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Abstract

Aims/hypothesis The aim of this study was to use lipidomics to determine if the lipid composition of apolipoprotein-B-containing lipoproteins is modified by dyslipidaemia in type 2 diabetes and if any of the identified changes potentially have biological relevance in the pathophysiology of type 2 diabetes.

Methods VLDL and LDL from normolipidaemic and dyslipidaemic type 2 diabetic women and controls were isolated and quantified with HPLC and mass spectrometry. A detailed molecular characterisation of VLDL triacylglycerols (TAG) was also performed using the novel ozone-induced dissociation method, which allowed us to distinguish vaccenic acid (C18:1 n-7) from oleic acid (C18:1 n-9) in specific TAG species.

Results Lipid class composition was very similar in VLDL and LDL from normolipidaemic type 2 diabetic and control participants. By contrast, dyslipidaemia was associated with significant changes in both lipid classes (e.g. increased diacylglycerols) and lipid species (e.g. increased C16:1 and C20:3 in phosphatidylcholine and cholesteryl ester and increased C16:0 [palmitic acid] and vaccenic acid in TAG). Levels of palmitic acid in VLDL and LDL TAG correlated with insulin resistance, and VLDL TAG enriched in palmitic acid promoted increased secretion of proinflammatory mediators from human smooth muscle cells.

Conclusions We showed that dyslipidaemia is associated with major changes in both lipid class and lipid species composition in VLDL and LDL from women with type 2 diabetes. In addition, we identified specific molecular lipid species that both correlate with clinical variables and are proinflammatory. Our study thus shows the potential of advanced lipidomic methods to further understand the pathophysiology of type 2 diabetes.

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Clinical dyslipidemia is associated with changes in the lipid composition and inflammatory properties of apolipoproteinB-containing lipoproteins from women with type 2 diabetes

Running title: Lipidomics on apoB-containing lipoproteins from type 2 diabetic subjects

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ABSTRACT

Aim/Hypothesis — The aim of this study was to use lipidomics to determine if the lipid composition of apolipoprotein B-containing lipoproteins is modified by dyslipidemia in type 2 diabetes and if any of the identified changes have potential biological relevance in the pathophysiology of type 2 diabetes.

Methods — VLDL and LDL from normolipidemic and dyslipidemic type 2 diabetic women and controls were isolated and quantified with high performance liquid chromatography and mass spectrometry. A detailed molecular characterization of VLDL triacylglycerols (TAG) was also performed using the novel OzID methodology, which allowed us to distinguish vaccenic acid (C18:1 n-7) from oleic acid (C18:1 n-9) in specific TAG species.

Results — Lipid class composition was very similar in VLDL and LDL from normolipidemic type 2 diabetic and control subjects. By contrast, dyslipidemia was associated with significant changes in both lipid classes (e.g. increased diacylglycerols) and lipid species [e.g. increased C16:1 and C20:3 in phosphatidylcholine and cholesteryl ester and increased C16:0 (palmitic acid) and vaccenic acid in TAG]. Levels of palmitic acid in VLDL and LDL TAG correlated with insulin resistance, and VLDL TAG enriched in palmitic acid promoted increased secretion of proinflammatory mediators from human smooth muscle cells.

Conclusions — We showed that dyslipidemia is associated with major changes in both lipid class and lipid species composition in VLDL and LDL from women with type 2 diabetes. In addition, we identified specific molecular lipid species that both correlate with clinical parameters and are proinflammatory. Our study thus shows the potential of advanced lipidomic methods to further understand the pathophysiology of type 2 diabetes.

Key words: • VLDL • LDL • apolipoprotein B • lipids • lipidomics • mass spectrometry

ABBREVIATIONS

CE	Cholesterol ester
TAG	Triacylglycerol
FC	Free cholesterol
DAG	Diacylglycerol
SM	Sphingomyelin
CER	Ceramide
LPC	Lyso-phosphatidylcholine
PC	Phosphatidylcholine
CID	Collision-induced dissociation
PKC	Protein kinase C
FID	Flame ionization detector
FAME	Fatty acid methyl esters
SMase	Sphingomyelinase
UKPDS	United Kingdom Prospective Diabetes Study
OzID	Ozone-induced dissociation

Introduction

Individuals with type 2 diabetes have an increased risk of cardiovascular events [1]. Although the mechanisms behind this increased risk are still not fully understood, dyslipidemia is common in patients with type 2 diabetes and is an established risk factor for cardiovascular disease [2]. Fatty liver has evolved as a key player in the pathogenesis of dyslipidemia and we have shown a strong relationship between increased liver fat, insulin resistance and the overproduction of large [triacylglycerol (TAG)-rich] VLDL particles [3, 4]. The increased secretion of large VLDL particles initiates a sequence of lipoprotein changes, resulting in additional abnormalities observed in dyslipidemia such as low levels of HDL and the appearance of small dense LDL [5]. In addition, hepatic uptake of VLDL and its metabolites [intermediate-density lipoprotein (IDL) and LDL] is decreased in patients with type 2 diabetes, resulting in increased plasma residence time of these lipoproteins and thus further contributing to the dyslipidemia [6, 7].

Traditionally, serum lipids have been studied in terms of the number of VLDL, LDL and HDL particles, and only the largest lipid classes have been quantified in an attempt to further characterize the lipoprotein particle. Although it is well known that the concentration of apolipoprotein B (apoB)-containing lipoproteins (VLDL, IDL and LDL) is a key determinant of atherogenicity, recent data suggests that compositional properties of lipoproteins are also important [8, 9]. With the advent of powerful analytical tools such as tandem mass spectrometry it is now possible to perform a more detailed characterization of the different lipoprotein particles. This analysis, which can be performed using a high-throughput approach [10], provides quantitative data about both highly abundant lipids as well as the less abundant bioactive lipids. These advances offer an exciting opportunity for the elucidation of mechanisms and for the identification of disease-specific, lipid-based biomarkers [11].

In this study, we used a lipidomics approach to characterize lipid classes and lipid species in apoB-containing lipoproteins isolated from control subjects and normolipidemic and dyslipidemic

subjects with type 2 diabetes. Our aim was to determine how clinical dyslipidemia affects the lipoprotein composition in subjects with insulin resistance and type 2 diabetes. We also wanted to investigate if insulin resistance without clinical dyslipidemia in type 2 diabetes was associated with changes in lipid composition in comparison with a healthy reference group. Finally we wanted to determine if any of the identified changes could have any biological relevance in the pathophysiology of type 2 diabetes.

Methods

Lipid annotation For explanation of lipid annotation, see Electronic Supplementary Material (ESM).

Study subjects The subjects in this study were all 64-year-old Caucasian women with the same ethnic background (Swedes) who originally participated in the Diabetes and Impaired glucose tolerance in Women and Atherosclerosis (DIWA) study [12]. From this population, we randomly chose twenty women per group according to the following criteria: (1) control subjects (HOMA < 1.35) with normal blood lipids (TAGs < 1.7 mmol/l and HDL > 1.29 mmol/l); (2) subjects with normal blood lipids (TAGs < 1.7 mmol/l and HDL > 1.29 mmol/l) but with type 2 diabetes as defined by WHO [13], insulin resistance (HOMA > 1.35) and glutamic acid decarboxylase antibodies < 4.6 units/ml; and (3) subjects with dyslipidemia (TAGs > 1.7 mmol/l and HDL < 1.29 mmol/l), but otherwise the same inclusion criteria as group two (Table 1). None of the subjects included in the study were on any medication for diabetes or dyslipidemia. In addition to the three groups above, we also complemented our characterization with a group containing non-treated, dyslipidemic non-diabetic subjects (Table 1). The study protocol was approved by the local ethics committee, and each participant provided written informed consent.

Lipoprotein isolation VLDL (including IDL) ($d < 1.019$) and LDL ($d = 1.019\text{--}1.063$) were isolated from 500 μ l plasma by ultracentrifugation [14]. The apoB concentrations were measured using a Konelab 20 autoanalyzer (Thermo Electron, Vantaa, Finland).

LDL sizing using gel electrophoresis LDL particle size was measured using non-denaturing polyacrylamide gel electrophoresis (2–16% gradient gels, Alamo, San Antonio, TX, USA) [15]. The gels were stained with Coomassie Brilliant Blue (Merck, Darmstadt, Germany), scanned and analyzed using the Quantity One[®] software (Bio-Rad, Richmond, CA, USA). The mean LDL size was measured as described [15].

Lipid extraction Lipids were extracted according to Folch *et al.* [16]. Internal standards were diluted

in chloroform and added during the extraction procedure.

Lipid analysis Lipids were analyzed using a combination of HPLC and mass spectrometry. For details see ESM.

Cell culture and lipoprotein incubation Fresh human smooth muscle cells (Lonza CC 2571) (Lonza, Basel, Switzerland) were seeded onto 6-well tissue culture plates after two passages. Cells were cultured in Lonza's special media cc318s and incubated for 24 h with VLDL (45 $\mu\text{g/ml}$) containing either high or low levels of TAG-palmitic acid, or in media with both free palmitic acid (100 μM) [17] and VLDL (45 $\mu\text{g/ml}$) containing low levels of TAG-palmitic acid (Low + P). All media contained 2 mg/mL BSA (fatty acid free and low endotoxin). Cytokine levels in the cell culture medium were analyzed with a SECTOR Imager 2400 reader (MesoScale Discovery, Gaithersburg, MD, USA). For further details see ESM.

Statistic evaluation ANOVA analysis followed by Tukey's post-hoc test was used for comparisons between groups. Correlation analysis was performed using Pearson correlation coefficient. To access the false discovery rate of large number of tests, q-values were calculated from the unadjusted p-values using QValue software package for R [18].

Results

Subject basic characteristics The dyslipidemic type 2 diabetic subjects showed several features of diabetic dyslipidemia including hypertriglyceridemia and low HDL (Table 1). There was a step-wise significant increase in BMI and waist between the control, normolipidemic and dyslipidemic type 2 diabetic groups but no significant differences in HOMA or HbA_{1c} between the two groups with type 2 diabetes (Table 1). Levels of plasma TAG, HDL and LDL were the same in the normolipidemic type 2 diabetic and control groups (Table 1).

Dyslipidemia is required to induce significant alterations in lipoprotein lipid class composition Using a combination of HPLC and mass spectrometry, we quantified eight lipid classes in both VLDL and LDL and showed that the lipid class composition was very similar in lipoproteins from normolipidemic type 2 diabetic and control subjects (Fig. 1a,b). By contrast, we showed that the VLDL and LDL from dyslipidemic type 2 diabetic subjects displayed significant changes in lipid class composition compared with VLDL and LDL from the other two groups (Fig. 1a,b). In VLDL, diacylglycerol (DAG) was significantly increased and the membrane lipid sphingomyelin (SM) was reduced (Fig. 1a). The changes in LDL were more profound: in parallel with VLDL, we observed increased DAG and decreased SM, but we also measured significantly reduced amounts of the core lipid cholesteryl ester (CE) and the membrane lipids free cholesterol (FC) and ceramide (CER) (Fig. 1b). The most abundant membrane lipid, phosphatidylcholine (PC), was unchanged, indicating an altered membrane composition.

To test the hypothesis that the compositional differences of LDL are linked to their size, we analyzed the correlation between the lipid composition and size of LDL. We showed that LDL isolated from dyslipidemic type 2 diabetic subjects was smaller than LDL isolated from the normolipidemic groups (Fig. 2a-h). Furthermore, the size of the LDL correlated negatively with the amount of TAG and DAG and positively with the amount of CE, FC, SM, CER, PC and lyso-PC (LPC).

Dyslipidemia amplifies alterations in PC and CE species composition observed in lipoproteins from type 2 diabetic subjects We comprehensively characterized the lipid species within each lipid class of the isolated lipoproteins using QqTOF mass spectrometry (ESM Table 1). These analyses revealed significantly increased relative amounts of PC 16:0-20:3 (in VLDL and LDL) and PC 18:0-20:3 (in LDL) in lipoproteins from normolipidemic type 2 diabetic subjects compared with controls (Fig. 3a). These alterations were even more significant in the dyslipidemic type 2 diabetic group, which also had an increased relative amount of PC 16:0-16:1. The alterations in the PC fatty acid composition observed in lipoproteins from dyslipidemic type 2 diabetic subjects were reflected in a similar change in the CE lipid class with significant increases in CE 16:1 (in VLDL and LDL) and CE 20:3 (in LDL) (Fig. 3b). A comparison with basic characteristics showed that the relative amount of PC species containing C16:1 and C20:3 fatty acids correlated significantly with important clinical parameters such as BMI and HDL levels (ESM Table 2).

Dyslipidemia is associated with increased palmitic acid-containing species in VLDL and LDL TAG and DAG Mass spectrometric analysis also revealed several significant changes in lipid species in the DAG and TAG fractions of VLDL from dyslipidemic type 2 diabetic subjects (ESM Table 1; Fig. 4). In DAG, these consisted mainly of increased palmitic acid (C16:0)-containing species such as DAG 16:0-16:0, DAG 16:0-16:1 and DAG 16:0-18:1. Similar patterns were observed in the TAG lipid class with significantly increased palmitic acid-containing species such as TAG 16:0-16:0-16:0, TAG 16:0-16:0-16:1 and TAG 16:0-16:0-18:1. In contrast, lipid species containing longer and more unsaturated fatty acids, especially linoleic acid (C18:2), were unaltered or decreased (with the exception of an increase in the doubly saturated TAG 16:0-16:0-18:2 and TAG 18:0-16:0-18:2). All changes detected in VLDL were closely mirrored in the LDL (ESM Table 1). A comparison with basic characteristics revealed several highly significant correlations between molecular TAGs and clinical parameters (ESM Table 3). A confirmatory GC/FID analysis of fatty acid methyl esters (FAME) from VLDL TAGs was also performed, which showed similar increases in the relative amount of palmitic acid and reduced amount of linoleic acid (ESM Table 4).

Dyslipidemia without type 2 diabetes is associated with alterations in VLDL and LDL lipid composition As the results show that dyslipidemia, in type 2 diabetic subjects, is associated with an altered VLDL and LDL lipid composition, we also performed a complementary analysis on a group of dyslipidemic, non-diabetic subjects (Table 1). The analysis of the VLDL and LDL lipid class composition showed alterations that were similar to the changes observed for the dyslipidemic type 2 diabetic subjects when compared to the control group (Fig. 1).

A characterization of molecular lipid species in the dyslipidemic non-diabetic subjects showed increased levels of C16:1 and C20:3 containing species in the CE and PC lipid class compared to the controls. However the increases were not as pronounced as for the dyslipidemic type 2 diabetic subjects (Fig. 3). In the TAG and DAG lipid class there was a tendency towards increased levels of palmitic acid-containing species compared to the control group. However, the results are not as prominent as for the dyslipidemic type 2 diabetic subjects (Fig. 4).

Dyslipidemia is linked to increased content of vaccenic acids in a specific molecular TAG Despite the lack of significant alterations of vaccenic acid when analyzing total fatty acids using FAME (ESM Table 4), we wanted to investigate whether the increased levels of palmitic acid and palmitoleic acid observed in VLDL TAG from the dyslipidemic group were reflected by increases in vaccenic acid in specific TAG species. For this we used a combination of CID and the novel OzID technology (see ESM for details). The results show that in a specific TAG, subjects with type 2 diabetes have an increased incorporation of the C18:1 fatty acid into the sn-2 position (Fig 5a). Furthermore, dyslipidemic type 2 diabetic subjects have a higher amount of vaccenic (as compared to oleic acid) (Fig. 5 b), and that this fatty acid is preferably incorporated into the sn-2 position of the investigated TAG (Fig. 5 c).

Increased palmitic acid in VLDL TAG is associated with increased proinflammatory activity To assess the biological significance of the increased palmitic acid in VLDL TAG, we investigated the effect of high and low VLDL TAG-palmitic acid on the inflammatory activity of human smooth

muscle cells. We showed that cells incubated with VLDL containing a high proportion of TAG-palmitic acid had a highly significant increase in the secretion of several cytokines involved in endothelial activation (Fig. 6a-i). Cells incubated with a combination of VLDL with low palmitic acid levels and free palmitic acid (100 μ M) also showed increased secretion of inflammatory markers. However, the response was in most cases lower than for the response generated by high palmitic acid VLDL incubation.

Assessment of 10 year risk of coronary heart disease from lipid data 10 year risk for CHD was calculated using the United Kingdom Prospective Diabetes Study (UKPDS) risk engine in the type 2 diabetic subjects [19]. Calculation of the UKPDS score is based on blood pressure, HbA1c, total and HDL cholesterol as well as age of onset and duration of type 2 diabetes, ethnicity, smoking and gender. Correlations between anthropometric data, blood lipids, selected lipid species and classes and CHD score were calculated (ESM Table 5). By definition, HbA1c ($r=0.82$, $p<0.001$) and LDL cholesterol ($r=0.68$, $p<0.001$) was positively, and HDL cholesterol negatively ($r=-0.62$, $p<0.001$), associated to CHD risk. In addition, total apoB ($r=0.90$, $p<0.001$), total TAG ($r=0.84$, $p<0.001$) and total apoCIII ($r=0.68$, $p<0.001$) showed strong associations with CHD risk. Furthermore, LDL SM ($r=-0.62$, $p<0.001$), CER ($r=-0.61$, $p<0.001$), FC ($r=-0.59$, $p<0.001$) and DAG ($r=0.56$, $p<0.001$) as well as VLDL CER ($r=0.55$, $p<0.01$) were also associated with CHD risk. Interestingly most of these measures were more strongly associated with CHD risk than HOMA-IR ($r=0.59$, $p<0.001$), waist ($r=0.37$, $p<0.05$) and BMI ($r=0.26$, NS). Furthermore, using a multivariate approach, combining the levels of several lipids and BMI, waist and HOMA-IR (variables in the UKPDS model (HDL and total cholesterol), or variables highly correlated to these (apoB, apoA1 and plasma triglycerides) were excluded), we obtained a high correlation (adjusted $r^2=0.72$) to the UKPDS score; variables in model: LDL total CER, waist, VLDL TAG 18:1/18:1/18:1 and VLDL palmitate. Including only lipid variables, we obtained an almost as strong correlation (adjusted $r^2=0.69$); variables in the model: LDL total CER, LDL DAG 18:0/18:0, VLDL CE 16:1, VLDL total DAG and VLDL DAG 16:1/18:1.

Discussion

In this study, we used a lipidomics approach to characterize apoB-containing lipoproteins from control and normolipidemic and dyslipidemic type 2 diabetic subjects. We show that dyslipidemia in subjects with type 2 diabetes is associated with significant changes in specific lipid classes and molecular species in VLDL and LDL. We identify several enriched molecular species in dyslipidemic VLDL and LDL that correlate with clinical parameters and we also show that palmitic acid-enriched VLDL TAG promotes inflammation.

Small dense LDL is a common characteristic of the dyslipidemic state [5], and LDL isolated from the dyslipidemic type 2 diabetic subjects in our study indeed had a smaller particle size compared with LDL from the normolipidemic subjects. A reduced particle size would explain reductions in the membrane lipids CER, SM and FC. However, the most abundant membrane lipid PC was unchanged, indicating that LDL would have an altered membrane composition. Because CER, SM and FC all have been shown to positively affect a closer lateral packing in LDL [20, 21], the altered lipid composition of LDL isolated from dyslipidemic type 2 diabetic subjects could be associated with higher membrane fluidity and higher freedom in lateral moving of lipoprotein-associated proteins. Although the metabolic consequences of the reduced lipoprotein SM levels remain to be elucidated, it has been proposed that a decreased SM/PC ratio could render these particles more susceptible to modifications by PLA₂ activity [22, 23].

VLDLs are metabolically heterogeneous, and the liver can secrete both large triglyceride-rich VLDL₁ and smaller cholesterol-rich VLDL₂. Variations in plasma triglyceride concentrations are mainly accounted for by differences in VLDL₁. We have earlier shown that increased liver fat in type 2 diabetic subjects is associated with increased VLDL₁ production and dyslipidemia [3]. A possible explanation for the reduced SM/PC, CER/PC and FC/PC ratio observed in dyslipidemic type 2 diabetic subjects could be a disturbed hepatic lipid metabolism that results in increased secretion of large VLDL₁ particles having a different lipid composition than the VLDL₂ particle.

Another explanation for the altered membrane composition is that lipoproteins from type 2 diabetic subjects with dyslipidemia are potentially more susceptible to modifications occurring in the circulation. Studies have shown that sphingomyelinase (SMase) activity is elevated in the serum of patients with type 2 diabetes [24], and also that LDL isolated from type 2 diabetic subjects is enriched with the SMase activator apolipoprotein CIII (apoCIII) [25]. Depletion of cell surface SM with SMase results in a simultaneous loss of FC [26], and thus increased SMase activity will result in parallel decreases in SM and FC.

Although TAG was slightly increased in VLDL and LDL isolated from dyslipidemic type 2 diabetic subjects, the differences were not significant. We expected to see greater changes in TAG, but it is likely that large TAG-rich VLDL characteristic of dyslipidemic subjects is rapidly metabolized to TAG-poor VLDL. Furthermore, our VLDL fraction did not differentiate between VLDL and IDL and therefore some of the changes in TAG may have been masked. By contrast, we observed clear increases in DAG in VLDL and LDL isolated from dyslipidemic type 2 diabetic subjects. A role for DAGs has been implicated in the etiology of insulin resistance in the liver through a mechanism involving activation of protein kinase C (PKC)- ϵ [27, 28]. However, it remains to be determined if hepatic uptake of DAG-enriched lipoproteins could contribute to this pathway.

We also showed that the PC lipid class in VLDL and LDL isolated from dyslipidemic type 2 diabetic subjects had increased levels of C16:1 and C20:3 that were reflected in CE. These parallel increases in PC and CE may be explained by the fact that CE can be synthesized by the lecithin-cholesterol acyltransferase-catalyzed transesterification of FC with the fatty acid attached to the *sn*-2 position of PC [29]. Increased levels of C16:1 and C20:3 esterified to PC and CE have previously been observed in subjects with type 2 diabetes [30, 31], and here we showed that the relative amount of PC and CE species containing C16:1 and C20:3 correlated significantly with clinical parameters.

Analysis of the lipid species composition of the DAG and TAG lipid classes revealed that VLDL and LDL from dyslipidemic type 2 diabetic subjects were enriched in palmitic acid (C16:0)

whereas linoleic acid (C18:2) was mainly unaffected or reduced. A recent study showed that serum TAGs containing palmitic acid correlate positively with insulin resistance whereas serum TAGs containing linoleic acid correlate negatively with insulin resistance [32]. Furthermore, they also showed that TAG 16:0-16:0-18:1 and TAG 16:0-18:1-18:0 are better markers of insulin resistance than total serum TAG concentrations [32]. We also showed that palmitic acid in TAG from VLDL and LDL correlated positively with insulin resistance whereas linoleic acid correlated negatively. However, both of these correlations were weaker than the correlation between total plasma TAGs and insulin resistance in our study.

Using a combination of CID and the novel OzID techniques, we showed an elevation of vaccenic acid in dyslipidemic type 2 diabetic subjects, specifically in the TAG 16:0-16:0-18:1. Furthermore, our results suggested that the *sn*-2 position of the glycerol backbone is the preferred position for its incorporation, which is in accordance with previously published data [33]. Since hepatic fat accumulation is associated with dyslipidemia in type 2 diabetes [34], we hypothesize that the increased content of vaccenic acid is an indication of altered hepatic lipid metabolism and a marker of hepatic steatosis. However, the reason for the selective incorporation into specific TAG or its potential role as a biomarker demands further investigation.

In addition to TAG 16:0-16:0-18:1, which was the TAG species that were most significantly changed between the dyslipidemic type 2 diabetic subjects and controls, we also did a similar characterization of the most common TAG species, TAG 18:1-18:1-16:0. Here we saw no alteration in the n-7/n-9 ratio. This result might be the reason for not detecting a difference in n-7/n-9 ratio when analyzing FAME using GC/FID. In that analysis, selective fatty acid incorporation into certain molecular TAG species will be masked by the signal from high abundant, non-changing, C18:1 n-7 and C18:1 n-9 containing TAG species (e.g. TAG 18:1-18:1-16:0).

It has previously been shown that VLDL isolated from subjects with dyslipidemia is proinflammatory and responsible for upregulation of several pathways involved in endothelial activation [35]. Although the mechanisms behind this are poorly understood, the composition of the

particle might play an important role. For example, TAG-rich lipoproteins (TRLs) isolated after a meal are more proinflammatory if enriched in saturated fatty acids rather than mono- and polyunsaturated fatty acids [36]. Furthermore, palmitic acid (in its free form) has been shown to be bioactive and proinflammatory [37, 38]. Because the TAGs in the VLDL particles isolated from dyslipidemic subjects in our study were enriched in palmitic acid, we tested the hypothesis that this would increase the proinflammatory properties of these particles. Incubation of smooth muscle cells with VLDL rich in TAG-palmitic acid resulted in a highly significant increase in the secretion of several important inflammatory mediators. However, although this supports our theory, we cannot exclude the possibility that other intrinsic factors of the VLDL particle could be responsible for the results.

In an attempt to evaluate the clinical relevance of our results, we correlated several of the lipid parameters to CHD risk as calculated by the UKPDS engine. Interestingly, the analysis showed that many of these correlations were more strongly associated to CHD risk than traditional risk markers such as HOMA-IR, waist and BMI. Furthermore, even stronger correlations could be obtained if several lipids were used in a multivariate approach. However, as this is not a definite estimate, further studies involving a larger cohort is needed to evaluate the importance of these parameters compared to the traditional risk factors.

We also performed a complementary analysis of lipoproteins isolated from a group of dyslipidemic subjects with normal glucose tolerance with the aim to further clarify the role of dyslipidemia for the observed changes in lipoprotein lipid composition. However, such a comparison was hampered by the fact that less than 5% (n=9) of the subjects with normal glucose tolerance and no lipid lowering treatment fulfilled the dyslipidemia criteria. Both hypertriglyceridemia and low HDL cholesterol are well-known predictors of diabetes [39]. Furthermore, all subjects had abdominal obesity with waist girth above 88 cm, seven out of nine subjects had insulin resistance, and 56% (n=5) had a first-degree relative with diabetes, compared with 18% (n=33, p=0.016) in those without dyslipidemia. As all the mentioned characteristics are

strong predictors of type 2 diabetes, it is obvious that these subjects with dyslipidemia represent a group with prediabetes, albeit still without hyperglycemia. This conclusion is supported by the striking similarity between the two dyslipidemic groups with and without diabetes regarding obesity, degree of dyslipidemia, C-reactive protein and apoCIII (Table 1). In line with these observations we found that, overall the lipid aberration in the dyslipidemic non-diabetic group was similar to that in the dyslipidemic type 2 diabetic group, although not as pronounced. Taken together these data support previous suggestions that dyslipidemia may precede the development of type 2 diabetes.

A possible limitation of our study is that only women of a certain age are included. Therefore it is not obvious that these results can be directly translated to the male population. However, the cohort of women that was investigated in the present study is of considerable interest given the fact diabetes prevalence start to increase steeply in this age category in women [40]. In addition, the diagnosis of diabetes is associated with a very high relative risk of future cardiovascular death in women that is much higher than among men [41].

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MS performed the lipid analyses, interpreted the data and wrote the manuscript. HTP, TWM and SJB performed lipid analysis, contributed to the data interpretation and the draft of the manuscript. MA performed the statistical analysis and interpreted the data. KE contributed to the study conception and design, and also to the draft of the manuscript. JB and BF contributed to the study conception and design, data analysis and interpretation, and draft of the manuscript. All authors revised the manuscript critically for intellectual content and approved of the final version.

Duality of interest The authors confirm that there is no duality of interest associated with this manuscript.

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FIGURE LEGENDS

Fig. 1. Lipid class composition of VLDL (A) and LDL (B) isolated from control subjects (black), type 2 diabetic subjects (grey), dyslipidemic type 2 diabetic subjects (white) and non-diabetic dyslipidemic subjects (striped). Values are average \pm SD. * p <0.05 vs. control; # p <0.05 vs. type 2 diabetic subjects; † p <0.05 vs. dyslipidemic type 2 diabetic subject.

Fig. 2. Correlations between lipid class amounts and the size of LDL. Triangles represent control subjects, circles normolipidemic type 2 diabetic subjects and crosses dyslipidemic type 2 diabetic subjects. All three groups are included in the correlations. * p <0.05, ** p <0.01, *** p <0.001.

Fig. 3. The relative abundance (mol% of total lipid class) of PC (A) and CE (B) species containing C16:1 and C20:3 fatty acids was compared between control subjects (black), type 2 diabetic subjects (grey), dyslipidemic type 2 diabetic subjects (white) and non-diabetic dyslipidemic subjects (striped). Values are average \pm SD. * p <0.05 vs. control; # p <0.05 vs. type 2 diabetic subjects; † p <0.05 vs. dyslipidemic type 2 diabetic subject.

Fig. 4. Lipid species composition of the lipid classes DAG (A) and TAG (B) in VLDL isolated from control subjects (black), type 2 diabetic subjects (grey), dyslipidemic type 2 diabetic subjects (white) and non-diabetic dyslipidemic subjects (striped). Values are average \pm SD. * p <0.05 vs. control; # p <0.05 vs. type 2 diabetic subjects; † p <0.05 vs. dyslipidemic type 2 diabetic subject.

Fig. 5. (A) Using CID, the ratio of the molecular species TAG 16:0/16:0/18:1 (C18:1 in *sn*-1(3) position) and the isomer TAG 16:0/18:1/16:0 (C18:1 in *sn*-2 position) were determined in the VLDL TAGs isolated from control subjects (black), normolipidemic type 2 diabetic (T2D) subjects (grey), and dyslipidemic T2D subjects with dyslipidemia (T2D+DL)(white) ($n=10$ in each group). Values are average \pm SD. (B,C) Using combinations of CID and OzID the ratio of vaccenic (C18:1 *n*-7) to oleic (C18:1 *n*-9) acid was determined in the molecular species TAG 50:1 (B) and TAG

16:0/16:0/18:1 (C). This was done in VLDL TAG isolated from control subjects, normolipidemic T2D subjects, and (T2D+DL subjects). ($n=10$ in each group). $*p<0.05$; $**p<0.01$

Fig. 6. Cells were incubated with VLDL containing either high (High) or low (Low) levels of palmitic acid in the triacylglycerols, or with a combination of VLDL containing low levels of palmitic acid and free palmitic acid (Low + P). The cytokine concentrations (pg/ml) were measured in the cell media and are expressed as average \pm SD ($n=5$). $*p<0.05$; $**p<0.01$; $***p<0.005$.

Figure 1

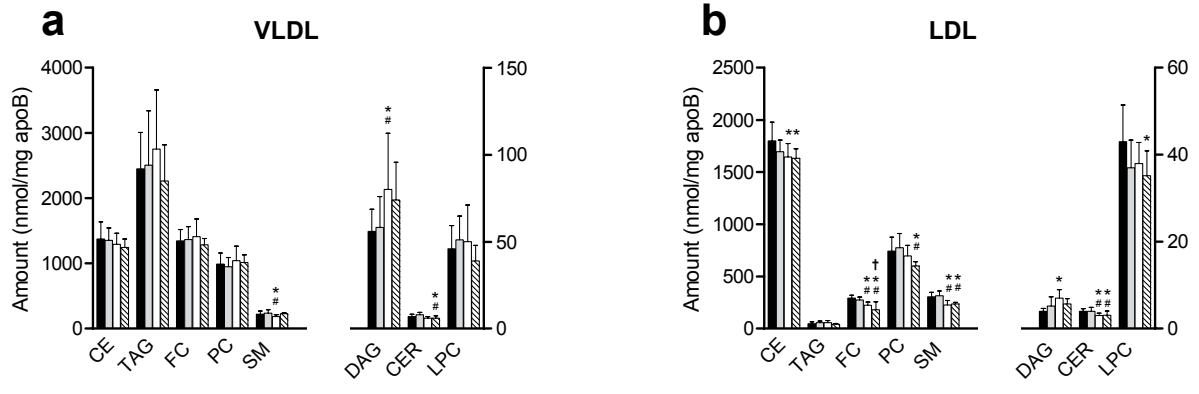


Figure 2

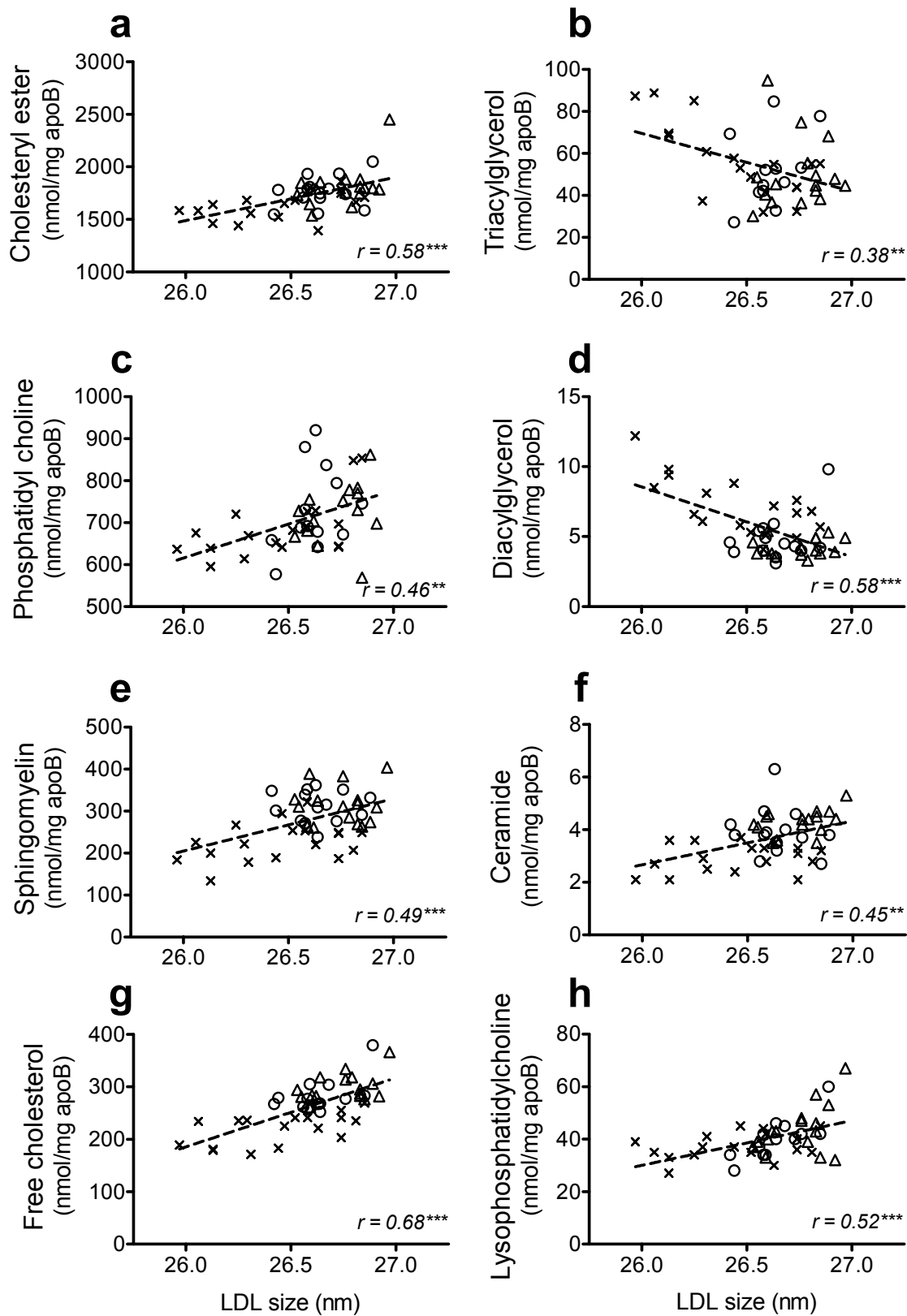


Figure 3

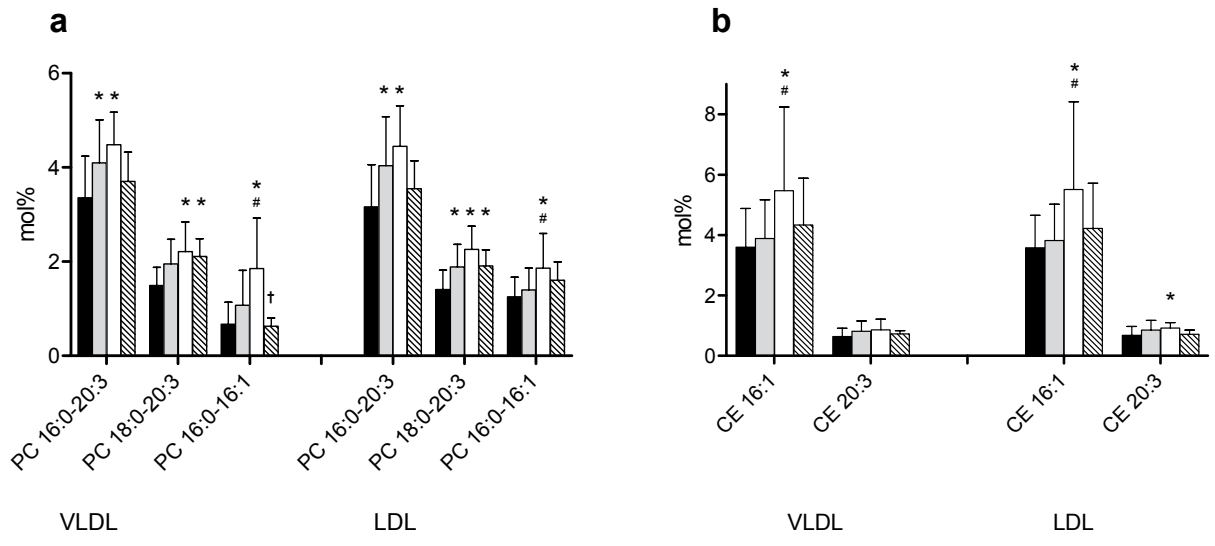


Figure 4

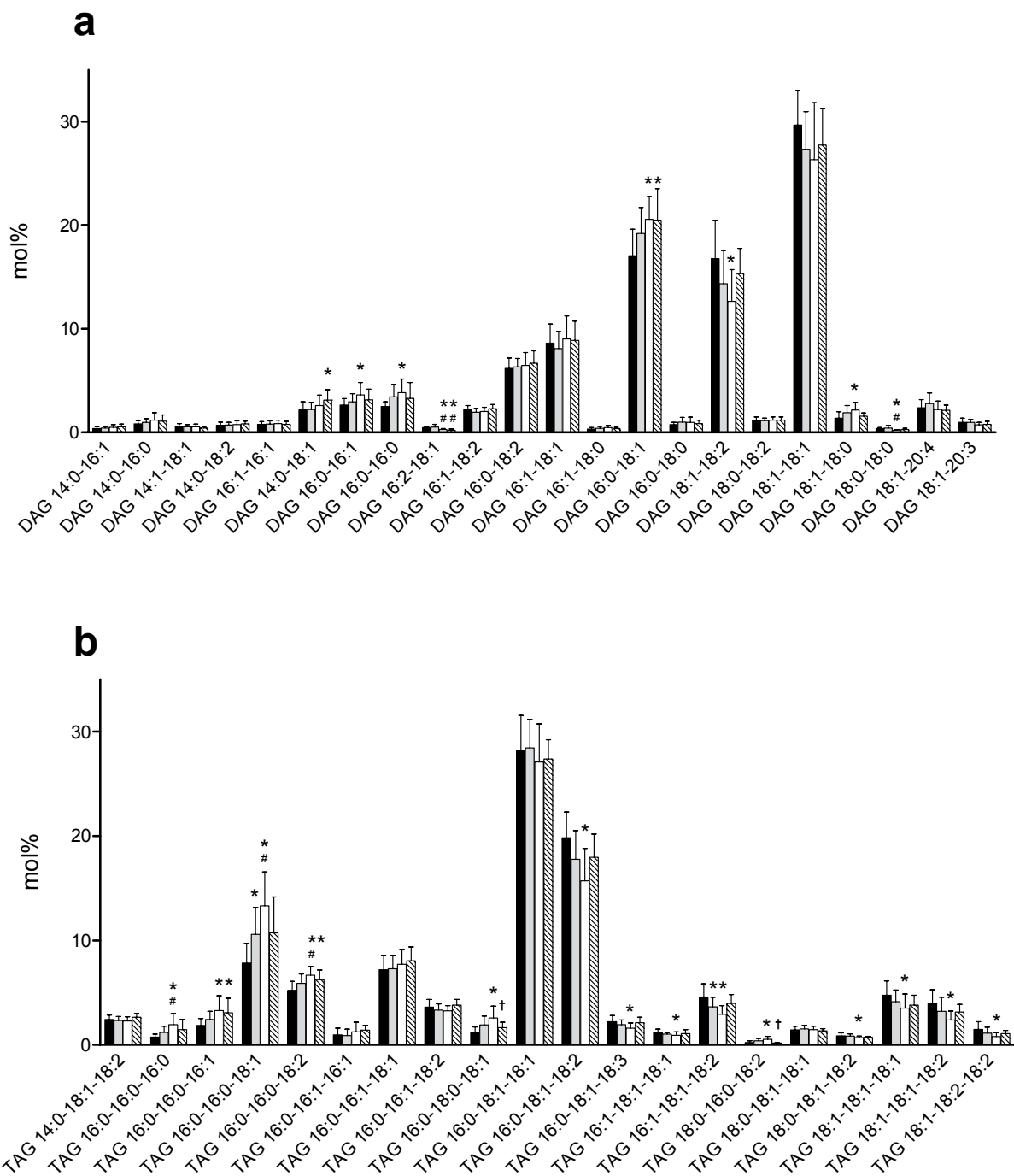


Figure 5

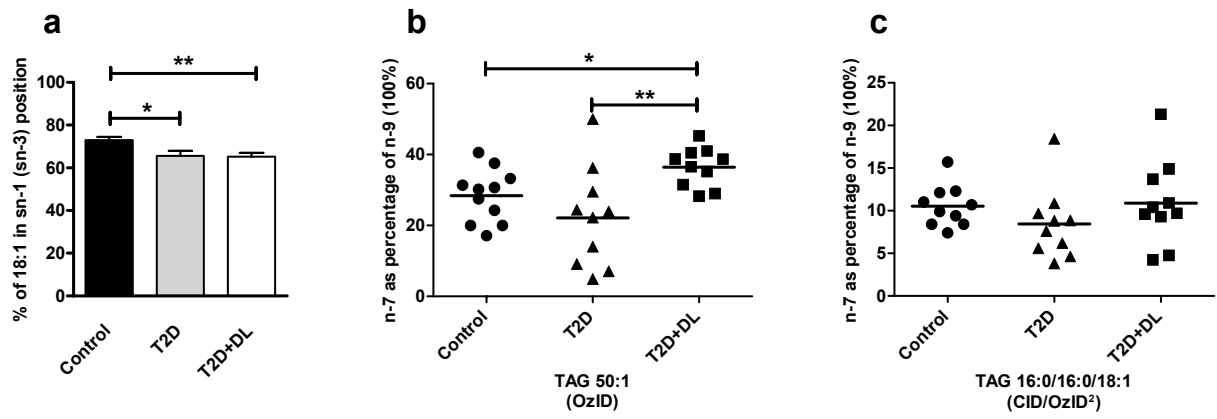


Figure 6

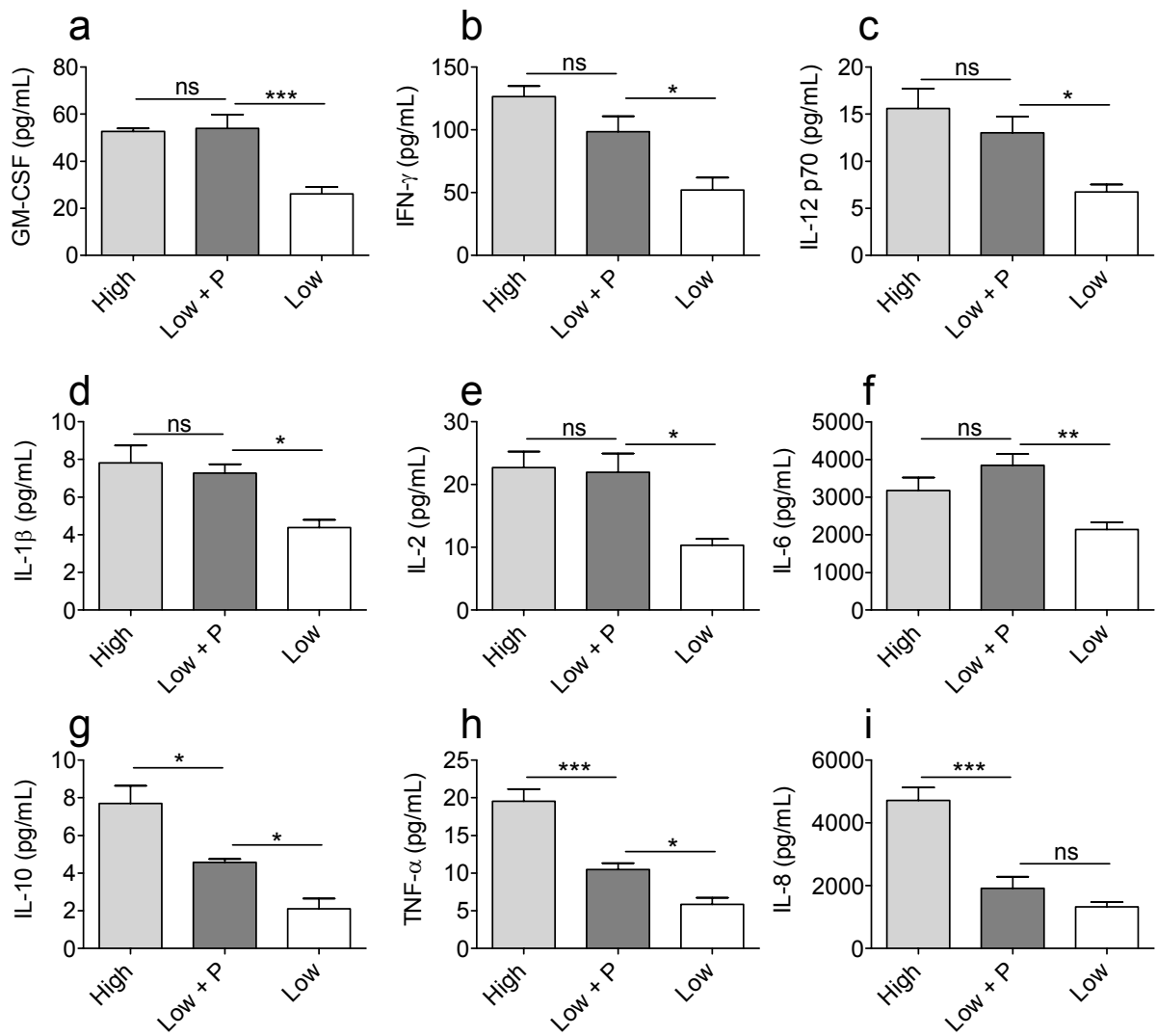


Table 1. Clinical characteristics of the study participants

	CTRL (n=20)	T2D (n=20)	T2D+DL (n=20)	DL (n=9)
BMI (kg/m²)	24 ± 3.3	28 ± 2.7 *	32 ± 4.5 * [#]	29.4 ± 2.7 *
Waist (cm)	85 ± 6.0	96 ± 8.7 *	107 ± 8.5 * [#]	98 ± 6.1 * [†]
Triacylglycerols (mmol/l)	1.0 ± 0.2	1.2 ± 0.3	2.8 ± 1.2 * [#]	2.1 ± 0.3 * [#]
HDL (mmol/l)	1.9 ± 0.2	1.7 ± 0.3 *	1.1 ± 0.2 * [#]	1.1 ± 0.1 * [#]
LDL (mmol/l)	3.3 ± 0.7	3.1 ± 1.1	4.0 ± 1.0 [#]	4.6 ± 1.4 * [#]
HOMA	0.85 ± 0.3	3.8 ± 1.6 *	5.4 ± 3.7 *	2.2 ± 0.9 [†]
HbA1c (% / mmol/mol)	5.5 ± 0.3 / 37 ± 2.9	6.2 ± 0.7 / 45 ± 7.5 *	7.0 ± 1.6 / 53 ± 18 *	5.6 ± 0.3 / 38 ± 3.6 [†]
ApoC3 (g/l)	0.13 ± 0.03	0.12 ± 0.03	0.18 ± 0.06 * [#]	0.16 ± 0.04
C-reactive protein (nmol/l)	14 ± 12	23 ± 36	36 ± 43	33 ± 35
Smoker (n)	0	0	0	2

T2D = Type 2 diabetes; T2D+DL = Type 2 diabetes and dyslipidemia; DL = Dyslipidemia, no diabetes.
Values are average ± SD. **p*<0.05 vs. CTRL; [#]*p*<0.05 vs. T2D; [†]*p*<0.05 vs. T2D+DL