Endotoxins are associated with visceral fat mass in type 1 diabetes

Mariann I. Lassenius1,2,3,*, Aila J. Ahola1,2,3,*, Valma Harjutsalo1,2,3,4, Carol Forsblom1,2,3, Per-Henrik Groop1,2,3,5 & Markku Lehto1,2,3

Bacterial lipopolysaccharides (LPS), potent inducers of inflammation, have been associated with chronic metabolic disturbances. Obesity is linked to dyslipidemia, increased body adiposity, and endotoxemia. We investigated the cross-sectional relationships between serum LPS activity and body adiposity as well as inflammation in 242 subjects with type 1 diabetes. Body fat distribution was measured by DXA and serum LPS activity by the limulus amebocyte lysate end-point assay. Since no interaction between visceral fat mass and sex was observed, data were pooled for the subsequent analyses. LPS was independently associated with visceral fat mass, when adjusted for traditional risk factors (age, sex, kidney status, hsCRP, insulin sensitivity). In the multivariate analysis, serum LPS activity and triglyceride concentrations had a joint effect on visceral fat mass, independent of these factors alone. A combination of high LPS and high hsCRP concentrations was also observed in those with the largest visceral fat mass. In conclusion, high serum LPS activity levels were associated with visceral fat mass in subjects with type 1 diabetes strengthening its role in the development of central obesity, inflammation and insulin resistance.

Bacterial endotoxins or lipopolysaccharides (LPS) are potent inducers of systemic inflammation. Endotoxin transport and clearance has been linked to lipoprotein metabolism. Endotoxins are redistributed towards VLDL and LDL particles especially in conditions of lower HDL cholesterol concentrations, like in atherosclerosis and insulin resistance3–5. Toll-like receptor 4 (TLR4) is a pattern recognition receptor that binds LPS and initiates an inflammatory response. Compared to lean individuals, TLR4 expression is up-regulated in the adipose tissue of obese subjects4. A low dose LPS infusion augments adiposity, inflammation, insulin resistance, hyperglycemia and dyslipidemia in mice4. In humans, a low dose LPS infusion induces insulin resistance and potentiates the expression of inflammatory markers in adipose tissue4.

Studies in non-diabetic children and obese adults, have shown an adverse association between endotoxin levels, central adiposity and insulin resistance6,7. We have previously shown that a high serum LPS activity is associated with features of the metabolic syndrome and the progression of kidney disease in type 1 diabetes6,9. Recent studies have further demonstrated a putative link between endotoxins and visceral fat10. Excess visceral fat is associated with impaired insulin sensitivity, and the development of vascular complications in diabetes11. It has also been suggested that the distribution of body fat could better predict insulin resistance than BMI itself12,13. Evidently, certain human genetic variants may also have a significant impact on BMI and body adiposity13.

Our aim was to investigate the association between serum LPS activity and visceral fat mass, measured by dual-energy x-ray absorptiometry (DXA), in subjects with type 1 diabetes.

Subjects and Methods

Data from individuals with type 1 diabetes were collected during the Finnish Diabetic Nephropathy (FinnDiane) Study visits taking place in Helsinki between years 2011 and 2016. In the current analyses, we included data from all individuals with completed LPS and body composition measurements, and either normal urinary albumin excretion rate (T1D-normo, n = 156), microalbuminuria (T1D-micro, n = 42) or macroalbuminuria (T1D-macro, n = 46). Renal status was assessed by the albumin excretion rate (AER) in two out of three consecutive timed urine collections using the following criteria: normal AER <20 μg/min or <30 μg/24 h, and macroalbuminuria ≥200 μg/min or ≥300 μg/24 h. Kidney transplant recipients and those undergoing dialysis were
### Table 1. Clinical characteristics of study participants divided by renal status.

Data are presented as frequency (percentage) for categorical variables, mean ± SD for normally distributed continuous variables, and median (25th–75th quartile) for non-normally distributed continuous variables. Significance across the three groups has been studied with Chi squared, ANOVA and Kruskal-Wallis test, respectively. AER, albumin excretion rate; LPS, lipopolysaccharides; eGDR, estimated glucose disposal rate; hsCRP, high-sensitive C-reactive protein.

|                      | Normal AER n = 154 | Microalbuminuria n = 42 | Macroalbuminuria n = 46 | p      |
|----------------------|--------------------|-------------------------|-------------------------|--------|
| Men, n (%)           | 66 (43)            | 19 (45)                 | 31 (67)                 | 0.013  |
| Age (years)          | 48.3 ± 12.7        | 49.0 ± 11.4             | 52.7 ± 8.2              | 0.085  |
| Diabetes duration (years) | 31.1 ± 28.7   | 35.9 ± 9.6              | 38.4 ± 9.7              | <0.001 |
| HbA1c, mmol/mol (%)  | 65 ± 12 (8.1 ± 1.1)| 68 ± 13 (8.4 ± 1.1)     | 66 ± 13 (8.2 ± 1.3)     | 0.241  |
| Cholesterol (mmol/l) | 4.5 (3.9–5.0)      | 4.7 (4.2–5.2)           | 4.2 (3.7–4.8)           | 0.046  |
| HDL cholesterol (mmol/l) | 1.58 (1.36–1.94) | 1.54 (1.20–2.01)        | 1.41 (1.12–1.85)        | 0.157  |
| Triglycerides (mmol/l) | 0.88 (0.67–1.16)  | 1.05 (0.70–1.76)        | 1.06 (0.84–1.77)        | <0.001 |
| LPS (EU/ml)          | 0.24 (0.20–0.28)   | 0.24 (0.20–0.31)        | 0.24 (0.20–0.31)        | 0.942  |
| Insulin dose (IU/kg) | 0.54 (0.43–0.71)   | 0.65 (0.49–0.76)        | 0.54 (0.47–0.74)        | 0.193  |
| Insulin pump, n (%)  | 32 (21)            | 10 (24)                 | 15 (33)                 | 0.252  |
| BMI (kg/m²)          | 24.9 (22.8–27.6)   | 26.5 (23.0–30.3)        | 25.5 (23.1–28.7)        | 0.087  |
| eGDR (mg/kg/min)     | 5.4 (4.0–8.3)      | 3.8 (3.0–5.1)           | 3.9 (2.8–4.7)           | <0.001 |
| hsCRP (mg/l)         | 1.11 (0.51–2.47)   | 1.63 (0.79–3.06)        | 1.01 (0.49–3.52)        | 0.391  |
| Visceral fat mass all (g) | 452 (194–1022)  | 737 (381–2020)          | 1096 (471–1881)         | <0.001 |
| Visceral fat mass, men (g) | 724 (372–1511) | 1298 (532–3039)         | 1100 (728–1932)         | 0.044  |
| Visceral fat mass, women (g) | 294 (140–737)  | 523 (300–1816)          | 644 (236–1556)          | 0.017  |
| Android fat mass, all (g) | 1866 (1131–2924) | 2292 (1456–4018)        | 2243 (1394–3321)        | 0.012  |
| Android fat mass, men (g) | 1913 (1189–2967) | 3047 (1470–4264)        | 2302 (1499–3028)        | 0.129  |
| Android fat mass, women (g) | 1569 (1096–2412) | 1958 (1417–3809)        | 1960 (759–3838)         | 0.149  |
| Gynoid fat mass, all (g) | 3642 (2945–4735) | 4089 (3333–5303)        | 3689 (2722–4590)        | 0.267  |
| Gynoid fat mass, men (g) | 3343 (2573–3907) | 3715 (2466–4889)        | 3557 (2735–4170)        | 0.407  |
| Gynoid fat mass, women (g) | 4300 (3210–5150) | 4860 (3517–5857)        | 4368 (2222–5496)        | 0.536  |
| Android/Gynoid ratio, all (g) | 0.45 (0.33–0.61) | 0.58 (0.40–0.75)        | 0.63 (0.49–0.78)        | <0.001 |
| Android/Gynoid ratio, men (g) | 0.57 (0.44–0.80) | 0.75 (0.50–0.95)        | 0.69 (0.53–0.84)        | 0.077  |
| Android/Gynoid ratio, women (g) | 0.38 (0.30–0.51) | 0.50 (0.35–0.66)        | 0.49 (0.33–0.71)        | 0.028  |

Statistical analyses. Normality of variable distribution was assessed with the Kolmogorov-Smirnov test. Normally distributed variables are reported as mean ± standard deviation, non-normally distributed variables as median (25th–75th quartile). Correlation coefficients were calculated by Spearman or Pearson’s correlation test as appropriate. Differences in frequencies were assessed by Pearson’s Chi squared analysis. Differences in variation between groups were calculated using Mann-Whitney U-test, Kruskal-Wallis test and ANOVA, as appropriate. The population was divided into tertiles based on serum LPS activity, triglyceride-, or hsCRP concentrations to further explore the role of endotoxins, dyslipidemia, and inflammation on visceral fat mass.

The interaction between LPS and gender on visceral fat mass was assessed using a generalized linear model. Since no interaction was evident, all data were analyzed together. The interaction between LPS and triglyceride concentration on visceral fat mass was analyzed similarly. As LPS and triglyceride concentration showed significant interaction, the interaction term was included in the multivariable model. Because LPS and triglycerides are highly correlated, we furthermore visualized their relative relationship using the generalized additive modeling (GAM) without a priori assumptions of the shape of the relation. The GAM modelling allows the inclusion of non-parametric smoothing functions to identify a potential non-linearity in the relationship between the independent and the dependent variables. The generalized cross-validation function (GCV) was used as a criterion
for selection of the smoothing parameters to determine an appropriate level of smoothing. Analyses were carried out using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA), and R open source software (http://www.r-project.org). GAM models were fitted using the mgcv library in R16.

Ethics. The study protocol was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa. The study was carried out in accordance with the approved guidelines. Participants gave their written informed consent prior to participating in the study.

Results
Patient characteristics divided by renal status are shown in Table 1. The proportion of men was highest among T1D-macro. The diabetes duration and visceral fat mass increased with worsening renal status. Insulin sensitivity (eGDR) was highest in T1D-normo. No differences were observed in HbA1c, HDL cholesterol concentration, LPS activity, insulin dose, use of insulin pump, BMI or hsCRP concentration amongst the three groups.

Serum LPS activity correlated positively with BMI (r = 0.274, p < 0.001), visceral fat mass (r = 0.248, p < 0.001), android to gynoid fat ratio (r = 0.231, p < 0.001), serum triglyceride concentration (r = 0.477, p < 0.001), and hsCRP concentration (r = 0.276, p < 0.001). Negative correlation was observed between insulin dose and eGDR (r = −0.205, p = 0.002). Supplementary Figure S1a and S1b show that triglyceride concentration seems to overrun the effect of LPS on the visceral fat mass. However, the relationship between LPS and triglycerides is complicated and interaction between LPS and triglyceride concentration was found, indicating that LPS has an effect on certain levels of triglyceride concentration. Therefore, the effect of serum LPS activity and triglyceride concentration on visceral fat mass is complicated and needs further investigation.

Figure 1. (A) Visceral fat mass (g) according to LPS and triglyceride tertiles (low/medium/high) in subjects with type 1 diabetes. Higher visceral fat mass was observed in those with a combination of high LPS activity/high triglyceride concentration (N = 50) compared to those with low LPS activity/low triglyceride concentration (N = 43, p < 0.001). (B) Visceral fat mass (g) according to LPS and hsCRP tertiles (low/medium/high) in subjects with type 1 diabetes. Higher visceral fat mass was observed in those with a combination of high LPS activity/high hsCRP concentration (N = 32) compared to low LPS activity/low hsCRP concentration (N = 38, p < 0.001).
LPS activity remained a significant predictor of visceral fat mass (Model 2: $B = 1253.2$, Wald Chi-squared $= 8.029$, $p = 0.005$). In this model, LPS activity was positively associated with the visceral fat mass ($B = 64.7$, Wald Chi-squared $= 23.299$, $p < 0.001$).

Subjects in the high triglyceride tertile presented the highest visceral fat mass. Notably, within each LPS tertile, the visceral fat mass was observed to increase towards the higher TG tertiles ($p < 0.001$). The Supplementary Figure S2 provides a deeper view on the interaction effect of LPS and triglyceride levels on the visceral fat mass focusing on the 75% of the population in the area with the densest accumulation of observations. Similarly, subjects in the high-LPS/high-hsCRP tertile presented greater visceral and triglyceride levels on the visceral fat mass focusing on the 75% of the population in the area with the densest accumulation of observations. Subjects in the high triglyceride tertile presented the highest visceral fat mass. Notably, within each LPS tertile, the visceral fat mass was observed to increase towards the higher TG tertiles ($p < 0.001$) (Fig. 1A). Moreover, the visceral fat mass among those with high LPS activity and high triglyceride level was higher compared to the group with both low LPS activity and low triglyceride level (high/high vs. low/low, median [IQR]: 1452 [767–2001] vs. 110 [53–198], Wald Chi-Squared $= 7.350$, $p = 0.007$), independently of the two factors entered in the model separately.

The independent association between LPS activity and visceral fat mass was analyzed in a number of multi-variable models (Table 2). With Model 1, we aimed at looking at this association while taking inflammation into account. In this model, LPS activity was positively associated with the visceral fat mass ($B = 1253.2$, Wald Confidence Interval 386.4–2120.0, Wald Chi-Squared $= 8.029$, $p = 0.005$). Further adjusting for insulin sensitivity (eGDR), LPS remained a significant predictor of visceral fat mass (Model 2: $B = 1253.2$, Wald Confidence Interval 386.4–2120.0, Wald Chi-Squared $= 8.029$, $p = 0.005$). However, after further adjusting for triglyceride concentration (Model 3), LPS no longer predicted visceral fat mass. To the final model (Model 4) we incorporated a LPS*triglyceride interaction term, which was significantly associated with the visceral fat mass ($B = -634.2$, 95% Wald CI $-1092.6$–$-175.7$, Wald Chi-Squared $= 7.350$, $p = 0.007$), independently of the two factors entered in the model separately.

### Discussion

Inflammation and insulin resistance are intertwined processes that are strongly linked to overweight and intra-abdominal obesity. We observed that serum LPS activity is associated with visceral fat mass in type 1 diabetes, independent of the traditional risk factors such as age, insulin sensitivity, and inflammation.

High serum LPS activity has been associated with central obesity and insulin resistance, and it has also been shown to predict the development of type 2 diabetes. Intestinal and oral microbial communities are the most likely sources of bacterial endotoxins in humans. Frequent use of antibiotics may have adverse effects on gut homeostasis accompanied by dysbiosis and increased intestinal permeability of bacterial compounds. Childhood hospitalizations related to infections have been associated with an increased risk of obesity and metabolic syndrome in adulthood. In conditions of intestinal inflammation, such as Crohn's disease, visceral fat accumulation is evident.
Dietary fat intake may increase systemic LPS activity, driving adipose inflammation. In a recent study, circulating LPS activity levels correlated with intra-abdominal fat volumes, but only modestly with the subcutaneous fat volumes in patients undergoing bariatric surgery. Notably, a significant decrease in serum endotoxin levels was still evident one year after the operation. Higher CRP levels may arise through a local inflammation of the adipose tissue associated with an increased bacterial burden.

Serum triglycerides and LPS are strongly correlated. Indeed, we observed an interaction between LPS and triglycerides on visceral fat mass. Importantly, the interaction term was independent of the individual factors alone, indicating that the combination of high serum LPS and triglycerides have additive effects on visceral fat mass. This observation is in concordance with previous rat studies, showing that a low dose intraperitoneal LPS injection increases liver triglyceride production, while high doses decrease triglyceride-rich lipoprotein clearance. Moreover, in healthy volunteers, a small intravenous dose of LPS acutely increased serum triglyceride levels, while in mice a subcutaneous infusion of LPS for four weeks significantly increased visceral fat mass. Cross-sectional nature and small study population are potential limitations of the current study. Moreover, due to the exclusion of individuals with end-stage renal disease, the results may not be generalizable to these individuals. Prospective studies are needed to establish the causalities related to the observations.

In conclusion, independent of traditional risk factors, serum LPS activity is associated with visceral fat mass in type 1 diabetes. The LPS-association with visceral fat is pronounced at high triglyceride concentrations. This strengthens the hypothesis that both bacterial endotoxins and dyslipidemia contribute to the development of central obesity, inflammation and insulin resistance and thus may subsequently be associated with adverse cardio-metabolic outcomes.

References
1. Feingold, K. R. & Grunfeld, C. The role of HDL in innate immunity. J. Lipid Res. 52, 1–3 (2011).
2. Vreugdenhil, A. C., Snoek, A. M., van’t Veer, C., Greve, J. W. & Bouhrman, W. A. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. J. Clin. Invest. 107, 225–234 (2001).
3. Boutagy, N. E., McMillan, R. P., Friesard, M. I. & Hulver, M. W. Metabolic endotoxemia with obesity: Is it real and is it relevant? Biochimie 124, 11–20 (2016).
4. Cani, P. D. et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56, 1761–1772 (2007).
5. Mehta, N. N. et al. Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. Diabetes 59, 172–181 (2009).
6. Moreira, A. P. et al. Higher plasma lipopolysaccharide concentrations are associated with less favorable phenotype in overweight/obese men. Eur. J. Nutr. 54, 1363–1370 (2014).
7. Samsell, L., Regier, M., Walton, C. & Cottrell, I. Importance of android/gynoid fat ratio in predicting metabolic and cardiovascular disease risk in normal weight as well as overweight and obese children. J. Obes. 846578 (2014).
8. Lassenius, M. I. et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care 34, 1809–1815 (2011).
9. Nymark, M. et al. Serum lipopolysaccharide activity is associated with the progression of kidney disease in Finnish patients with type 1 diabetes. Diabetes Care 32, 1689–1693 (2009).
10. Lam, Y. et al. Role of the gut in visceral fat inflammation and metabolic disorders. Obesity 19, 2113–2120 (2011).
11. Gustafson, B. & Smith, U. Regulation of white adipogenesis and its relation to ectopic fat accumulation and cardiovascular risk. Atherosclerosis 241, 27–35 (2015).
12. Peterson, M. D., Al Snih, S., Serra-Rexach, J. A. & Burant, C. Android adiposity and lack of moderate and vigorous physical activity are associated with insulin resistance and diabetes in aging adults. J. Gerontol. A. Biol. Sci. Med. Sci. 70, 1009–1017 (2015).
13. Lu, Y. et al. New loci for body fat percentage reveal link between adiposity and cardiometabolic disease risk. Nat. Commun. 7, 10495 (2016).
14. Hastie, T. & Tibshirani, R. Exploring the nature of covariate effects in the proportional hazards model. Biometrics 46, 1005–1016 (1990).
15. Wood, S. N. Generalized additive models: An introduction with R. (Chapman & Hall/CRC, Boca Raton, Florida, 2006).
16. Wood, S. Mixed GAM computation vehicle with GVC/AIC/REML smoothness estimation. R package version 1. (2016).
Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: Professor Per-Henrik Groop has received research grants from Eli Lilly and Roche, is an advisory board member for AbbVie, Astra Zeneca, Boehringer-Ingelheim, Cebix, Eli Lilly, Janssen, Medscape, MSD, and Novartis. He also has received lecture fees from Astra Zeneca, Boehringer-Ingelheim, Eli Lilly, Genzyme, MSD, Novartis, Novo Nordisk, Sanofi. All other authors declare that there is no conflict of interest.

How to cite this article: Lassenius, M. I. et al. Endotoxins are associated with visceral fat mass in type 1 diabetes. Sci. Rep. 6, 38887; doi: 10.1038/srep38887 (2016).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016