 diabetes (The FinnDiane Study)

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Abstract Diabetic kidney disease, diagnosed by urinary albumin excretion rate (AER), is a critical symptom of chronic vascular injury in diabetes, and is associated with dyslipidemia and increased mortality. We investigated serum lipids in 326 subjects with type 1 diabetes: 56% of patients had normal AER, 17% had microalbuminuria (20 < AER < 200 µg/min or 30 < AER < 300 mg/24 h) and 26% had overt kidney disease (macroalbuminuria AER ≥ 200 µg/min or AER ≥ 300 mg/24 h). Lipoprotein subclass lipids and low-molecular-weight metabolites were quantified from native serum, and individual lipid species from the lipid extract of the native sample, using a proton NMR metabonomics platform. Sphingomyelin (odds ratio 2.53, \( P < 10^{-7} \)), large VLDL cholesterol (odds ratio 2.36, \( P < 10^{-10} \)), total triglycerides (odds ratio 1.88, \( P < 10^{-6} \)), omega-9 and saturated fatty acids (odds ratio 1.82, \( P < 10^{-5} \)), glucose disposal rate (odds ratio 0.44, \( P < 10^{-9} \)), large HDL cholesterol (odds ratio 0.39, \( P < 10^{-9} \)) and glomerular filtration rate (odds ratio 0.19, \( P < 10^{-10} \)) were associated with kidney disease. No associations were found for polyunsaturated fatty acids or phospholipids. Sphingomyelin was a significant regressor of urinary albumin (\( P < 0.0001 \)) in multivariate analysis with kidney function, glycemic control, body mass, blood pressure, triglycerides and HDL cholesterol. Kidney injury, sphingolipids and excess fatty acids have been linked in animal models—our exploratory approach provides independent support for this relationship in human patients with diabetes.

Keywords Diabetic nephropathy · NMR metabonomics · Fatty acids · Sphingolipids · Phospholipids · Lipoprotein subclasses

1 Introduction

Type 1 diabetes follows the autoimmune destruction of pancreatic beta-cells at a young age. Acute symptoms can be controlled by insulin injections, but the diabetes-related
disease burden increases with time due to chronic complications (Lithovius et al. 2011). An estimated third of patients with long-standing type 1 diabetes are affected by kidney disease, which is characterized by increased urinary albumin excretion, high blood pressure and a gradual decline in glomerular filtration (Gross et al. 2005; Mäkinen et al. 2008a). For individual prognosis, kidney disease is the crucial feature: it is associated with a ten-fold increase in mortality, often from cardiovascular disease or stroke before end-stage renal disease is reached (Forsblom et al. 2011; Groop et al. 2009; Morrish et al. 2001; Stadler et al. 2006).

Obesity, selective insulin resistance and lipotoxicity are recognized as important aspects of end-organ damage in diabetes (Groop et al. 2005). Adipose tissue in a state of over-nutrition can trigger the release of pro-inflammatory agents (Kennedy et al. 2009; Surmi and Hasty 2010), which could explain some of the observed chronic inflammation in atherosclerosis and diabetic kidney disease (Best et al. 2005; Cao et al. 2007; Saraheimo et al. 2003). Furthermore, a large proportion of type 1 diabetic patients satisfy the metabolic syndrome definition and particularly the dyslipidemia component of the definition increases the likelihood of complications and premature death (Mäkinen et al. 2008a; Thorn et al. 2005; Tolonen et al. 2009). Accordingly, the triglyceride-rich VLDL and cholesterol-carrying HDL subclasses are important covariates of diabetic complications (Jenkins et al. 2003; Soedamah-Muthu et al. 2003).

Various and partly conflicting differences in serum lipid species have been detected in high-risk children and type 1 diabetic patients (Fievet et al. 1990; Jain et al. 2000; Oresic et al. 2008; Seigneur et al. 1994). Serum phospholipids have been studied as structural components of lipoproteins, but their relevance for type 1 diabetes complications is not fully known (Bagdade and Subbaiah 1989; Watala and Józwiak 1990); sphingolipids have been associated with numerous diseases (Boini et al. 2010; Fox et al. 2011; Hicks et al. 2009; Pifieri et al. 2004; Summers 2010), but their significance for human diabetic kidney disease needs clarification. Dietary polyunsaturated fatty acids are considered beneficial (Lee et al. 2010; Mori et al. 1989) whereas saturated fatty acids are likely to be harmful also in type 1 diabetes. However, information on serum lipid concentrations in type 1 diabetic subjects remains fragmented.

Metabonomics has the potential to recognize previously unconsidered associations between metabolic products and clinical end-points (Ala-Korpela 2007; Mäkinen et al. 2006). In this study, we investigate a number of lipids and other biochemical measures in a set of human subjects with long-standing type 1 diabetes with and without complications. Our aim is to find those biochemical quantities that are associated with the (i) diagnostic classification and (ii) continuous markers of kidney injury. The findings provide clues on the lipid mediators of vascular damage and biomarker candidates for further mechanistic and epidemiological studies.

2 Methods

We studied 326 type 1 diabetic patients (218 men and 108 women) as part of the Finnish Diabetic Nephropathy Study (Mäkinen et al. 2008a). The data collection was cross-sectional (serum and urine samples), but with longitudinal records of albuminuria and clinical history. Type 1 diabetes mellitus was defined as an age of onset below 35 years and initiation of insulin treatment within a year of onset.

The clinical characteristics are listed in Table 1. The normal range of urinary albumin excretion rate (AER)
was defined as AER < 20 µg/min for night urine or AER < 30 mg/24 h for a timed circadian collection. Overt kidney disease was diagnosed if AER ≥ 200 µg/min or AER ≥ 300 mg/24 h (macroalbuminuria). The intermediary range (microalbuminuria, 20 ≤ AER < 200 µg/min or 30 ≤ AER < 300 mg/24 h) represents a clinically challenging borderline with no clear consensus on pathology. In this study, microalbuminuria was regarded as not having kidney disease, since we could not see reduced kidney function. AER exhibits a large daily variance and the kidney diagnosis was based on at least two out of three consecutive albumin tests. We also measured urinary albumin centrally from a single 24 h collection to obtain a continuous renal status (denoted by 24 h-AER). Laser treatment of the retina was used as an indicator of proliferative retinopathy.

The metabolic syndrome was defined according to the NCEP ATP III criteria and glomerular filtration rate was estimated by the Cockcroft-Gault formula. Efficient glucose disposal rate was estimated according to the Williams-Orchard formula 24.4–12.97 × waist-hip ratio − 3.39 × hypertension − 0.60 × hemoglobin A1c, where hypertension was defined as systolic/diastolic > 140/90 mmHg or anti-hypertensive medication.

The proton NMR experiments were performed on a platform with three molecular windows (Mäkinen et al. 2008b; Tukiainen et al. 2008). Two windows were applied to native serum: the LIPO window yields information on lipoprotein subclasses and the LMWM window on a number of low-molecular-weight metabolites. The third window, denoted by LIPID, was applied to serum lipid extract to measure the serum lipid constituents and the diversity of fatty acid saturation. The current methodological and metabolite details for the serum NMR metabolomics, including spectral characteristics and metabolite assignments for all the three molecular windows, have been published previously (Inouye et al. 2010). Information on other biochemical and clinical variables can be found in (Mäkinen et al. 2008a).

Odds ratios for kidney disease were calculated by univariate logistic regression for each continuous variable (clinical, biochemical and NMR), respectively. Before analysis, the variables were rank transformed to produce comparable results. Adjustments for age, diabetes duration and gender were made by linear regression. Statistical significance and 95% confidence intervals were estimated by bootstrap simulation. The reported P-values are not corrected for multiple testing, but the Bonferroni significance limit is listed in the figure and table captions. The associations between continuous variables and 24 h-AER were estimated by the Spearman correlation coefficient.

Multivariate linear regression was applied to two sets of variables that were chosen based on prior biological knowledge. The first set includes multivariate formulas for the metabolic syndrome, glomerular filtration and glucose disposal rate. The second set contains directly measured quantities such as blood pressure, hemoglobin A1c and serum creatinine that reflect established vascular risk factors. Set 1 represents a clinically motivated dimension reduction, whereas Set 2 works as an a priori variable selection. We also tested ridge regression and projection to latent structures (PLS), however, neither method could produce robust estimates for the regression coefficients due to the collinearity between the variables (data not shown).

3 Results and discussion

Figure 1 shows the logistic odds ratios (OR) for kidney disease—adjusted by diabetes duration, age and gender—for a subset of variables. The full list is available in Supplement 1. Serum creatinine, cystatin-C and urea are cleared by the kidneys, so a reduction in the filtration capacity has a direct effect on their concentrations (OR ≥ 3.25, P ≤ 3.4 × 10⁻⁶), and on the creatinine-based estimate of glomerular filtration rate (OR = 0.19, P = 8.2 × 10⁻¹¹).

Sphingomyelin (OR = 2.53, P = 1.5 × 10⁻⁸) and large HDL particles (OR ≤ 0.40, P ≤ 3.1 × 10⁻¹⁰) are the strongest regressors after the established kidney biomarkers. Furthermore, medium HDL particles (OR ≤ 0.60, P ≤ 6.6 × 10⁻⁵) and lipids in small HDL (OR = 0.62, P = 0.00031) are inversely associated with kidney disease. Extra large and large VLDL particles have the highest ORs after sphingomyelin (OR ≥ 1.95, P ≤ 1.4 × 10⁻⁷). Monounsaturated 16:1 and 18:1 and omega-9 and saturated fatty acids have a stronger association with kidney disease (OR ≥ 1.82, P ≤ 3.5 × 10⁻⁶) than omega-6 and 7 (OR = 1.59, P = 0.00029), 18:2 (no association), omega-3 (no association), 22:6 (no association) or other polyunsaturated fatty acids (OR = 0.76, P = 0.020).

Odds ratios for retinopathy were also estimated by logistic regression: only 24 h-AER (OR = 1.75, P = 9.3 × 10⁻⁶), serum creatinine (OR = 1.52, P = 0.00028), efficient glucose disposal rate (OR = 0.55, P = 2.6 × 10⁻⁶) and glomerular filtration (OR = 0.62, P = 5.4 × 10⁻⁵) were significant regressors after adjusting for diabetes duration, age and gender.

To complement the categorical analysis, we also calculated correlations between the centrally measured continuous 24 h-AER, and the other clinical and biochemical variables. Table 2 highlights the important correlations and the full list is available in Supplement 2. Serum creatinine is strongly correlated with 24 h-AER (r = 0.73, P < 10⁻¹⁷), as expected. Sphingomyelin is again the top lipid variable (r = 0.42, P = 1.5 × 10⁻¹⁴), followed by systolic blood pressure, total triglycerides and large VLDL.
cholesterol. Glomerular filtration rate, cholesterol in large HDL and efficient glucose disposal rate are inversely associated with 24 h-AER.

We chose two sets of variables that represent established risk factors for diabetic kidney disease to explain the covariance between sphingomyelin and 24 h-AER (Table 3). Set 1 includes derived clinical traits (such as the metabolic syndrome), whereas Set 2 includes measures that represent diagnostic components: waist circumference for obesity, systolic blood pressure for hypertension, hemoglobin A1c for glycemic control, triglycerides and HDL cholesterol for dyslipidemia and serum creatinine for kidney function. Sphingomyelin is a statistically significant trait in both models ($P < 0.00038$), which suggests that it may reflect previously undetected biological phenomena that are specific to albuminuria.

Models of the binary kidney disease diagnosis and retinopathy are available in Supplement 3. Kidney disease is
Sphingomyelin is associated with kidney disease

Table 2. A representative set of variables that are significantly correlated with 24 h-AER. Correlation was measured by the Spearman coefficient and the Bonferroni multiple testing limit is at $P < 0.00038$

|                          | Adjusted by diabetes duration, age and gender | Unadjusted |
|--------------------------|---------------------------------------------|------------|
|                          | Correlation | $P$-value | Correlation | $P$-value |
| Serum creatinine         | 0.73        | $<10^{-17}$ | 0.52        | $<10^{-17}$ |
| Sphingomyelin            | 0.42        | $1.5 \times 10^{-14}$ | 0.43        | $6.7 \times 10^{-16}$ |
| Systolic blood pressure  | 0.31        | $4.7 \times 10^{-8}$  | 0.32        | $1.5 \times 10^{-8}$  |
| Triglycerides            | 0.30        | $3.7 \times 10^{-7}$  | 0.38        | $1.6 \times 10^{-10}$ |
| Large VLDL cholesterol   | 0.30        | $1.2 \times 10^{-7}$  | 0.29        | $1.4 \times 10^{-7}$  |
| Free cholesterol         | 0.29        | $3.0 \times 10^{-7}$  | 0.32        | $9.2 \times 10^{-9}$  |
| Omega-9 and saturated fatty acids | 0.26 | $3.3 \times 10^{-6}$  | 0.33        | $1.8 \times 10^{-9}$  |
| Omega-6 and 7 fatty acids | 0.26        | $5.3 \times 10^{-6}$  | 0.26        | $4.3 \times 10^{-6}$  |
| IDL triglycerides        | 0.22        | $0.00011$ | 0.27        | $1.8 \times 10^{-6}$  |
| Serum adiponectin        | 0.15        | 0.017  | 0.26        | $2.4 \times 10^{-5}$  |
| Phosphoglycerides        | 0.12        | 0.036  | 0.16        | 0.0041 |
| Diabetes duration        | 0.03        | 0.64    | 0.38        | $4.2 \times 10^{-12}$ |
| Phosphatidylcholine      | -0.02       | 0.71    | 0.00        | 0.96    |
| Omega-3 fatty acids      | -0.05       | 0.39    | -0.03       | 0.60    |
| Fatty acid length        | -0.20       | 0.00040 | -0.25       | $6.6 \times 10^{-6}$ |
| Medium HDL cholesterol   | -0.25       | $1.2 \times 10^{-5}$ | -0.28       | $5.7 \times 10^{-7}$ |
| HDL cholesterol          | -0.31       | $2.1 \times 10^{-7}$ | -0.27       | $4.9 \times 10^{-6}$ |
| Large HDL cholesterol    | -0.33       | $3.6 \times 10^{-9}$ | -0.32       | $7.7 \times 10^{-9}$ |
| Efficient glucose disposal rate | -0.33 | $8.2 \times 10^{-9}$ | -0.51       | $<10^{-17}$ |
| Glomerular filtration rate | -0.45    | $3.3 \times 10^{-16}$ | -0.43       | $1.2 \times 10^{-14}$ |

associated with glomerular filtration and sphingomyelin in Set 1, and with serum creatinine, HDL cholesterol, sphingomyelin and diabetes duration in Set 2. Retinopathy is strongly related to diabetes duration, but not to sphingomyelin.

Sphingomyelin is correlated with glomerular filtration rate ($r = -0.32$, $P < 10^{-6}$), but this study cannot ascertain the causal relationships between albuminuria, declining kidney function and lipotoxicity. The selection of study subjects is another limitation: although matched for gender, the patients without kidney disease had a shorter diabetes duration. It is therefore possible that some of the controls were in an early phase of kidney disease, which could have diluted the results.

A recent animal study demonstrated how the manipulation of the sphingolipid pathway could alleviate kidney disease: untreated mice on a high-fat diet developed insulin resistance and albuminuria, but a pharmacological intervention reduced the effects (Boini et al. 2010). By inhibiting the conversion of sphingomyelin into ceramide, the authors were able to significantly decrease glomerular injury and restore urinary albumin excretion rate to normal range.

The proportional increase of sphingomyelin in the kidney disease group was modest (+17% when adjusted by aging and gender) and much of the variance was shared with other clinical characteristics, which suggests that excess circulating sphingomyelin might be a by-product of the disease process rather than an active initiator. In fact, excess saturated fatty acids may be the underlying cause: they are substrates for sphingolipid synthesis, they promote lipotoxicity (production of ceramides) and are also linked to insulin resistance (Bunn et al. 2010; Kennedy et al. 2009; Yang et al. 2009). The NMR method cannot distinguish between omega-9 and saturated fatty acid chains, but the combination was nevertheless significantly correlated with clinical kidney disease (Fig. 1) and 24 h-AER (Table 2), whereas polyunsaturated fatty acids such as 18:2 or 22:6 showed no association.

4 Concluding remarks

Sphingomyelin emerged as a significant biochemical covariate of urinary albumin excretion in human type 1 diabetes, and the strongest lipid regressor for kidney disease. We also reproduced the associations between macroalbuminuria and high triglycerides, excess serum fatty acids and altered VLDL-HDL balance. Phospholipids or polyunsaturated fatty acids were not covariates of albuminuria. Our results are supported by animal data, which suggests that sphingolipids may reflect some of the...
molecular links between microvascular injury, insulin resistance, and saturated fatty acids. Further research of the sphingolipid pathway may thus yield more sensitive phenotyping measures and new pharmacological options to ease the burden of diabetic complications.

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Table 3 Regression coefficients for biologically motivated models of 24 h-AER

| Model 1 of 24 h-AER | Beta | P-value |
|---------------------|------|---------|
| Sphingomyelin       | 0.22 | 1.8 × 10^{-5} |
| T1DM duration       | 0.011 | 0.42 |
| Male gender         | 0.00012 | 0.50 |
| Waist               | 0.057 | 0.173 |
| Systolic BP         | 0.084 | 0.047 |
| Hemoglobin A1c      | 0.15 | 0.0011 |
| Triglycerides       | 0.031 | 0.33 |
| HDL cholesterol     | -0.13 | 0.0046 |
| Serum creatinine    | 0.30 | 7.8 × 10^{-6} |

All data were rank transformed and normalized prior to analyses and the regression coefficients are in standardized units. Model 1 explained 44.1%, and Model 2 explained 41.9% of 24 h-AER variance. Without sphingomyelin (i.e., sphingomyelin values were shuffled randomly), the two models explained 40.5 and 38.8% of the 24 h-AER variance, respectively. Abbreviations: type 1 diabetes (T1DM), efficient glucose disposal rate (eGDR), efficient glucose disposal rate (eGDR).

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