Articles in PresS. Am J Physiol Endocrinol Metab (October 20, 2015). doi:10.1152/ajpendo.00338.2015

Diabetes prevalence in NZO females depends on estrogen action on liver fat content

Running head: Estrogen modulates T2DM prevalence by influencing liver fat

Marko Lubura* [a,b], Deike Hesse* [a,b], Maria Kraemer [a,b], Nicole Hallahan [a,b], Michael Schupp [c], Christian von Löffelholz [b,d,e], Jennifer Kriebel [b,f], Natalia Rudovich [b,d], Andreas Pfeiffer [b,d], Clara John [g], Ludger Scheja [g], Joerg Heeren [g], Chryssi Koliaki [b,h,i], Michael Roden [b,h,i] and Annette Schürmann [a,b]

*contributed equally

[a] Department of Experimental Diabetology, German Institute of Human Nutrition (DIfE), Arthur-Scheunert Allee 114-116, D-14558 Nuthetal, Germany;

[b] German Center of Diabetes Research, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany;

[c] Institute of Pharmacology, Center for Cardiovascular Research, Charité University Medicine, D-10115 Berlin;

[d] Department of Clinical Nutrition, German Institute of Human Nutrition (DIfE), Arthur-Scheunert Allee 114-116, D-14558 Nuthetal, Germany;

[e] Integrated Research and Treatment Center, Center for Sepsis Control and Care (CSCC), Friedrich Schiller University, Jena and Department of Anaesthesiology and Intensive Care, Jena University Hospital, Jena, Germany;

[f] Research Unit of Molecular Epidemiology, Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, German Center for Diabetes Research (DZD) and Institute of Epidemiology II, Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, Neuherberg, Germany

[g] Department of Biochemistry and Molecular Cell Biology, University Medical Center Hamburg-Eppendorf, Martinistraße 52, D-20246 Hamburg, Germany;

[h] Institute for Clinical Diabetology, German Diabetes Center, Leibniz Institute for Diabetes Research, Heinrich Heine University, Düsseldorf, Germany;

[i] Department of Endocrinology and Diabetology, Medical Faculty, Heinrich-Heine University Düsseldorf, Düsseldorf, Germany

*Correspondence to:

Dr. A. Schürmann
Department of Experimental Diabetology
German Institute of Human Nutrition
Arthur-Scheunert-Allee 114-116
D-14558 Nuthetal
Germany
schuermann@dife.de
Tel. (49) 33200-882368
Fax (49) 33200-882334

Abstract

In humans and rodents risk of metabolic syndrome is sexually dimorphic, with an increased incidence in males. Additionally, the protective role of female gonadal hormones is ostensible as prevalence of type 2 diabetes mellitus (T2DM) increases after menopause. Here, we investigated the influence of estrogen (E2) on the onset of T2DM in female New Zealand Obese (NZO) mice. Diabetes prevalence (defined as blood glucose levels >16.6 mmol/l) of NZO females on high-fat diet (60kcal% fat) at week 22 was 43%. This was markedly dependent on liver fat content in week 10, as detected by computed tomography. Only mice with a liver fat content >9% in week 10 plus glucose levels >10 mmol/l in week 9 developed hyperglycaemia by week 22. In addition, at 11 weeks diacylglycerols were elevated in livers of diabetes-prone mice compared to controls. Hepatic expression profiles obtained from diabetes-prone and -resistant mice at 11 weeks revealed increased abundance of two transcripts in diabetes-prone mice: *Mogat1* which catalyzes the synthesis of diacylglycerols from monoacylglycerol and fatty acyl-CoA and the fatty acid transporter *Cd36*. E2-treatment of diabetes-prone mice for 10 weeks prevented any further increase in liver fat content, reduced diacylglycerols and the abundance of *Mogat1* and *Cd36* leading to a reduction of diabetes prevalence and an improved glucose tolerance compared to untreated mice. Our data indicates that early elevation of hepatic *Cd36* and *Mogat1* associates with increased production and accumulation of triglycerides and diacylglycerols, presumably resulting in reduced hepatic insulin sensitivity and leading to later onset of T2DM.

Keywords

type 2 diabetes, hepatic steatosis, diacylglycerol, estrogen, NZO mice

Key messages

- Early liver fat combined with early blood glucose can be used as a predictor for later onset of T2DM in NZO mice.
- Estrogen supplementation averts fat accumulation in the liver under HFD and prevents from T2DM.
- Diabetes-prone animals have increased abundance of transcripts involved in hepatic fatty acid metabolism (MOGAT1 and CD36) prior to the onset of T2DM.

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by an impaired glucose homeostasis. The prevalence of this disease has been increasing rapidly in recent decades and with 382 million people affected worldwide it is already considered pandemic (21). Several studies indicated that men have slightly higher prevalence for T2DM than women. However, if premenopausal women are compared with age-matched men the differences are striking (6, 50). In rodents, a sexual dimorphism in T2DM development can also be observed (37, 39). The New Zealand Obese (NZO) mouse, a model of polygenic obesity and T2DM (25), shows that females are protected from T2DM unless fed with an extremely high-fat diet (40, 52). Moreover, ovariectomy of NZO females increased their diabetes prevalence to the level of male mice (52). Collectively, these data indicate the importance of gonadal hormones as a protective factor against the development of T2DM in females, however, the exact mechanisms are yet to be elucidated.

Obesity is a major risk factor for the development of the metabolic syndrome and related diseases such as T2DM, fatty liver and cardiovascular complications (14). However, increased fat accumulation in the visceral compared to the subcutaneous compartment correlates better with overt T2DM than the total amount of fat (33). Fat distribution is also sexually dimorphic, with an increased accumulation of intra-abdominal fat in men compared to women (13). When the deposition of excess fat in visceral fat depots exceeds its capacity and insulin resistance develops, lipids are stored in other organs and tissues such as liver and muscle (35, 51) which further deteriorates the insulin sensitivity of these organs (41). However, it is still not clear how early the differences in fat distribution occur and if they can be used as a predictor for later onset of T2DM.

98 The aim of this study was to compare diabetic and non-diabetic NZO females in respect to
99 their fat distribution, in order to establish criteria for an early prediction of the onset of T2DM.
100 Additionally, the goal was to clarify the impact of estrogen on the development of T2DM. We
101 show that the early liver fat content and the expression of *Mogat1* and *Cd36* play an
102 important role for the later onset of the disease. Furthermore, we demonstrate that E2
103 reduces hepatic fat accumulation and thereby contributes to improved insulin sensitivity.

Material and Methods

Animals. Female NZO/HlBomDife mice (R. Kluge, German Institute of Human Nutrition, Nuthetal, Germany) from our own colony were housed in Sealsafe™-VC Type 1284L cages (Tecniplast, Buguggiate, Italy) at a temperature of 20 ± 2 °C with a 12-h light-dark cycle (lights on at 06:00 h) and had free access to water and diet. All experiments were approved by the ethics committee of the State Agency of Environment, Health and Consumer Protection (State of Brandenburg, Germany).

Diets. After weaning animals were kept on standard diet (SD; V153x R/M-H, Ssniff, Soest, Germany) until the age of 5 weeks when some of the mice were switched to high-fat diet (HFD; 60 kcal% fat, D12492, Research Diets, New Brunswick, USA) **(Figure 1A)**. To generate plasma and tissue samples mice were sacrificed either at 11 or at 22 weeks of age. From week 11 estradiol-treated groups received 17β-estradiol (E2) orally over 10 weeks (800 µg/kg HFD, Sigma Aldrich, Munich, Germany) **(Figure 1B)**.

Computed tomography. Body fat distribution and liver fat content were measured by CT (Hitachi-Aloka LCT-200, Tokyo, Japan) as described earlier (31). The correlation between liver fat content, body fat distribution and onset of T2DM was assessed by CT scans at weeks 10, 16 and 22 **(Figure 1A)**. Influence of E2 treatment on accumulation of liver fat was examined by CT scans of the liver at weeks 10 and 20 **(Figure 1B)**. During measurements animals were anesthetized by isoflurane.

Transcriptome analysis. Total RNA was isolated from snap-frozen livers of 11-weeks old mice by RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). Before labeling, the integrity of the samples was checked using an Agilent 2100 Bioanalyzer. Microarray analysis of mRNA was performed using SurePrint G3 Mouse GE 8x60K Microarray gene chips (Agilent

Technologies, Santa Clara, CA, USA). The human study was conducted in accordance with the Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. 16 subjects from the cross-sectional INSIGHT (German Clinical Trials-Register: DRKS00005450) study who gave written informed consent were included. Subject characteristics are given in table 3. Blood samples were obtained after overnight fasting and clinical chemistry was assessed using standard methods in certified Clinical Chemistry laboratories (9). Liver specimens were harvested during hepatic surgery. Samples were flash-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. A part of the liver sample was used for histopathological analysis after fixing in 4% formalin (Histofix, Roth, Germany), embedding in paraffin, and staining with hematoxylin & eosin. NAFLD was diagnosed according to standard criteria (24). Exclusively healthy liver tissue was used for analyses, as a blinded clinical expert pathologist considered all harvested liver samples to be histologically normal with respect to pathologies except for NAFLD.

Real time PCR. Real time PCRs (RT-qPCR) were performed with TaqMan® assays, as described earlier (26). The expression level of target genes was normalized to the housekeeping reference gene TATA-Box Binding Protein (*Tbp*) by the ΔΔCt method (30). The following TaqMan® gene expressions assays were used *Cd36* (Mm01135198_m1), *Fasn* (Mm00662319_m1), *G6pc* (Mm00491176_m1), *Mogat1* (Mm00503358_m1), *Pepck* (Mm01247058_m1), *Pparg* (Mm00440945_m1) and *Scd1* (Mm01197142_m1).

Western blotting. MOGAT1 and CD36 were analyzed in liver (50 mg) homogenates by Western blotting that was performed as described earlier (4) with an anti-MOGAT1 antibody (Novus Biologicals, Littleton, USA) in a dilution of 1:1000 and an anti-CD36 antibody (R&D Systems, Minneapolis, USA; 1:1000) in combination with horseradish peroxidase-labeled secondary antibodies. PKC-ε activity was determined as PKC-ε association with plasma membranes as described earlier (BD Transduction Laboratories, Heidelberg, Germany; 1:2000; (22)).

Quantification of diacylglycerols and ceramides. Liver samples were prepared as described earlier (34). The procedure included lipid extraction with MeOH/$CHCl_3$ (2:1, v/v), homogenization and incubation for 12 h at 48°C. After centrifugation (10 min, 5°C, 3.500 x g) supernatant was separated and vacuum dried. Subsequently, the pellet was resolved in eluent for liquid chromatography and the mixture was injected into the LC column, Kinetex XBC18 (Phenomenex, Aschaffenburg, Germany). Separation of compounds was performed by UPLC, Ultimate 3000 System (Dionex, Idstein, Germany). Upon separation single components were detected by mass spectrometer ESI-qToF, maXis® 3G (Bruker, Bremen, Germany).

Glucose tolerance. Oral glucose tolerance tests were performed after overnight fasting as previously described (43). Insulin measurements were performed by ELISA (DRG Diagnostics, Marburg, Germany) according to manufacturer's instruction.

Statistical analysis. Data, if not indicated otherwise, are presented as means ± standard error of the mean (SEM). Statistical analysis and graphical presentation of results were performed by GraphPad Prism version 6.05 for Windows (GraphPad Software, San Diego, USA).

Results

Early liver fat content as a predictor for the development of hyperglycemia

NZO females on HFD (n = 42) had significantly higher body weight (week 22: 76.6 ± 1.3 g vs. 44.6 ± 2.1 g, $p < 0.001$) and random blood glucose values (14.8 ± 0.9 mmol/l vs. 7.2 ± 0.4 mmol/l, $p < 0.001$) than animals on SD (n = 9). Diabetes prevalence (defined as blood glucose levels > 16.6 mmol/l for at least three weeks) after 22 weeks reached 43 % in the HFD-fed group (blood glucose concentration: 20.3 ± 0.7 mmol/l) while none of the mice on SD became diabetic during this period (10.8 ± 0.6 mmol/l, $p < 0.001$) (**Figure 2**). The body weight of diabetic and non-diabetic females within the HFD group did not show significant differences (78.0 ± 2.1 g vs. 75.6 ± 1.6 g, n.s.).

In order to test if differences in fat distribution were responsible for diabetes development in NZO females, fat distribution was quantified by CT at the weeks 10, 16 and 22 (**Figure 1A**). No differences in amounts of total and visceral fat in the abdominal region were detected between diabetic and non-diabetic mice within the HFD group **(Figure 3A** and **3B)**. However, quantification of ectopic fat accumulation in the liver showed more intrahepatic fat in diabetic than in non-diabetic mice, at all three time points **(Figure 3C)**. Earlier CT measurements at week 5 indicated no initial differences in mice that were later defined as diabetes-prone or resistant (DR: 3.37 ± 0.49 % vs. DP: 3.74 ± 0.48 %, n = 5, n.s.). A positive correlation was found between early liver fat content at week 10, prior to the onset of T2DM, and later random blood glucose values at week 22 ($r^2 = 0.303$, **Figure 3F**). We determined a threshold for liver fat content of 9 % at week 10 that could predict later onset of diabetes with 70 % probability. Combining the increased liver fat content at week 10 with blood glucose values at week 9 (> 10 mmol/l), which itself predicts later hyperglycemia with 63 % probability, resulted in an even more precise prediction quotient of 79 %. Early total (**Figure 3D**) and visceral fat (**Figure 3E**) mass, however, was not related to later hyperglycemia.

Livers of diabetes-prone mice exhibited higher diacylglycerol levels and elevated expression of lipogenic enzymes

Diacylglycerol species were measured in livers of designated diabetes-prone or -resistant mice as determined by our pre-defined criteria at the age of 11 weeks. Diabetes-prone mice exhibited a significantly elevated diacylglycerol concentration compared to the diabetes-resistant group (**Figure 4A**). However, the increase was not specific for individual diacylglycerol species (**Figure 4B**). In contrast, hepatic ceramides were not altered between diabetes-prone and -resistant mice (DR: 365.46 ± 27.15 nmol/g, n = 5; DP: 350.84 ± 23.70 nmol/g, n = 6). In order to clarify whether alterations in the expression of lipogenic enzymes are responsible for elevated hepatic triglycerides and diacylglycerols, the transcriptome of the livers of the same animals that were used for diacylglycerol analysis (diabetes-prone n = 5; diabetes-resistant n = 6) was analyzed by microarray analysis. We identified 28 significantly (Student's t-test: $p < 0.05$) differentially expressed genes exhibiting $|log2(fold\ change)| > 0.7$ (**Table 1**). Among these genes two (*Mogat1* and *Cd36*) could be linked to hepatic triglyceride synthesis according to their known function (16, 19). MOGAT1 catalyzes the synthesis of diacylglycerols (8), as an intermediate product of triglyceride synthesis. CD36, also known as fatty acid translocase, has been suggested to act as a fatty acid transporter in various tissues (20, 56). Their differential expression could be confirmed by RT-qPCR analysis (**Figure 5 A, B,** upper panels). Moreover, Western blot analysis confirmed the higher abundance of MOGAT1 and CD36 proteins in livers DP mice (**Figure 5 A, B,** lower panels). Furthermore, a closer look on the expression of other genes involved in the generation and degradation of lipid stores revealed no further relevant alterations of mRNA levels between diabetes-resistant and diabetes-prone mice (**Table 2**). Besides these alterations in lipogenic transcripts, the expression of *Pck1* and *G6pc* was elevated in livers of diabetes-prone mice (**Figures 6**) pointing towards an elevated hepatic glucose production in response to insulin resistance. In order to test if MOGAT and/or CD36 expression are altered in human subjects with a fatty liver we analyzed microarray data obtained from human liver biopsies of controls and patients from the cross-sectional INSIGHT study suffering from non-

alcoholic fatty liver disease (NAFLD). In contrast to mice not *MOGAT1* but *MOGAT2* revealed a significantly higher expression ($p = 0.05$) in livers of NAFLD patients. The two other genes, MOGAT1 and MOGAT3 showed only a tendency towards elevated expression (**Figure 7**). However, *CD36* expression appeared not to be different between the patients and controls.

Treatment with E2 suppresses the development of T2D and prevents fat accumulation in the liver

In order to test if estrogen exhibits protective potential and prevents T2DM we treated female diabetes-prone mice with E2 from the age of 11 weeks (**Figure 1B**). Ten weeks of dietary supplementation with E2 (800 µg/kg HFD) did not result in any significant differences in body weight compared to control groups (week 22: 81.1 ± 1.9 g vs. 80.5 ± 1.1 g, n.s.). However, E2-treated mice exhibited significantly lower random blood glucose values than control mice (15.0 ± 2.2 mmol/l vs. 27.7 ± 1.1 mmol/l, Student's t-test, $p < 0.01$). As expected, all diabetes-prone control mice developed T2DM, whereas E2 treatment reduced diabetes prevalence from 100 % to 42 % at week 21 (**Figure 8A**). Prior to E2 treatment the liver fat content was similar in both groups at week 10 (E2-treated mice: 11.4 ± 0.3 % vs. control mice: 11.8 ± 0.7 %, n.s). We next tested whether the protective effects of estrogen against T2DM could be mediated by a limited hepatic lipid accumulation. In fact, supplementation of E2 completely prevented the increase in liver fat with 11.4 ± 0.9 % fat in week 20, whereas liver fat content increased to 26.5 ± 0.8 % in the control group (Student's t-test, $p < 0.001$) (**Figure 8B**). In addition, E2-treated mice exhibited significantly lower total diacylglycerol concentrations in the liver (**Figure 8C**), which was caused by a general reduction in diacylglycerol species (**Figure 8D**). To clarify a possible mechanism by which E2 can prevent the impairment of insulin sensitivity in the liver, PKC-ε activity was assessed by isolation of plasma membranes and its detection by Western blotting. Livers from DP-E2 mice showed a reduced plasma membrane localization of PKC-ε in comparison with DP-C livers indicating a

reduced PKC-ε activity (**Figure 8E**). In order to test if E2 treatment influences the expression of *Mogat1* and *Cd36* we analyzed their mRNA in E2- and non-treated NZO females. After E2 treatment we detected a reduced mRNA expression of both genes (**Figures 8F** and **8G**), which could contribute to lower hepatic triglyceride and diacylglycerol levels in these livers. These results were also confirmed on protein level by Western blot analysis (**Figures 8F** and **8G**, lower panels).

E2 treatment improves glucose tolerance and prevents β-cell loss

Oral glucose tolerance tests (oGTT) at week 13 displayed a slightly but significantly increased glucose tolerance of E2-treated mice in comparison to diabetes-prone control animals. This was demonstrated by reduced blood glucose and increased plasma insulin levels at all time-points in the E2-supplemented group (**Figure 9 A, C, E**). At week 18, glucose tolerance had further deteriorated in diabetes-prone control mice; their plasma insulin levels during the test dropped markedly in comparison to the test performed in week 13, indicating β-cell loss. In contrast, oGTT results of diabetes-prone E2 treated mice were unchanged in comparison to week 13 (**Figure 9 B, D, F**). Furthermore, random plasma insulin levels at week 22 were markedly reduced in diabetes-prone control (3.4 ± 0.8 µg/l) compared to diabetes-prone E2-treated mice (23.6 ± 5.5 µg/l, Student's t-test, $p < 0.01$). One other proposed mechanism for the amelioration of glucose homeostasis is the estrogen-induced reduction of the expression of lipogenic genes such as *Scd1*, *Fasn* and *Pparg* (3, 12). The effect of E2 on *Fasn* and *Pparg* expression could not be confirmed in our experiment, whereas *Scd1* was significantly increased in E2-treated animals (**Figure 10**).

Discussion

The present study demonstrates that **(1)** early elevated liver fat content is a valuable prediction marker for T2DM, **(2)** this parameter associated with increased hepatic *Mogat1* and *Cd36* levels, and **(3)** E2 treatment prevented an increase in liver fat and the development of T2DM in NZO mice. The accumulation of ectopic fat in the liver and the development of NAFLD in humans are associated with up to four times increased risk of developing T2DM (5, 28, 29, 38). Several studies in rodents also support these findings (1, 53). The elevated amount of intrahepatic fat in NZO females that later become diabetic was accompanied by increased diacylglycerol concentrations which have been shown to disrupt the insulin signaling pathway (2, 47). As a consequence of insulin resistance in hepatocytes, expression of enzymes involved in gluconeogenesis (glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pepck*)) are not efficiently suppressed by the insulin-activated transcription factor FoxO1 (55). This leads to increased gluconeogenesis and an elevated glucose concentration in plasma (36). Indeed, the expression of both enzymes, *G6pc* and *Pepck* was increased in livers of diabetes-prone mice at week 11 (**Table 1** and **Figure 6**).

Transcriptome analysis of livers at week 11 revealed the increased expression of two genes involved in hepatic lipid metabolism (*Mogat1* and *Cd36*). *Mogat1* encodes for a liver-specific monoacylglycerol O-acyltransferase 1 (MOGAT1), an enzyme which is involved in the alternative triglyceride synthesis pathway where it catalyzes the formation of diacylglycerols from monoacylglycerols (8), which were elevated in diabetic NZO females in our study. *Cd36* encodes for the membrane protein fatty acid translocase (CD36) which is responsible for the uptake of long-chain fatty acids into hepatocytes (57). Two recent studies indicated the importance of *Mogat1* for insulin sensitivity in the liver. They showed that silencing of hepatic *Mogat1* expression by siRNA results in significant improvement in glucose tolerance and hepatic insulin signaling, as well as a reduction of hepatic fat as well as body weight (19, 46).

Furthermore, in our own human samples and data by Hall (17) the expression of *MOGAT2* was associated with non-alcoholic fatty liver disease (NAFLD). Increased expression of *CD36* in humans and rodents is associated with obesity, insulin resistance and T2DM (27, 44). The abundance of CD36 is increased in patients with NAFLD (15) and is postulated as a cause of an increased flux of free fatty acids into hepatocytes (57). However, our own data did not show this trend for *CD36,* which might be caused by the low sample size.

Diacylglycerols are not only intermediates in triglyceride synthesis but also important signaling molecules in the cell (8). In hepatocytes diacylglycerols can bind to cytosolic PKC-ε to form a complex that is translocated to the plasma membrane where it binds to and inhibits the insulin receptor tyrosine kinase. This leads to decreased insulin-stimulated glycogen synthesis and a reduced insulin suppression of hepatic gluconeogenesis (45) and can thereby contribute to the development of T2DM. Increased abundance of *Mogat1* and *Cd36* in livers of diabetes-prone mice might explain the increase in hepatic diacylglycerols. In addition, E2 treatment of diabetes-prone NZO females resulted in a lower expression of *Mogat1* and *Cd36*, lower hepatic diacylglycerols as well as in a reduced PKC-ε activity which can contribute to the amelioration of T2DM prevalence in this group. In contrast to our data Hall and colleagues detected an elevated diacylglycerol concentration in the liver after suppression of *Mogat1* expression by siRNA treatment (18). This discrepancy might be explained by a compensatory decreased diacylglycerol O-acyltransferase (*Dgat*) expression that occurred simultaneously to a decreased *Mogat1* expression in the Hall study.

Interestingly, E2 treatment of females that exhibit elevated blood glucose and liver fat levels at week 9 and 10, respectively, reduced diabetes prevalence by almost 60%, confirming that estrogen mediates beneficial effects on glucose homeostasis. These results are in accordance with previous studies where E2 administration was shown to decrease insulin resistance and diabetes prevalence in rodents (5, 48), whereas ovariectomy increased insulin resistance and the risk of developing T2DM (49, 52, 54). Human data also suggest that hormone replacement therapy in menopausal women is associated with a decreased risk of developing insulin resistance and T2DM (7, 42).

Improved glucose homeostasis detected after E2 treatment might be a consequence of reduced ectopic fat accumulation in the liver (**Figures 8B and 8C**). This phenomenon was previously observed in postmenopausal women receiving hormone-replacement therapy (11, 32) and estrogen-treated mice (5, 23).

As E2 treatment suppressed the expression of *Mogat1* and *Cd36* it is possible that E2 acts via these two targets resulting in a reduced uptake of long-chain fatty acids and decreased hepatic triglyceride synthesis by the alternative monoacylglycerol pathway. The analysis of the promoter region of *Mogat1* and *Cd36* by the transcription factor binding analysis tool Matinspector (http://www.genomatix.de/matinspector.html) revealed the presence of 10 and 2 putative estrogen responsive elements (EREs), respectively (10). This fact indicates that the expression of *Mogat1* and *Cd36* is at least partially controlled by estrogen.

In summary, we conclude that an increase in early liver fat content combined with a slight increase in plasma glucose concentrations can be used as a valuable predictor for the later onset of T2DM in NZO females. Moreover, estrogen treatment reduced the abundance of *Mogat1* and *Cd36*, leading to a triglyceride and diacylglycerol accumulation in the liver and finally to the prevention of T2DM. However, as estrogens cannot be used systematically over a long period of time due to their tumor promoting action on the uterus it will be important to develop novel therapeutic approaches by which estrogen could be target-delivered only into the liver.

Acknowledgments and Funding Information

We thank Andrea Teichmann, Malte Neubauer, Christine Gumz, Reinhart Kluge and Markus Jähnert for their skillful technical assistance. This work was supported by the German Ministry of Education and Research (BMBF, NEUROTARGET: grant 01GI0847 and DZD: grant 01GI0922). J.H. is supported by the EU FP7 project RESOLVE (FP7-HEALTH-2012-305707).

Disclosure

The authors declare that they have no conflict of interest.

Literature

1. **Anstee QM, Goldin RD**. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *Int J Exp Pathol* 87: 1–16, 2006.

2. **Birkenfeld AL, Shulman GI**. Non alcoholic fatty liver disease, hepatic insulin resistance and type 2 diabetes. *Hepatology*, 2013.

3. **Bryzgalova G, Lundholm L, Portwood N, Gustafsson J-A, Khan A, Efendic S, Dahlman-Wright K**. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *Am J Physiol Endocrinol Metab* 295: E904–12, 2008.

4. **Buchmann J, Meyer C, Neschen S, Augustin R, Schmolz K, Kluge R, Al-Hasani H, Jürgens H, Eulenberg K, Wehr R, Dohrmann C, Joost HG, Schürmann A**. Ablation of the cholesterol transporter adenosine triphosphate-binding cassette transporter G1 reduces adipose cell size and protects against diet-induced obesity. *Endocrinology* 148: 1561–1573, 2007.

5. **Camporez JPG, Jornayvaz FR, Lee H-Y, Kanda S, Guigni B a, Kahn M, Samuel VT, Carvalho CRO, Petersen KF, Jurczak MJ, Shulman GI**. Cellular mechanism by which estradiol protects female ovariectomized mice from high-fat diet-induced hepatic and muscle insulin resistance. *Endocrinology* 154: 1021–8, 2013.

6. **Carr MC**. The emergence of the metabolic syndrome with menopause. *J Clin Endocrinol Metab* 88: 2404–11, 2003.

7. **Catalano D, Trovato GM, Spadaro D, Martines GF, Garufi G, Tonzuso a, Grasso D, Sciacchitano SG**. Insulin resistance in postmenopausal women: concurrent effects of hormone replacement therapy and coffee. *Climacteric* 11: 373–82, 2008.

8. **Coleman R, Lee D**. Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res* 43: 134–176, 2004.

9. **Döcke S, Lock JF, Birkenfeld AL, Hoppe S, Lieske S, Rieger A, Raschzok N, Sauer IM, Florian S, Osterhoff MA, Heller R, Herrmann K, Lindenmüller S, Horn P, Bauer M, Weickert MO, Neuhaus P, Stockmann M, Möhlig M, Pfeiffer AFH, von Loeffelholz C**. Elevated hepatic chemerin mRNA expression in human non-alcoholic fatty liver disease. *Eur J Endocrinol* 169: 547–57, 2013.

10. **Driscoll MD, Sathya G, Muyan M, Klinge CM, Hilf R, Bambara RA**. Sequence Requirements for Estrogen Receptor Binding to Estrogen Response Elements. *J Biol Chem* 273: 29321–29330, 1998.

11. **Florentino GS de A, Cotrim HP, Vilar CP, Florentino AV de A, Guimarães GMA, Barreto VST**. Nonalcoholic fatty liver disease in menopausal women. *Arq Gastroenterol* 50: 180–5, 2013.

12. **Gao H, Bryzgalova G, Hedman E, Khan A, Efendic S, Gustafsson J-åke, Dahlman-wright K**. Long-Term Administration of Estradiol Decreases Expression of Hepatic Lipogenic Genes and Improves Insulin Sensitivity in ob / ob Mice : A Possible Mechanism Is through Direct Regulation of Signal Transducer and Activator of Transcription 3. *Mol Endocrinol* 20: 1287–1299, 2006.

13. **Geer EB, Shen W**. Gender differences in insulin resistance, body composition, and energy balance. *Gend Med* 6: 60–75, 2009.

14. **Gesta S, Tseng Y-H, Kahn CR**. Developmental origin of fat: tracking obesity to its source. *Cell* 131: 242–56, 2007.

15. **Greco D, Kotronen A, Westerbacka J, Puig O, Arkkila P, Kiviluoto T, Laitinen S, Kolak M, Fisher RM, Hamsten A, Auvinen P, Yki-Järvinen H**. Gene expression in human NAFLD. *Am J Physiol Gastrointest Liver Physiol* 294: G1281–7, 2008.

16. **Hajri T, Han X, Bonen A, Abumrad NA**. Defective fatty acid uptake modulates insulin responsiveness and metabolic responses to diet in CD36-null mice. *J Clin* 109: 1381–1389, 2002.

17. **Hall a. M, Kou K, Chen Z, Pietka T a., Kumar M, Korenblat KM, Lee K, Ahn K, Fabbrini E, Klein S, Goodwin B, Finck BN**. Evidence for regulated monoacylglycerol acyltransferase expression and activity in human liver. *J Lipid Res* 53: 990–999, 2012.

18. **Hall AM, Soufi N, Chambers KT, Chen Z, Schweitzer GG, McCommis KS, Erion DM, Graham MJ, Su X, Finck BN**. Abrogating Monoacylglycerol Acyltransferase Activity in Liver Improves Glucose Tolerance and Hepatic Insulin Signaling in Obese Mice. *Diabetes* : 1–35, 2014.

19. **Hayashi Y, Suemitsu E, Kajimoto K, Sato Y, Akhter A, Sakurai Y, Hatakeyama H, Hyodo M, Kaji N, Baba Y, Harashima H**. Hepatic Monoacylglycerol O-acyltransferase 1 as a Promising Therapeutic Target for Steatosis, Obesity, and Type 2 Diabetes. *Mol Ther Nucleic Acids* 3: e154, 2014.

20. **Ibrahimi A, Abumrad N a**. Role of CD36 in membrane transport of long-chain fatty acids. *Curr Opin Clin Nutr Metab Care* 5: 139–145, 2002.

21. **International Diabetes Federation**. *IDF Diabetes Atlas, 6th Edition*. 2013.

22. **Jelenik T, Sequaris G, Kaul K, Ouwens DM, Phielix E, Kotzka J, Knebel B, Weiss J, Reinbeck AL, Janke L, Nowotny P, Partke HJ, Zhang D, Shulman GI, Szendroedi J, Roden M**. Tissue-specific differences in the development of insulin resistance in a mouse model for type 1 diabetes. *Diabetes* 63: 3856–3867, 2014.

23. **Kamada Y, Kiso S, Yoshida Y, Chatani N, Kizu T, Hamano M, Tsubakio M, Takemura T, Ezaki H, Hayashi N, Takehara T**. Estrogen deficiency worsens steatohepatitis in mice fed high-fat and high-cholesterol diet. *Am J Physiol Gastrointest Liver Physiol* 301: G1031–43, 2011.

24. **Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ**. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 41: 1313–1321, 2005.

25. **Kluge R, Scherneck S, Schürmann A, Joost H-G**. Pathophysiology and genetics of obesity and diabetes in the New Zealand obese mouse: a model of the human metabolic syndrome. *Methods Mol Biol* 933: 59–73, 2012.

26. **Kluth O, Matzke D, Schulze G, Schwenk RW, Joost H-G, Schurmann a.** Differential transcriptome analysis of diabetes resistant and sensitive mouse islets reveals significant overlap with human diabetes susceptibility genes. *Diabetes* 63: db14–0425–, 2014.

27. **Koonen DPY, Jacobs RL, Febbraio M, Young ME, Soltys CM, Ong H, Vance DE, Dyck JRB**. Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. *Diabetes* 56: 2863–71, 2007.

28. **Korenblat KM, Fabbrini E, Mohammed BS, Klein S**. Liver, Muscle, and Adipose Tissue Insulin Action Is Directly Related to Intrahepatic Triglyceride Content in Obese Subjects. *Gastroenterology* 134: 1369–1375, 2008.

29. **Koska J, Stefan N, Permana PA, Weyer C, Sonoda M, Bogardus C, Smith SR, Joanisse DR, Funahashi T, Krakoff J, Bunt JC**. Increased fat accumulation in liver may link insulin resistance with subcutaneous abdominal adipocyte enlargement, visceral adiposity, and hypoadiponectinemia in obese individuals. *Am J Clin Nutr* 87: 295–302, 2008.

30. **Livak KJ, Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402–8, 2001.

31. **Lubura M, Hesse D, Neumann N, Scherneck S, Wiedmer P, Schürmann A**. Non-Invasive Quantification of White and Brown Adipose Tissues and Liver Fat Content by Computed Tomography in Mice. *PLoS One* 7: e37026, 2012.

32. **McKenzie J, Fisher BM, Jaap AJ, Stanley A, Paterson K, Sattar N**. Effects of HRT on liver enzyme levels in women with type 2 diabetes: a randomized placebo-controlled trial. *Clin Endocrinol (Oxf)* 65: 40–4, 2006.

33. **Meisinger C, Döring A, Thorand B, Heier M, Löwel H**. Body fat distribution and risk of type 2 diabetes in the general population: are there differences between men and women? The MONICA/KORA Augsburg cohort study. *Am J Clin Nutr* 84: 483–9, 2006.

34. **Merrill AH, Sullards MC, Allegood JC, Kelly S, Wang E**. Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods* 36: 207–24, 2005.

35. **Nagle C, Klett EL, Coleman R a**. Hepatic triacylglycerol accumulation and insulin resistance. *J Lipid Res* 50 Suppl: S74–9, 2009.

36. **Nakae J, Oki M, Cao Y**. The FoxO transcription factors and metabolic regulation. *FEBS Lett* 582: 54–67, 2008.

37. **Ohta T, Katsuda Y, Miyajima K, Sasase T, Kimura S, Tong B, Yamada T**. Gender differences in metabolic disorders and related diseases in Spontaneously Diabetic Torii-Lepr(fa) rats. *J Diabetes Res* 2014: 841957, 2014.

38. **Ortiz-Lopez C, Lomonaco R, Orsak B, Finch J, Chang Z, Kochunov VG, Hardies J, Cusi K**. Prevalence of prediabetes and diabetes and metabolic profile of patients with nonalcoholic fatty liver disease (NAFLD). *Diabetes Care* 35: 873–8, 2012.

39. **Ortlepp JR, Kluge R, Giesen K, Plum L, Radke P, Hanrath P, Joost HG**. A metabolic syndrome of hypertension, hyperinsulinaemia and hypercholesterolaemia in the New Zealand obese mouse. *Eur J Clin Invest* 30: 195–202, 2000.

40. **Plum L, Kluge R, Giesen K, Altmüller J, Ortlepp JR, Joost HG**. Type 2 diabetes-like hyperglycemia in a backcross model of NZO and SJL mice: characterization of a susceptibility locus on chromosome 4 and its relation with obesity. *Diabetes* 49: 1590–6, 2000.

41. **Roden M**. Mechanisms of Disease: hepatic steatosis in type 2 diabetes--pathogenesis and clinical relevance. *Nat Clin Pract Endocrinol Metab* 2: 335–48, 2006.

42. **Salpeter SR, Walsh JME, Ormiston TM, Greyber E, Buckley NS, Salpeter EE**. Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. *Diabetes Obes Metab* 8: 538–54, 2006.

43. **Schwenk RW, Baumeier C, Finan B, Kluth O, Brauer C, Joost H, DiMarchi RD, Tschöp MH, Schürmann A**. GLP-1-oestrogen attenuates hyperphagia and protects from beta cell failure in diabetes-prone New Zealand obese (NZO) mice. *Diabetologia* 58: 604–14, 2015.

44. **Sheedfar F, Sung MM, Aparicio-Vergara M, Kloosterhuis NJ, Miquilena-Colina ME, Vargas-Castrillón J, Febbraio M, Jacobs RL, de Bruin A, Vinciguerra M, García-Monzón C, Hofker MH, Dyck JR, Koonen DPY**. Increased hepatic CD36 expression with age is associated with enhanced susceptibility to nonalcoholic fatty liver disease. *Aging (Albany NY)* 6: 281–95, 2014.

45. **Shulman GI**. Ectopic Fat in Insulin Resistance, Dyslipidemia, and Cardiometabolic Disease. *N Engl J Med* 371: 1131–41, 2014.

46. **Soufi N, Hall AM, Chen Z, Yoshino J, Collier SL, Mathews JC, Brunt EM, Albert CJ, Graham MJ, Ford D a, Finck BN**. Inhibiting monoacylglycerol acyltransferase 1 ameliorates hepatic metabolic abnormalities but not inflammation and injury in mice. *J Biol Chem* 289: 30177–88, 2014.

47. **Stefan N, Häring H-U**. The metabolically benign and malignant fatty liver. *Diabetes* 60: 2011–7, 2011.

48. **Stubbins RE, Holcomb VB, Hong J, Núñez NP**. Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance. *Eur. J. Nutr,* 2011.

49. **Stubbins RE, Najjar K, Holcomb VB, Hong J, Núñez NP**. Oestrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance. *Diabetes Obes Metab* 14: 58–66, 2012.

50. **Szmuilowicz ED, Stuenkel C a, Seely EW**. Influence of menopause on diabetes and diabetes risk. *Nat Rev Endocrinol* 5: 553–8, 2009.

51. **Tchernof A, Després J-P**. Pathophysiology of human visceral obesity: an update. *Physiol Rev* 93: 359–404, 2013.

52. **Vogel H, Mirhashemi F, Liehl B, Taugner F, Kluth O, Kluge R, Joost H-G, Schürmann A**. Estrogen Deficiency Aggravates Insulin Resistance and Induces β-Cell Loss and Diabetes in Female New Zealand Obese Mice. *Horm. Metab. Res*, 2013.

53. **Williams KH, Shackel NA, Gorrell MD, McLennan S V, Twigg SM**. Diabetes and nonalcoholic Fatty liver disease: a pathogenic duo. *Endocr Rev* 34: 84–129, 2013.

54. **Yonezawa R, Wada T, Matsumoto N, Morita M, Sawakawa K, Ishii Y, Sasahara M, Tsuneki H, Saito S, Sasaoka T**. Central versus peripheral impact of estradiol on the impaired glucose metabolism in ovariectomized mice on a high-fat diet. *Am J Physiol Endocrinol Metab* 303: E445–56, 2012.

55. **Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Xiao X, Franks R, Heidenreich K a., Sajan MP, Farese R V., Stolz DB, Tso P, Koo SH, Montminy M, Unterman TG**. FoxO1 regulates multiple metabolic pathways in the liver effects on gluconeogenic, glycolytic, and lipogenic gene expression. *J Biol Chem* 281: 10105–10117, 2006.

56. **Zhang X, Fitzsimmons RL, Cleland LG, Ey PL, Zannettino ACW, Farmer E-A, Sincock P, Mayrhofer G**. CD36/fatty acid translocase in rats: distribution, isolation from hepatocytes, and comparison with the scavenger receptor SR-B1. *Lab Invest* 83: 317–332, 2003.

57. **Zhou J, Febbraio M, Wada T, Zhai Y, Kuruba R, He J, Lee JH, Khadem S, Ren S, Li S, Silverstein RL, Xie W**. Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPARgamma in promoting steatosis. *Gastroenterology* 134: 556–67, 2008.

Figure legends

Figure 1:

Experimental design**. (A)** Female NZO mice were either fed with a standard diet (SD, n = 9) during the whole experiment or were switched to HFD (n = 42) at week 5. Body fat distribution and liver fat content were measured by computed tomography at the indicated time points (CT1, CT2, CT3). **(B)** Female NZO mice received HFD from week 5. After applying prediction criteria for later onset of T2DM selected diabetes prone animals were divided into control (DP-C, n = 12) and estradiol-treated (DP-E, n = 12, 800 µg/kg HFD) groups. Five diabetes-prone and six diabetes-resistant mice were sacrificed at week 11 and livers were taken for further analysis (microarray, diacylglycerol/ceramide content). oGTT1 and oGTT2 – oral glucose tolerance tests. CT1 and CT2 – Quantification of hepatic fat content by computed tomography. Body weight and blood glucose were measured weekly.

Figure 2:

Female NZO mice exhibit heterogeneous diabetes prevalence. Animals fed with standard diet (SD) (n = 9) did not develop T2DM. However, feeding with high-fat diet (HFD) that contains 60 kcal% fat (n = 42, diet switch at week 5) causes T2DM from 10 weeks of age and results in diabetes prevalence of 43 % at week 22.

Figure 3:

Distribution of abdominal white adipose tissue was measured by computed tomography at week 10, 16 and 22. There were no significant differences in amounts of total **(A)** and visceral **(B)** fat in abdominal region (lumbar vertebrae L4-L5) between diabetic and non-diabetic animals. **(C)** Liver fat content at week 10, 16 and 22 as measured by computed tomography. Early total **(D)** and visceral abdominal fat **(E)** at week 10, as measured by computed tomography, did not correlate with later blood glucose values in non-fasted animals at week 22 and therefore cannot be used as predictors for onset of T2DM. Accumulation of liver fat was increased in diabetic animals at all three time points and correlates with later blood glucose values in non-fasted animals at week 22 **(F)**. Data are presented as mean fat weight per CT-slice (600 µm). Student's t-test: * $p < 0.05$, *** $p < 0.001$. Mice: NZO females on HFD (60 kcal% fat), n = 41. r^2 = Pearson's correlation coefficient.

Figure 4:

(A) LC-MS/MS quantification of diacylglycerols in livers of 11 weeks old NZO female mice indicated increased diacylglycerol levels in diabetes-prone (DP, n = 5) compared to diabetes-resistant (DR, n = 6) NZO females. **(B)** Diacylglycerol profile of livers from 11 weeks old diabetes-prone (DP, n = 5) and diabetes-resistant (DR, n = 6) NZO females revealed a general increase of diacylglycerol species in diabetes-prone group compared to diabetes-resistant animals. Student's t-test: ***** $p < 0.05$.

Figure 5:

Expression of *Mogat1* **(A)** and *Cd36* **(B)** mRNA was increased in livers of diabetes-prone (DP, n = 5) female NZO mice at week 11 compared to diabetes-resistant (DR, n = 6) females. MOGAT1 and CD36 protein abundance indicated the same trend, as identified by Western blot analysis. Student's t-test: * $p < 0.05$, ** $p < 0.01$.

Figure 6:

Expression of genes encoding for gluconeogenic enzymes *Pck1* and *G6pc* was increased in diabetes-prone (DP, n = 5) compared to diabetes-resistant (DR, n = 6) females at week 11. Student's t-test: ***** $p < 0.05$.

Figure 7:

Microarray expression data for *Cd36* and *Mogat* isoformes of liver biopsies from patients with non-alcoholic fatty liver disease (NAFLD, n = 8) and controls (n = 8). Student's t-test.

Figure 8:

(A) At week 22 all diabetes-prone control mice (DP-C, n = 12) suffered from T2DM while only 41.7 % of estrogen-treated diabetes-prone (DP-E, n = 12) animals became diabetic. Both groups received high-fat diet containing 60 kcal% fat from the age of 5 weeks; E2-treated animals received the same diet supplemented with 800 µg estradiol per kg diet from the age of 11 weeks. **(B)** Estrogen treatment averts ectopic accumulation of fat in livers of diabetes-prone NZO females. Liver fat content was

quantified by CT at weeks 10 and 20. **(C)** LC-MS/MS quantification of diacylglycerols in livers of 22 weeks old diabetes-prone mice by LC-MS/MS indicated reduced diacylglycerol levels after estrogen treatment (DP-E, n = 11) compared to control (DP-C, n = 10) NZO females. **(D)** Estrogen supplementation of diabetes-prone (DP-E) mice resulted in a reduction of all hepatic diacylglycerol species compared to controls (DP-C). **(E)** PKC-ε activity was higher in control livers (DP-C) compared to estrogen treated mice. PKC-ε activity was determined by localization of PKC-ε to the plasma membrane in DP-C and DP-E livers (n = 4). Expression of *Mogat1* **(F)** and *Cd36* **(G)** mRNA was lower in livers of estrogen-treated (DP-E, n = 6) female NZO mice at week 22 compared to controls (DP-C, n = 6). MOGAT1 and CD36 protein abundance indicated the same trend, as determined by Western blot analysis. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 9:

Estrogen treatment improved glucose tolerance in diabetes-prone mice at the age of 13 and 18 weeks. Left panel - oGTT at week 13; right panel – oGTT at week 18; blood glucose **(A, B)** and plasma insulin **(C, D)** concentrations during oGTT; area under the **(E)** glucose and **(F)** insulin curves during oGTT was calculated using the trapezoidal rule. DP-C: diabetes-prone controls (n = 12); DP-E: diabetes-prone estrogen-treated mice (n = 12). Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 10:

RT-qPCR analysis could not confirm earlier findings that estrogen reduces hepatic expression of lipogenic genes *Fasn* **(C)** and *Pparg* **(D)**, while the expression of *Scd1* **(E)** was slightly increased at week 22 upon E2 treatment. DP-C: diabetes-prone control mice, n = 6; DP-E: diabetes-prone E2-treated mice, n = 6; Student's t-test: * $p < 0.05$, **n.s.** – non-significant ($p > 0.05$).

Table 1:

Differentially expressed annotated genes from transcriptome analysis. Microarray analysis was performed in livers of 11 weeks old diabetes-prone and diabetes-resistant NZO females and genes were selected according to the stringent criteria: $p < 0.05$, $|\log2(\text{fold change})| > 0.7$; mean signal intensity > 90 (in at least one group); DR - mean signal intensity in diabetes resistant group (n = 6), DP - mean signal intensity in diabetes prone group (n = 5), p-value - according to the Student's t-test.

Table 2:

Expression of transcripts involved in hepatic lipid metabolism as determined by microarray analysis in livers of 11 weeks old diabetes-prone and diabetes-resistant NZO females. Genes were regarded as significantly altered according to these criteria: $p < 0.05$, $|\log2(\text{fold change})| > 0.7$; mean signal intensity > 90 (in at least one group); DR - mean signal intensity in diabetes resistant group (n = 6), DP - mean signal intensity in diabetes prone group (n = 5), p-value - according to the Student's t-test.

Table 3:

Characteristics of human study subjects. Subjects were paired according to clinical parameters of NAFLD or no NAFLD for microarray data analysis. mean ± SD.

Figure 1

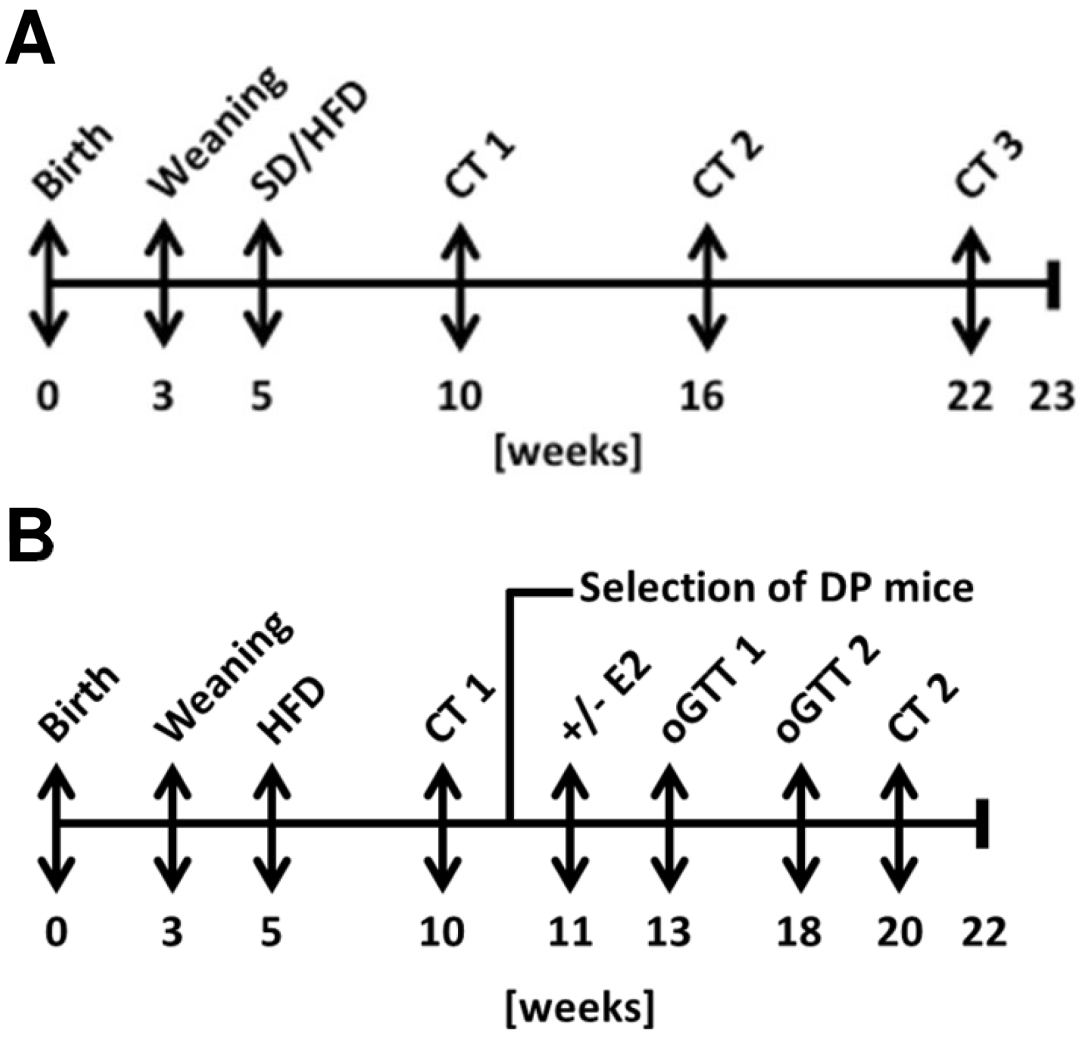
A
Birth
Weaning
SD/HFD
CT 1
CT 2
CT 3
0
3
5
10
16
22
23
[weeks]
B
Selection of DP mice
Birth
Weaning
HFD
CT 1
+/- E2
oGTT 1
oGTT 2
CT 2
0
3
5
10
11
13
18
20
22
[weeks]

Figure 2

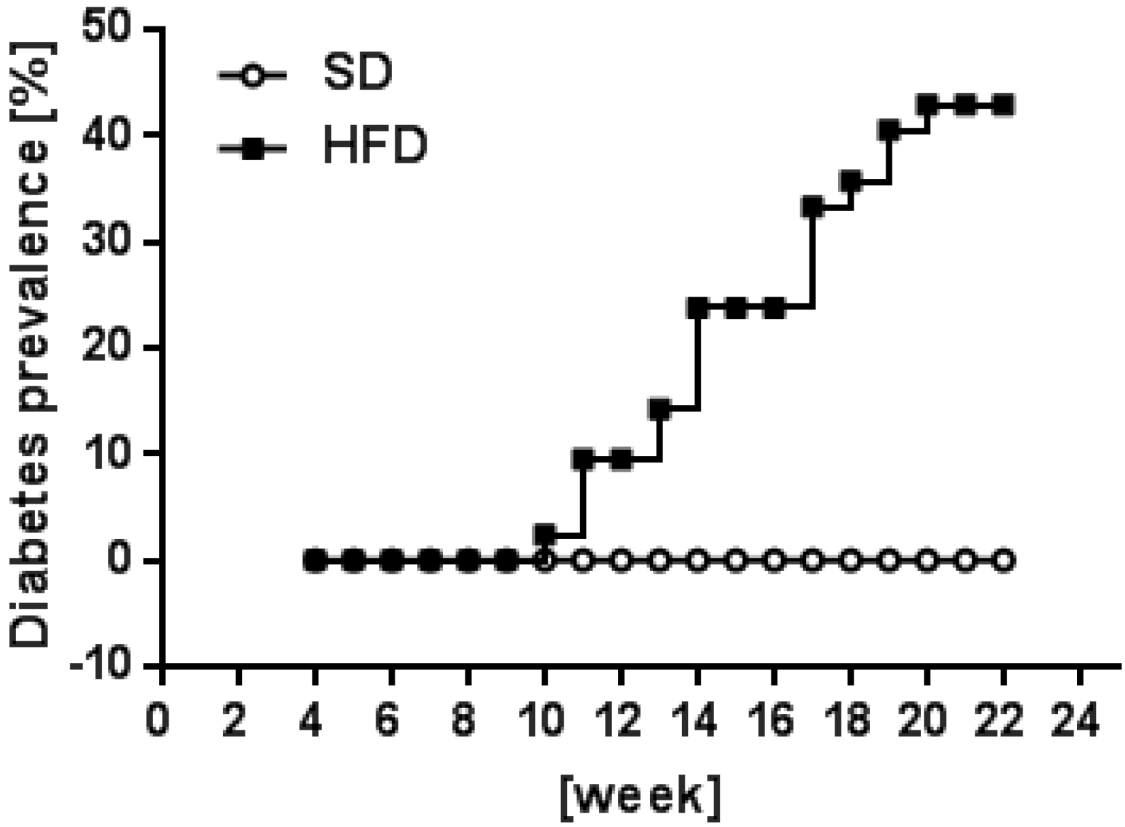
Diabetes prevalence [%]
50
40
30
20
10
0
-10
SD
HFD
0
2
4
6
8
10
12
14
16
18
20
22
24
[week]

Figure 3

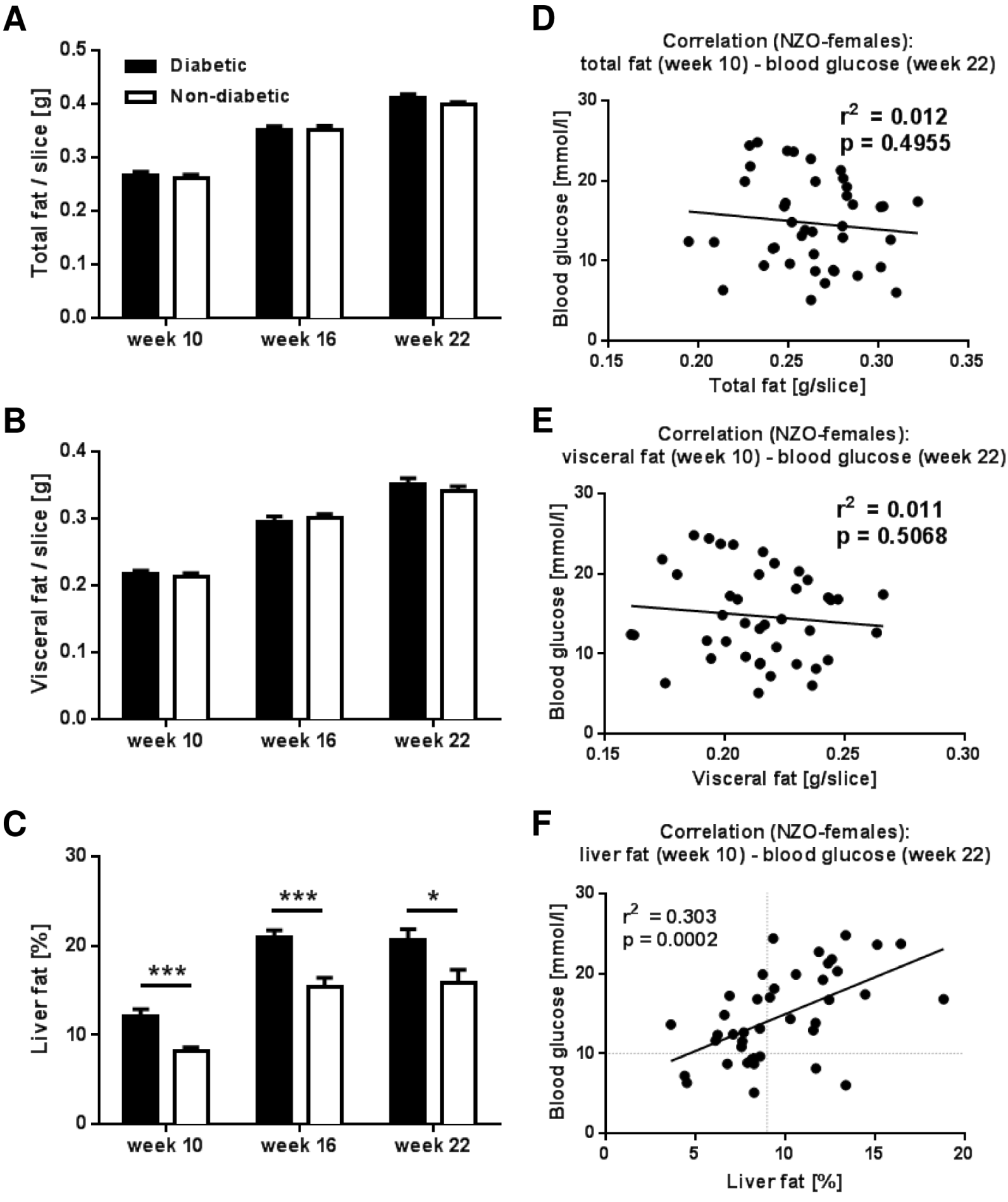
A
Diabetic
Non-diabetic
Total fat / slice [g]
0.5
0.4
0.3
0.2
0.1
0.0
week 10
week 16
week 22
B
Visceral fat / slice [g]
0.4
0.3
0.2
0.1
0.0
week 10
week 16
week 22
C
Liver fat [%]
30
20
10
0
***
***
*
week 10
week 16
week 22
D
Correlation (NZO-females):
total fat (week 10) - blood glucose (week 22)
r² = 0.012
p = 0.4955
Blood glucose [mmol/l]
30
20
10
0
0.15
0.20
0.25
0.30
0.35
Total fat [g/slice]
E
Correlation (NZO-females):
visceral fat (week 10) - blood glucose (week 22)
r² = 0.011
p = 0.5068
Blood glucose [mmol/l]
30
20
10
0
0.15
0.20
0.25
0.30
Visceral fat [g/slice]
F
Correlation (NZO-females):
liver fat (week 10) - blood glucose (week 22)
r² = 0.303
p = 0.0002
Blood glucose [mmol/l]
30
20
10
0
0
5
10
15
20
Liver fat [%]

Figure 4

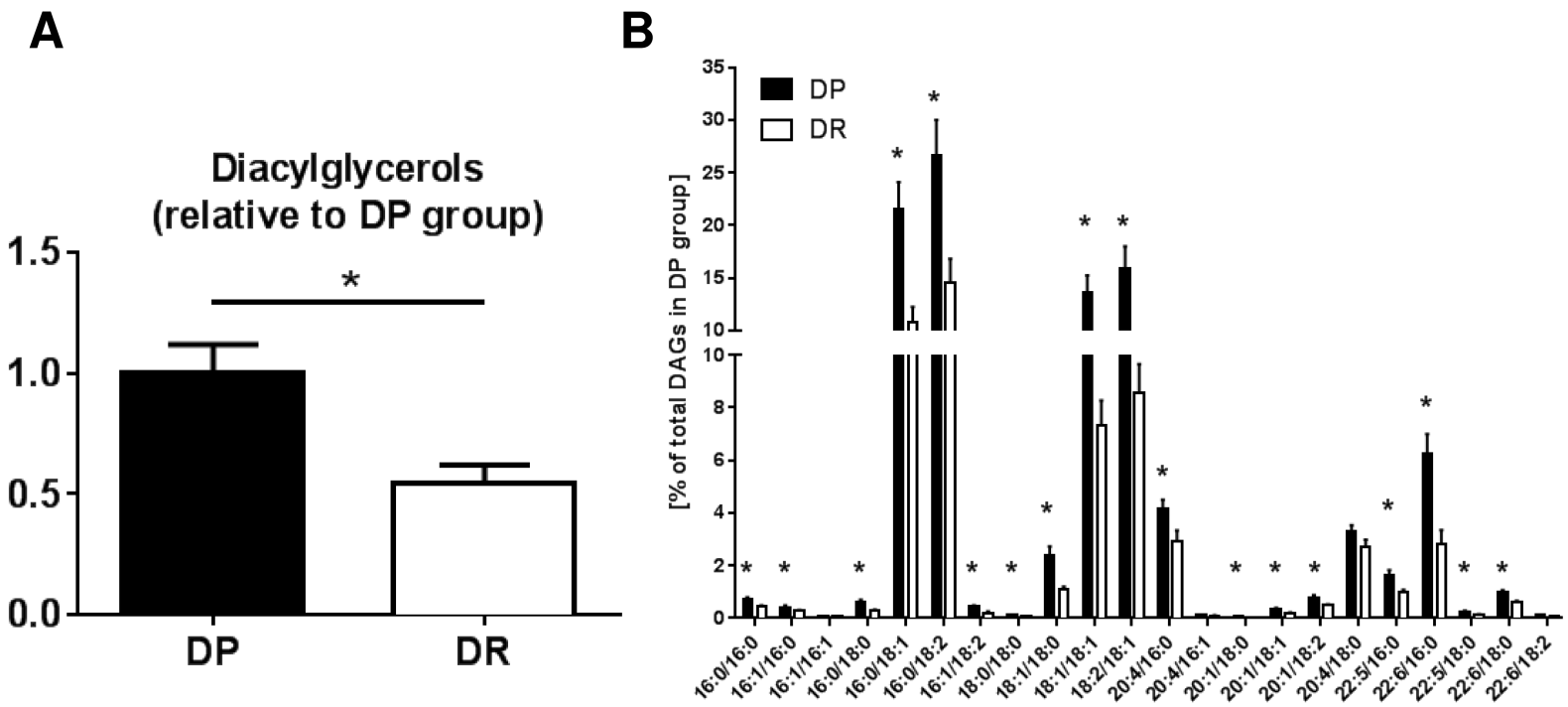
A
Diacylglycerols
(relative to DP group)
1.5
1.0
0.5
0.0
*
DP
DR
B
DP
DR
[% of total DAGs in DP group]
35
30
25
20
15
10
8
6
4
2
0
16:0/16:0
16:1/16:0
16:1/16:1
16:0/18:0
16:0/18:1
16:0/18:2
16:1/18:2
18:0/18:0
18:1/18:0
18:1/18:1
18:2/18:1
20:4/16:0
20:4/16:1
20:1/18:0
20:1/18:1
20:1/18:2
20:4/18:0
22:5/16:0
22:6/16:0
22:5/18:0
22:6/18:0
22:6/18:2

Figure 5

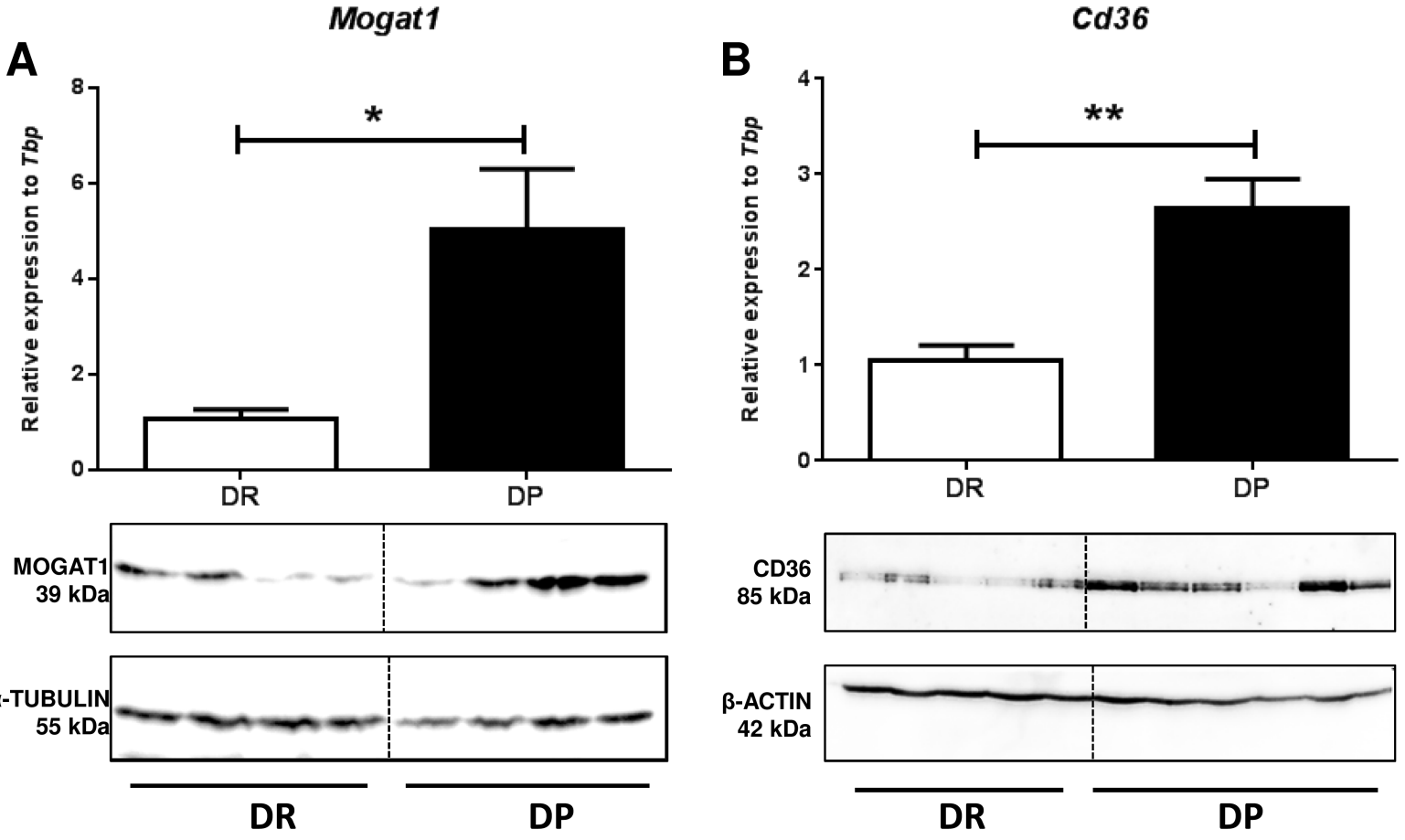
A
Mogat1
Relative expression to Tbp
8
6
4
2
0
*
DR
DP
MOGAT1
39 kDa
α-TUBULIN
55 kDa
DR
DP
B
Cd36
Relative expression to Tbp
4
3
2
1
0
**
DR
DP
CD36
85 kDa
β-ACTIN
42 kDa
DR
DP

Figure 6

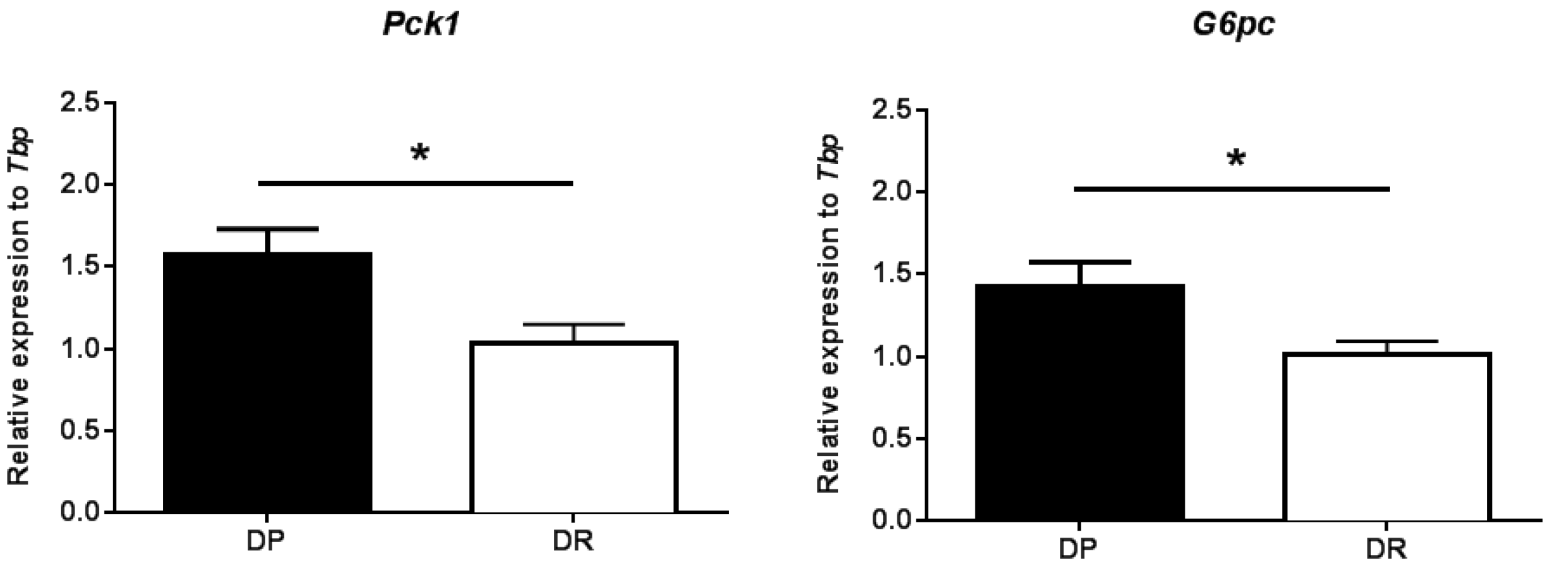
Pck1
Relative expression to Tbp
2.5
2.0
1.5
1.0
0.5
0.0
*
DP
DR
G6pc
Relative expression to Tbp
2.5
2.0
1.5
1.0
0.5
0.0
*
DP
DR

Figure 7

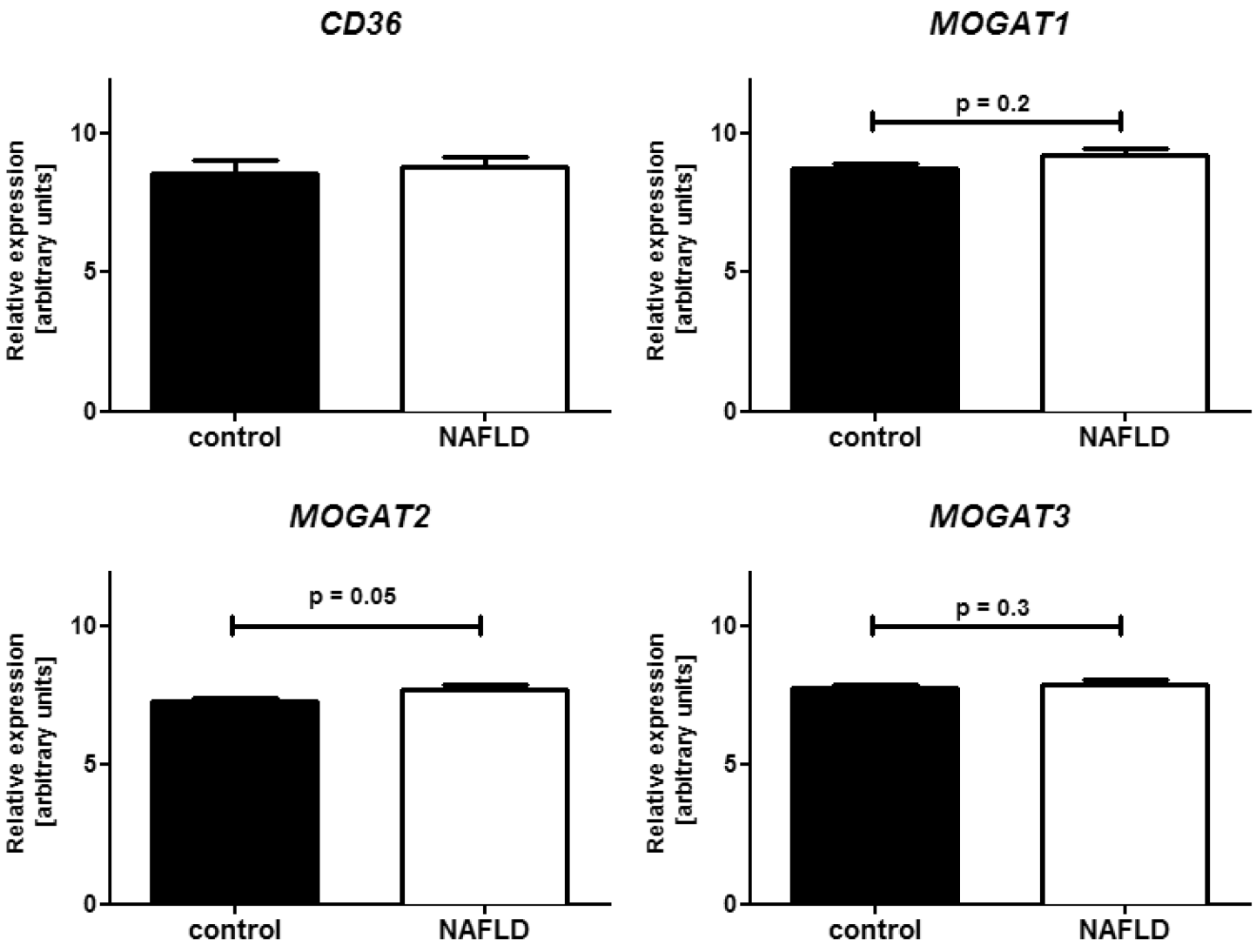
CD36
MOGAT1
MOGAT2
MOGAT3
Relative expression [arbitrary units]
10
5
0
control
NAFLD
p = 0.2
p = 0.05
p = 0.3

Figure 8

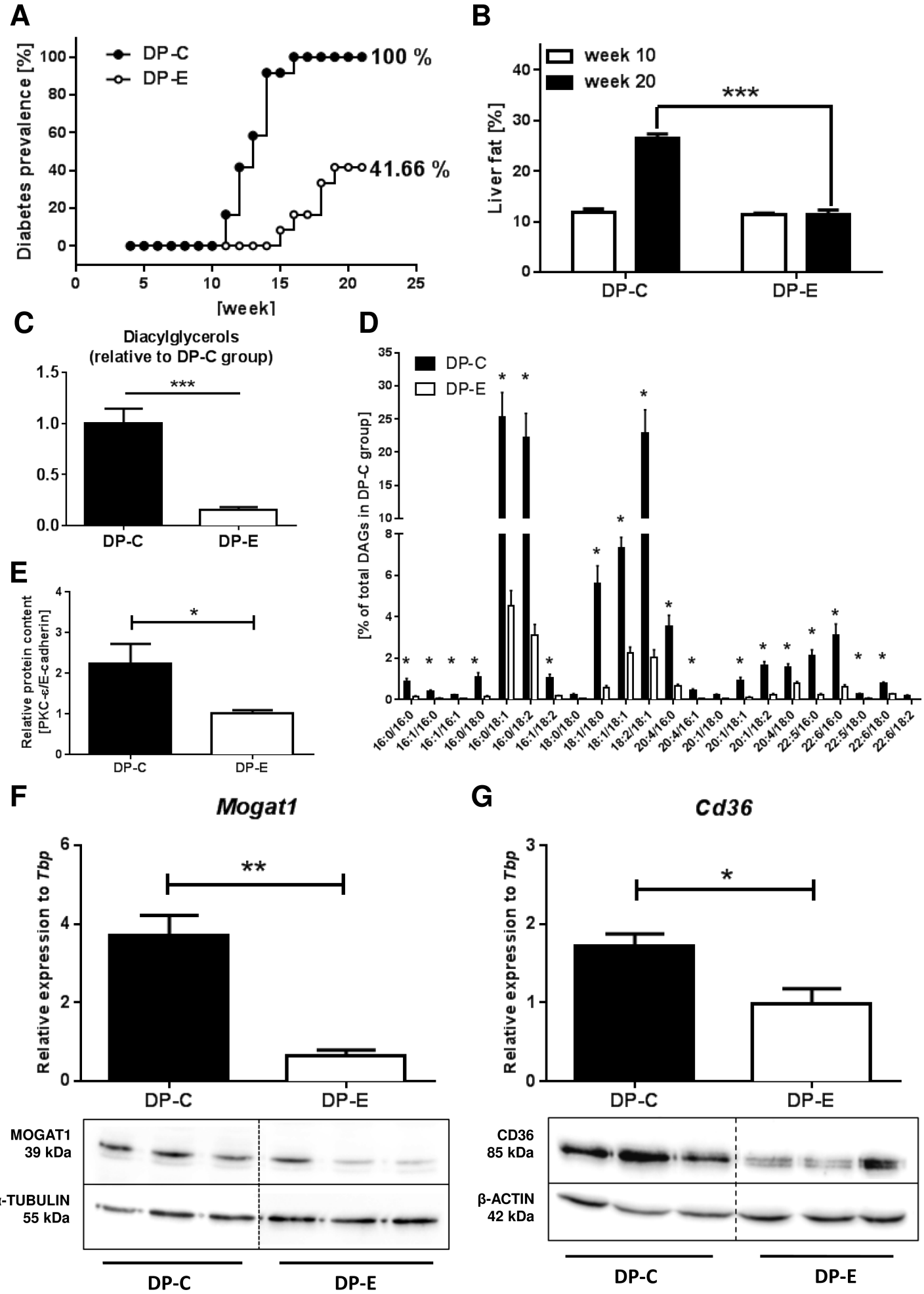
A
Diabetes prevalence [%]
DP-C
DP-E
100 %
41.66 %
[week]
B
week 10
week 20
***
Liver fat [%]
DP-C
DP-E
C
Diacylglycerols
(relative to DP-C group)
***
DP-C
DP-E
D
DP-C
DP-E
[% of total DAGs in DP-C group]
16:0/16:0
16:1/16:0
16:1/16:1
16:0/18:0
16:0/18:1
16:0/18:2
16:1/18:2
18:0/18:0
18:1/18:0
18:1/18:1
18:2/18:1
20:4/16:0
20:4/16:1
20:1/18:0
20:1/18:1
20:1/18:2
20:4/18:0
22:5/16:0
22:6/16:0
22:5/18:0
22:6/18:0
22:6/18:2
E
Relative protein content
[PKC-ε/E-cadherin]
*
DP-C
DP-E
F
Mogat1
Relative expression to Tbp
**
DP-C
DP-E
MOGAT1
39 kDa
α-TUBULIN
55 kDa
DP-C
DP-E
G
Cd36
Relative expression to Tbp
*
DP-C
DP-E
CD36
85 kDa
β-ACTIN
42 kDa
DP-C
DP-E

Figure 9

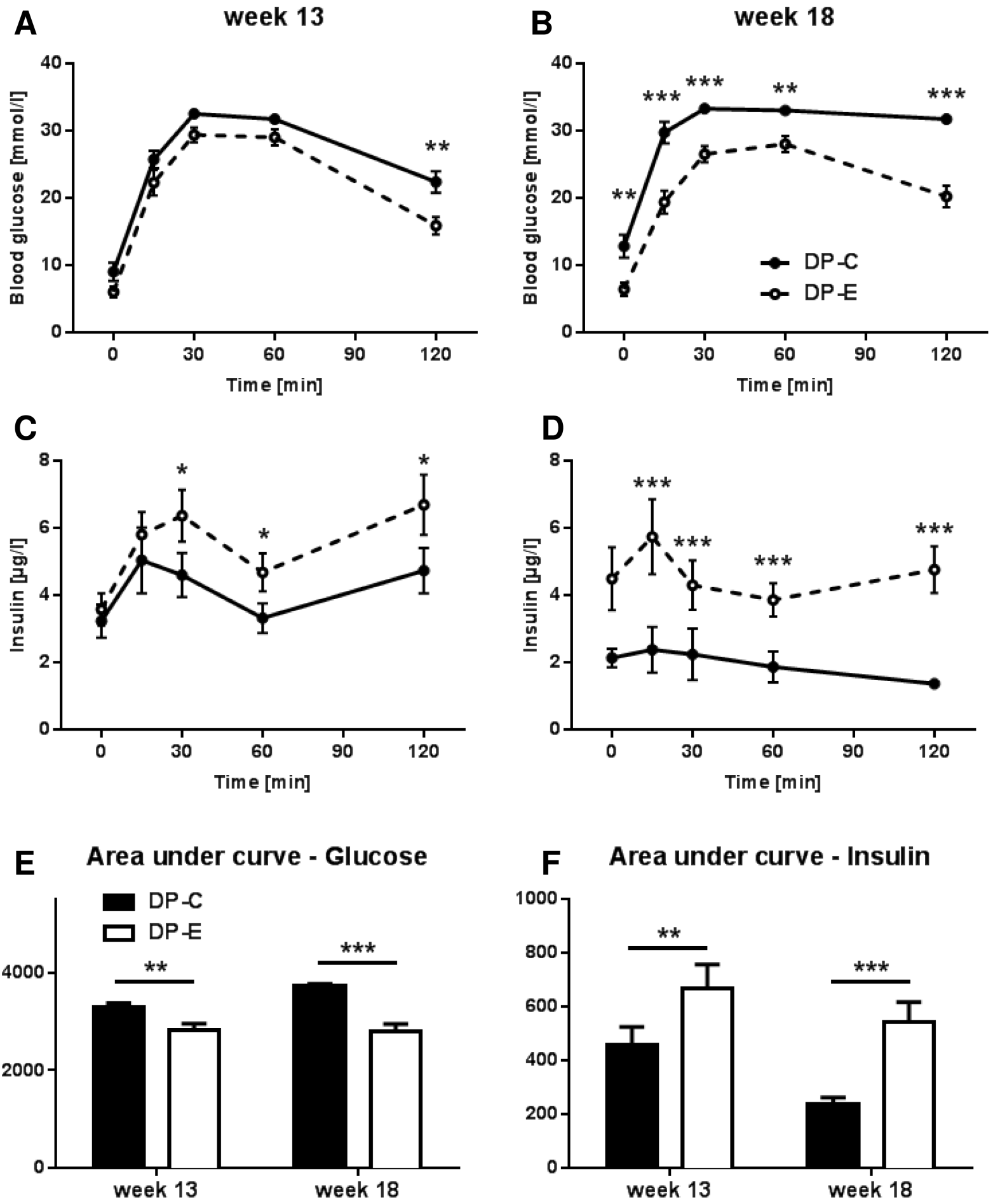
A
week 13
Blood glucose [mmol/l]
Time [min]
B
week 18
DP-C
DP-E
C
Insulin [µg/l]
D
E
Area under curve - Glucose
F
Area under curve - Insulin
week 13
week 18

Figure 10

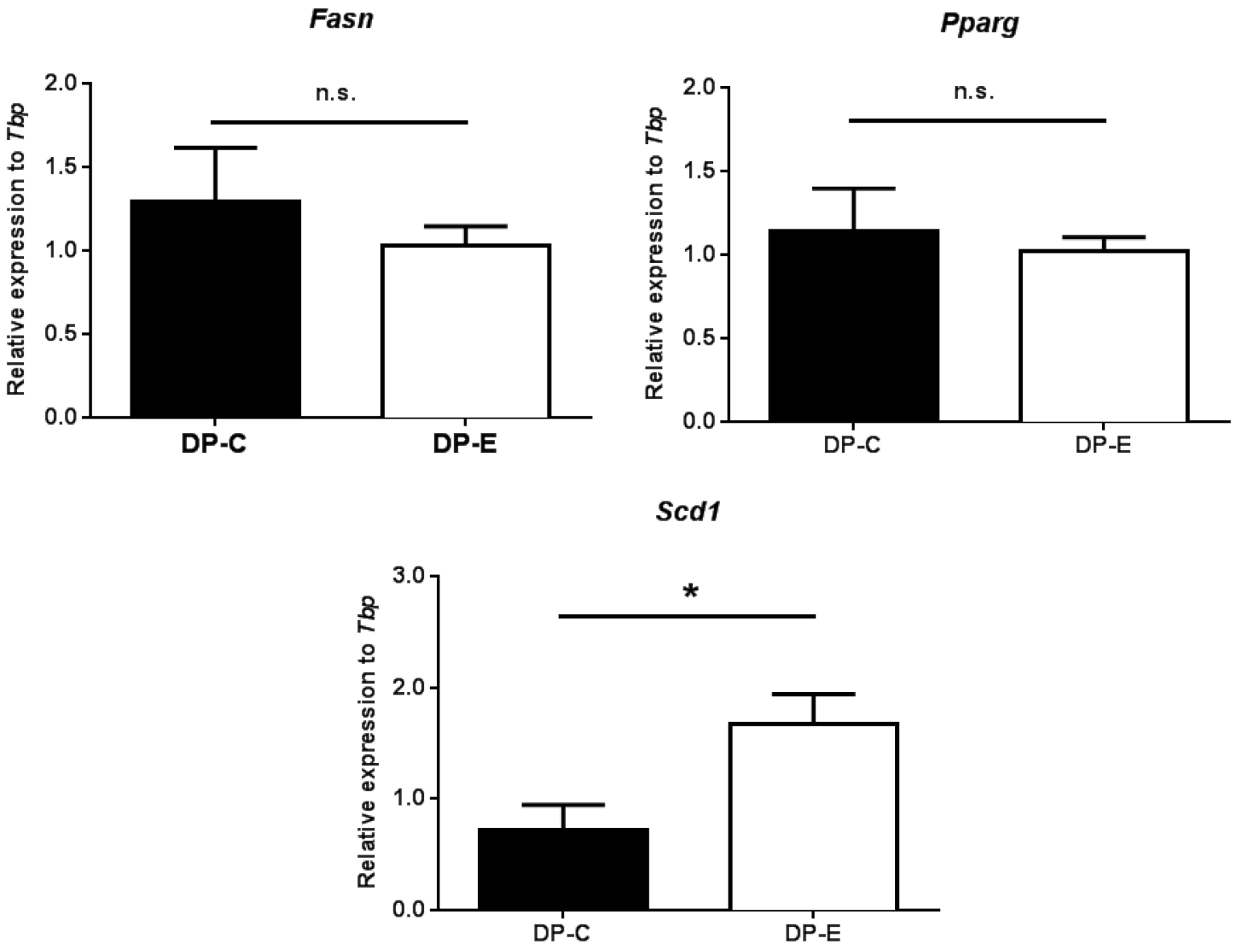
Fasn
n.s.
Relative expression to Tbp
2.0
1.5
1.0
0.5
0.0
DP-C
DP-E
Pparg
n.s.
Relative expression to Tbp
2.0
1.5
1.0
0.5
0.0
DP-C
DP-E
Scd1
*
Relative expression to Tbp
3.0
2.0
1.0
0.0
DP-C
DP-E

Table 1

GeneSymbol	Gene-ID	mean DR	mean DP	log2fold	fold change	pval
Mogat1	NM_026713	52.6	170.13	1.6935	3.2345	0.0497
Ly6d	NM_010742	88.54	271.76	1.6179	3.0695	0.0491
Cml5	NM_023493	69.79	187.82	1.4283	2.6912	0.0319
Cyp4a14	NM_007822	13963.23	35024.81	1.3267	2.5084	0.0213
Shbg	NM_011367	138.15	58.62	-1.2368	-2.3567	0.0154
Cyp3a44	NM_177380	6954.4	3023.32	-1.2018	-2.3003	0.0062
Atp6v0d2	NM_175406	253.49	117.87	-1.1047	-2.1506	0.0025
Dlx6as2	NR_002839	58.6	125.84	1.1026	2.1475	0.0336
Apoa4	NM_007468	5183.6	10956.2	1.0797	2.1136	0.0184
Cyp4a31	NM_001252539	1102.77	2289.74	1.0541	2.0764	0.0133
A4gnt	NM_001077424	65.74	132.14	1.0072	2.0101	0.0359
Scn4b	NM_001013390	50.73	100.5	0.9863	1.981	0.0264
Atp1b2	NM_013415	92.39	46.83	-0.9803	-1.9731	0.0068
Col5a3	NM_016919	103.29	203.35	0.9773	1.9687	0.032
Fmo3	NM_008030	6890.9	3596.3	-0.9382	-1.9161	0.019
Cyp4a32	NM_001100181	1816.31	3478.7	0.9375	1.9153	0.0183
Igfbp1	NM_008341	1540.36	2912.48	0.9190	1.8908	0.0317
Slc22a26	NM_146232	1855.37	1019.67	-0.8636	-1.8196	0.0022
Clstn3	NM_153508	169.73	307.72	0.8584	1.8131	0.0239
Hamp	NM_032541	15203.45	8411.96	-0.8539	-1.8074	0.0201
Fitm1	NM_026808	179.29	316.07	0.8179	1.7629	0.0218
Cyp2g1	NM_013809	1027.7	583.95	-0.8155	-1.7599	0.0021
Cyp4a10	NM_010011	13392.05	22618.64	0.7561	1.689	0.0369
Gpr110	NM_133776	131.09	79.27	-0.7257	-1.6537	0.0394
Trpm1	NM_001039104	246.44	149.11	-0.7249	-1.6528	0.0155
Cmya5	NM_023821	158.88	96.16	-0.7244	-1.6522	0.0289
Cd36	NM_007643	476.48	787.15	0.7242	1.652	0.0101
Aym1	NM_001012726	80.7	131.4	0.7033	1.6283	0.0149
				|log2(fold change)| < 0.7		
Pck1	NM_011044	20341.13	30161.63	**0.5683**	1.4828	0.0041
G6pc	NM_008061	8137	12600.82	**0.6309**	1.5486	0.0352

Table 2

GeneSymbol	Gene-ID	mean DR	mean DP	log2fold	fold change	pval
			TG Synthesis			
Gpam	NM_008149	110.69	133.27	0.26785	1.2040	0.1090
Gpat2	NM_001081089	106.39	93.07	-0.19297	-1.1431	0.1400
Agpat1	NM_001163379	171.76	152.99	-0.16696	-1.1227	0.0371
Agpat2	NM_026212	3652.35	4547.19	0.31615	1.2450	0.0638
Agpat3	NM_053014	10694.07	9935.12	-0.10620	-1.0764	0.1966
Agpat4	NM_026644	128.21	109.79	-0.22374	-1.1678	0.0224
Agpat5	NM_026792	545.48	562.14	0.04339	1.0305	0.3869
Agpat6	NM_018743	6038.75	6277.38	0.05591	1.0395	0.5717
Lclat1	NM_001081071	621.42	661.12	0.08934	1.0639	0.1564
Agpat9	NM_172715	1661.60	1776.97	0.09685	1.0694	0.1581
Lpin1	NM_001130412	229.23	254.89	0.15307	1.1119	0.5038
Lpin2	NM_001164885	331.23	375.52	0.18107	1.1337	0.3089
Mogat1	NM_026713	52.60	170.13	1.69353	3.2345	0.0497
Mogat2	NM_177448	42.47	50.06	0.23719	1.1787	0.3215
Ppap2c	NM_015817	1318.62	1022.74	-0.36660	-1.2893	0.0029
Dgat1	NM_010046	492.49	513.58	0.06051	1.0428	0.3985
Dgat2	NM_026384	46458.21	43908.51	-0.08143	-1.0581	0.2643
			Fatty Acid Synthesis			
Fasn	NM_007988	703.00	876.13	0.3176	1.2463	0.3106
Acaca	NM_133360	1248.97	1246.13	-0.0033	-1.0023	0.9811
Scd1	NM_009127	5984.96	10108.73	0.7562	1.6890	0.0858
Acacb	NM_133904	891.01	1185.03	0.4114	1.3300	0.0364
Mcat	NM_001030014	1704.97	1702.39	-0.0022	-1.0015	0.9576
Pdha1	NM_008810	533.03	703.05	0.3994	1.3190	0.0104
Pdha2	NM_008811	1.72	1.89	0.1315	1.0954	0.3976
Pdhb	NM_024221	6677.90	6498.61	-0.0393	-1.0276	0.6408
Pdk1	NM_172665	172.67	181.83	0.0746	1.0531	0.4737
Pdk2	NM_133667	2327.40	2249.41	-0.0492	-1.0347	0.6635
Pdk3	NM_145630	7.15	7.22	0.0134	1.0094	0.9613
Pdk4	NM_013743	195.02	296.48	0.6044	1.5203	0.0728
Pdp1	NM_001098230	35.41	29.61	-0.2580	-1.1958	0.1277
Pdp2	NM_001024606	596.14	609.19	0.0312	1.0219	0.7094
Dlat	NM_145614	323.16	313.22	-0.0451	-1.0318	0.6138
Dld	NM_007861	1265.16	1519.55	0.2643	1.2011	0.0017
Elovl6	NM_130450	1437.22	2468.09	0.7801	1.7173	0.0589
			TG Hydrolysis			
Lipe	NM_010719	37.07	33.49	-0.1465	-1.1069	0.4359
Pnpla2	NM_001163689	1498.10	1365.47	-0.1337	-1.0971	0.0948
			Transcription factors			
Srebf1	NM_011480	703.60	718.77	0.0308	1.0216	0.8345
Ppara	NM_011144	4685.93	4631.22	-0.0169	-1.0764	0.9104
Pparg	NM_011146	367.85	535.41	0.5415	1.4555	0.0461
			Fatty acid transporters			
Slc27a2	NM_011978	46096.47	41158.16	-0.1635	-1.0764	0.4849
Slc27a5	NM_009512	70194.59	68594.14	-0.0333	-1.0764	0.3783
Cd36	NM_007643	476.48	787.15	0.7242	1.6520	0.0101
Fabp1	NM_017399	135958.71	143369.63	0.0766	1.0545	0.1785
Acsl1	NM_007981	15340.11	16955.73	0.14446	1.1053	0.0317
Acsl4	NM_207625	1792.81	1696.05	-0.0800	-1.0764	0.4999
Acsl5	NM_027976	3822.18	4252.40	0.1539	1.1126	0.0777

Table 3

	NAFLD	No NAFLD	P-value
n (male) (n)	8 (3)	8 (5)	-
Age (years)	57 ± 5	49 ± 5	0.33
BMI (kg/m²)	30.5 ± 3.7	24.7 ± 1.5	0.28
CRP (mg/dl)	1.9 ± 0.9	1.6 ± 0.6	0.85
Fasting glucose (mg/dl)	91.5 ± 6.2	90.7 ± 2.2	0.80
HbA1c (%)	5.4 ± 0.2	5.6 ± 0.2	0.61
Triglycerides (mg/dl)	126.6 ± 0.4	93.3 ± 8.3	0.05
High density lipoprotein (mg/dl)	45.9 ± 2.7	44.0 ± 4.1	0.88
NAFLD activity score (0-8)	2.9 ± 0.4	-	0.01
Histological liver steatosis (%)	38.7 ± 8.1	1.2 ± 1.2	<0.001