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

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Lubura M, Hesse D, Kraemer M, Hallahan N, Schupp M, von Löffelholz C, Kriebel J, Rudovich N, Pfeiffer A, John C, Scheja L, Heeren J, Koliaki C, Roden M, Schürmann A. Diabetes prevalence in NZO females depends on estrogen action on liver fat content. *Am J Physiol Endocrinol Metab* 309: E968–E980, 2015. First published October 20, 2015; doi:10.1152/ajpendo.00338.2015.—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 (E_2) 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 (60 kcal% fat) in *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 hyperglycemia by *week 22*. In addition, at 11 wk, diacylglycerols were elevated in livers of diabetes-prone mice compared with controls. Hepatic expression profiles obtained from diabetes-prone and -resistant mice at 11 wk 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*. E_2 treatment of diabetes-prone mice for 10 wk prevented any further increase in liver fat content and reduced diacylglycerols and the abundance of *Mogat1* and *Cd36*, leading to a reduction of diabetes prevalence and an improved glucose tolerance compared with untreated mice. Our data indicate 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.

type 2 diabetes; hepatic steatosis; diacylglycerol; estrogen; New Zealand obese mice

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 they are fed 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 with 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 with 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 whether they can be used as a predictor for later onset of T2DM.

The aim of this study was to compare diabetic and nondiabetic NZO females with respect to their fat distribution to establish criteria for an early prediction of the onset of T2DM. Additionally, the goal was to clarify the impact of estrogen on the development of T2DM. We show that the

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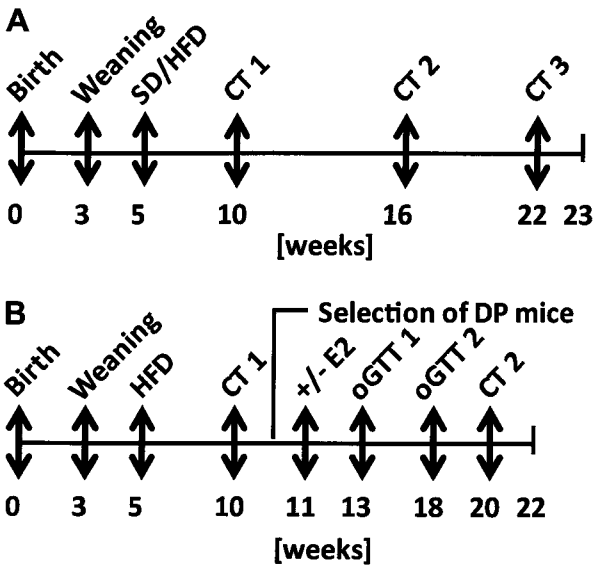


Fig. 1. Experimental design. *A*: female New Zealand obese (NZO) mice were either fed a standard diet (SD; $n = 9$) during the whole experiment or switched to high-fat diet (HFD; $n = 42$) at *week 5*. Body fat distribution and liver fat content were measured by computed tomography (CT) at the indicated time points (CT1, CT2, and CT3). *B*: female NZO mice received HFD from *week 5*. After prediction criteria for later onset of T2DM were applied, selected diabetes-prone (DP) animals were divided into control ($n = 12$) and 17β-estradiol (E_2)-treated ($n = 12$; 800 μg/kg HFD) groups. Five DP and 6 diabetes-resistant (DR) mice were euthanized at *week 11*, and livers were taken for further analysis (microarray, diacylglycerol/ceramide content). OGTT1 and OGTT2, oral glucose tolerance tests 1 and 2. Body weight and blood glucose were measured weekly.

early liver fat content and the expression of monoacylglycerol *O*-acyltransferase 1 (*Mogat1*) and *Cd36* play an important role for the later onset of the disease. Furthermore, we demonstrate that 17β-estradiol (E_2) reduces hepatic fat accumulation and thereby contributes to improved insulin sensitivity.

MATERIALS AND METHODS

Animals. Female NZO/HIBomDife 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 0600) 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; V153 × R/M-H; Ssniff, Soest, Germany) until the age of 5 wk, when some of the mice were switched to a high-fat diet (HFD; 60 kcal% fat, D12492; Research Diets, New Brunswick, NJ) (Fig. 1*A*). To generate plasma and tissue samples, mice were euthanized either at 11 or at 22 wk of age. From *week 11* estradiol-treated groups received 17β-estradiol (E_2) orally over 10 wk (800 μg/kg HFD; Sigma Aldrich, Munich, Germany) (Fig. 1*B*).

Computed tomography. Body fat distribution and liver fat content were measured by computed tomography (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* (Fig. 1*A*). Influence of E_2 treatment on accumulation of liver fat was examined by CT scans of the liver at *weeks 10* and *20* (Fig. 1*B*). During measurements, animals were anesthetized by isoflurane.

Transcriptome analysis. Total RNA was isolated from snap-frozen livers of 11-wk-old mice by an 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 8 × 60K Microarray gene chips (Agilent Technologies, Santa Clara, CA). 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. Sixteen subjects from the cross-sectional INSIGHT (German Clinical Trials Register: DRKS00005450) study who gave written informed consent were included. 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 and eosin. Nonalcoholic fatty liver disease (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 $\Delta\Delta C_T$ method (30). The following TaqMan gene expressions assays were used: *Cd36* (Mm01135198_m1), *Fasn* (Mm00662319_m1), *G6pc* (Mm00491176_m1), *Mogat1* (Mm00503358_m1), *Pck1* (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, CO) in a dilution of 1:1,000 and an anti-CD36 antibody (1:1,000; R & D Systems, Minneapolis, MN) in combination with horseradish peroxidase-labeled secondary antibodies. PKCε activity was determined as PKCε association with plasma membranes, as described earlier (1:2,000; BD Transduction Laboratories, Heidelberg, Germany) (22).

Quantification of diacylglycerols and ceramides. Liver samples were prepared as described earlier (34). The procedure included

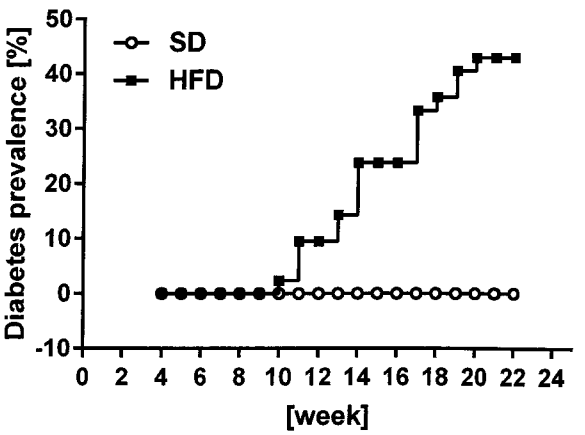


Fig. 2. Female NZO mice exhibit heterogeneous diabetes prevalence. Animals fed SD ($n = 9$) did not develop type 2 diabetes mellitus (T2DM). However, feeding with HFD that contains 60 kcal% fat ($n = 42$, diet switch at *week 5*) causes T2DM from 10 wk of age and results in diabetes prevalence of 43% at *week 22*.

lipid extraction with MeOH/$CHCl_3$ (2:1, vol/vol), homogenization, and incubation for 12 h at 48°C. After centrifugation (10 min, 5°C, 3,500 *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 the UPLC Ultimate 3000 System (Dionex, Idstein, Germany). Upon separation, single components were detected by mass spectrometer ESI-qToF maXis 3G (Bruker, Bremen, Germany).

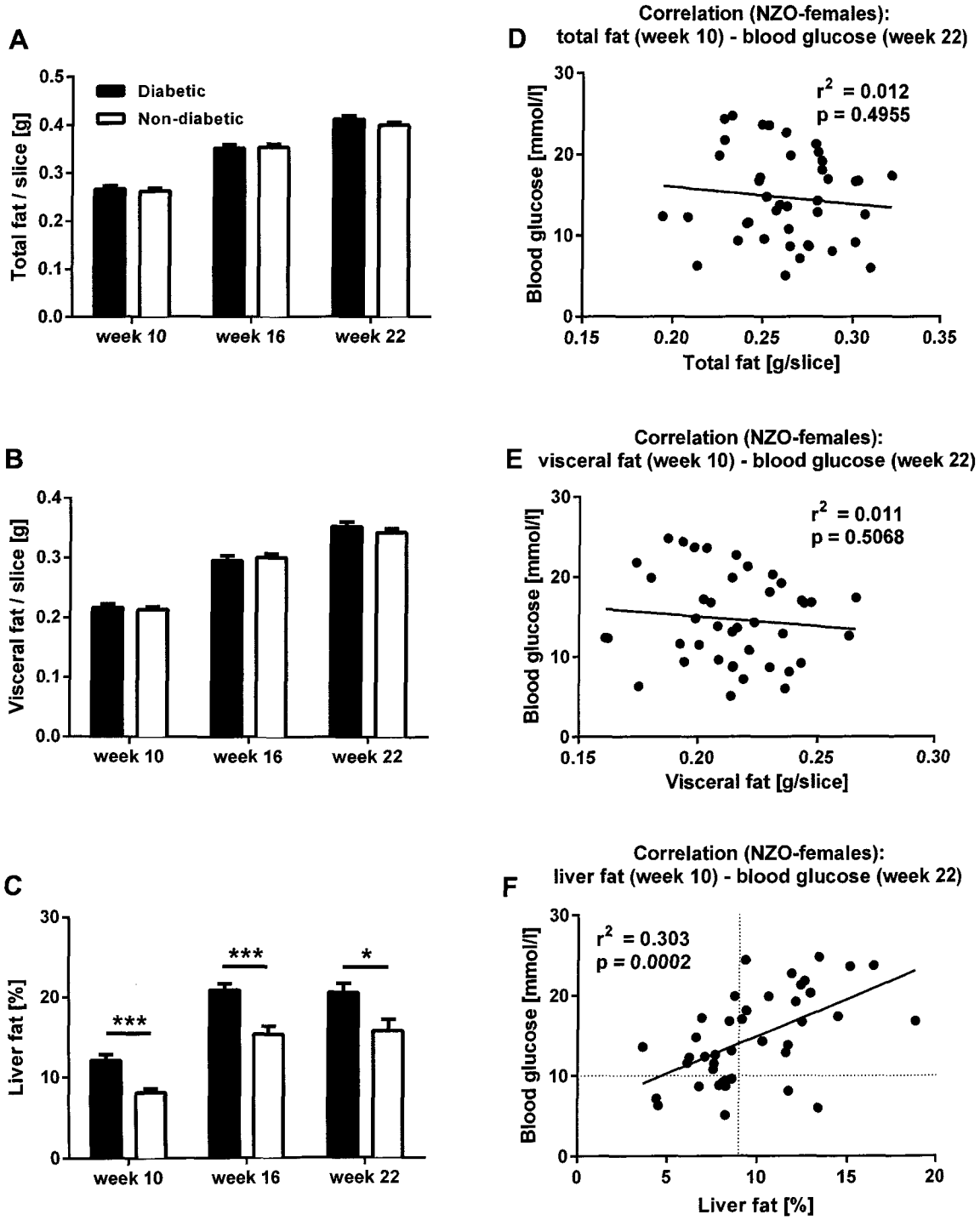


Fig. 3. Distribution of abdominal white adipose tissue was measured by CT at *weeks 10*, *16*, and *22*. *A* and *B*: there were no significant differences in amounts of total (*A*) or visceral fat (*B*) in the abdominal region (lumbar vertebrae L4–L5) between diabetic and nondiabetic animals. *C*: liver fat content at *weeks 10*, *16*, and *22*, as measured by CT, was elevated in diabetic mice. *D* and *E*: early total (*D*) and visceral abdominal fat (*E*) at *week 10*, as measured by CT, did not correlate with later blood glucose values in nonfasted animals at *week 22*; therefore, they cannot be used as predictors for the onset of T2DM. *F*: liver fat correlates with later blood glucose values in nonfasted animals at *week 22*. 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.

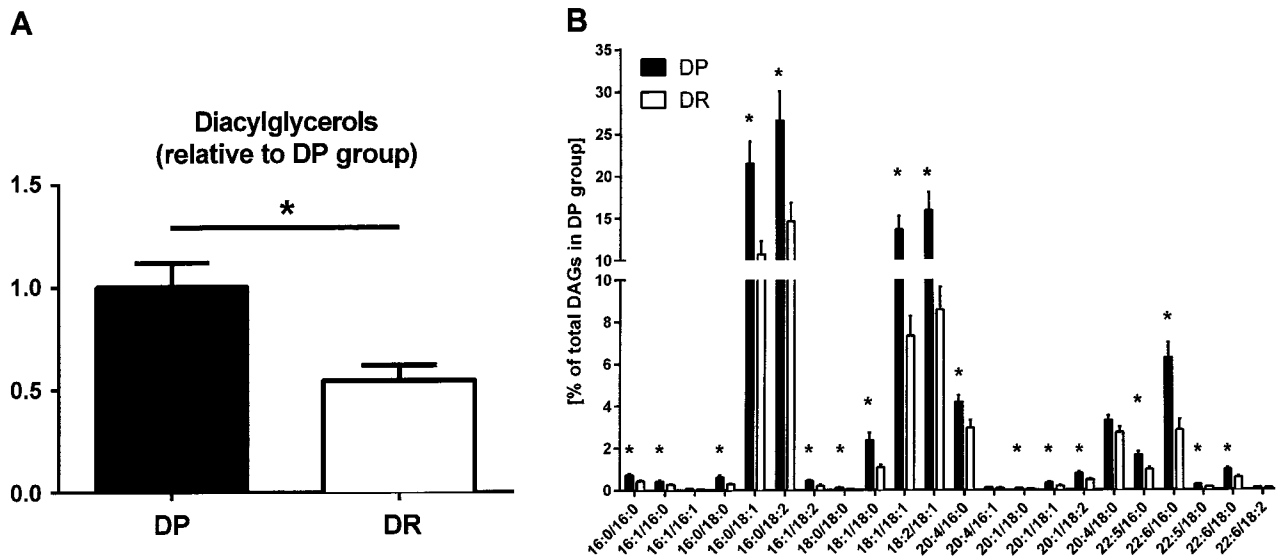


Fig. 4. *A*: liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) quantification of diacylglycerols in livers of 11-wk-old NZO female mice indicated increased diacylglycerol levels in DP (n = 5) compared with DR (n = 6) NZO females. *B*: diacylglycerol profile of livers from 11-wk-old DP (n = 5) and DR (n = 6) NZO females revealed a general increase in diacylglycerol species in DR group compared with DR animals. Student's t-test: $^*P < 0.05$.

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

Statistical analysis. Data, if not indicated otherwise, are presented as means ± SE. Statistical analysis and graphic presentation of the results were performed by GraphPad Prism version 6.05 for Windows (GraphPad Software, San Diego, CA).

Table 1. *Differentially expressed annotated genes from transcriptome analysis*

Gene Symbol	Gene ID	Mean DR	Mean DP	Log2 Fold	Fold Change	*P* Value
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	13,963.23	35,024.81	1.3267	2.5084	0.0213
Shbg	NM_011367	138.15	58.62	−1.2368	−2.3567	0.0154
Cyp3a44	NM_177380	6,954.4	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	5,183.6	10,956.2	1.0797	2.1136	0.0184
Cyp4a31	NM_001252539	1,102.77	2,289.74	1.0541	2.0764	0.0133
A4 gnt	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	6,890.9	3,596.3	−0.9382	−1.9161	0.019
Cyp4a32	NM_001100181	1,816.31	3,478.7	0.9375	1.9153	0.0183
Igfbp1	NM_008341	1,540.36	2,912.48	0.9190	1.8908	0.0317
Slc22a26	NM_146232	1,855.37	1,019.67	−0.8636	−1.8196	0.0022
Clstn3	NM_153508	169.73	307.72	0.8584	1.8131	0.0239
Hamp	NM_032541	15,203.45	8,411.96	−0.8539	−1.8074	0.0201
Fitm1	NM_026808	179.29	316.07	0.8179	1.7629	0.0218
Cyp2 g1	NM_013809	1,027.7	583.95	−0.8155	−1.7599	0.0021
Cyp4a10	NM_010011	13,392.05	22,618.64	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	20,341.13	30,161.63	0.5683	1.4828	0.0041
G6pc	NM_008061	8,137	12,600.82	0.6309	1.5486	0.0352

DR, diabetes resistant; DP, diabetes prone. Microarray analysis was performed in livers of 11-wk-old DP (n = 5) and DR (n = 6) New Zealand obese (NZO) females, and genes were selected according to the stringent criteria: $P < 0.05$, |log2 (fold change)| >0.7; mean signal intensity >90 (in at least 1 group). P values are according to Student's t-test.

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 vs. 44.6 ± 2.1 g, $P < 0.001$) and random blood glucose values (14.8 ± 0.9 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 ≥3 wk) after 22 wk reached 43% in the HFD-fed group (blood glucose concentration: 20.3 ± 0.7 mmol/l), whereas none of the mice on SD became diabetic during this period (10.8 ± 0.6 mmol/l, $P < 0.001$) (Fig. 2). The body weight of diabetic and nondiabetic females within the HFD group did not show significant differences (78.0 ± 2.1 vs. 75.6 ± 1.6 g; not significant).

To test whether differences in fat distribution were responsible for diabetes development in NZO females, fat distribution was quantified by CT at *weeks 10*, *16*, and *22* (Fig. 1*A*). No differences in amounts of total and visceral fat in the abdominal region were detected between diabetic and nondiabetic mice within the HFD group (Fig. 3, *A* and *B*). However, quantification of ectopic fat accumulation in the liver showed more intrahepatic fat in diabetic than in nondiabetic mice at all three time points (Fig. 3*C*). Earlier CT measurements in *week 5* indicated no initial differences in mice that were later defined as diabetes prone (DP) or diabetes resistant (DR) (DR: 3.37 ± 0.49% vs. DP: 3.74 ± 0.48%, n = 5; not significant). 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; Fig. 3*F*). 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%. However, early total (Fig. 3*D*) and visceral fat (Fig. 3*E*) mass were not related to later hyperglycemia.

Livers of DP mice exhibited higher diacylglycerol levels and elevated expression of lipogenic enzymes. Diacylglycerol species were measured in livers of designated DP or DR mice, as determined by our predefined criteria at the age of 11 wk. DP mice exhibited a significantly elevated diacylglycerol concentration compared with the DR group (Fig. 4*A*). However, the increase was not specific for individual diacylglycerol species (Fig. 4*B*). In contrast, hepatic ceramides were not altered between DP and DR mice (DR: 365.46 ± 27.15 nmol/g, n = 5; DP: 350.84 ± 23.70 nmol/g, n = 6). 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 (DP, n = 5; DP, 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 (Fig. 5, *A* and *B*, *top*). Moreover, Western blot analysis con-

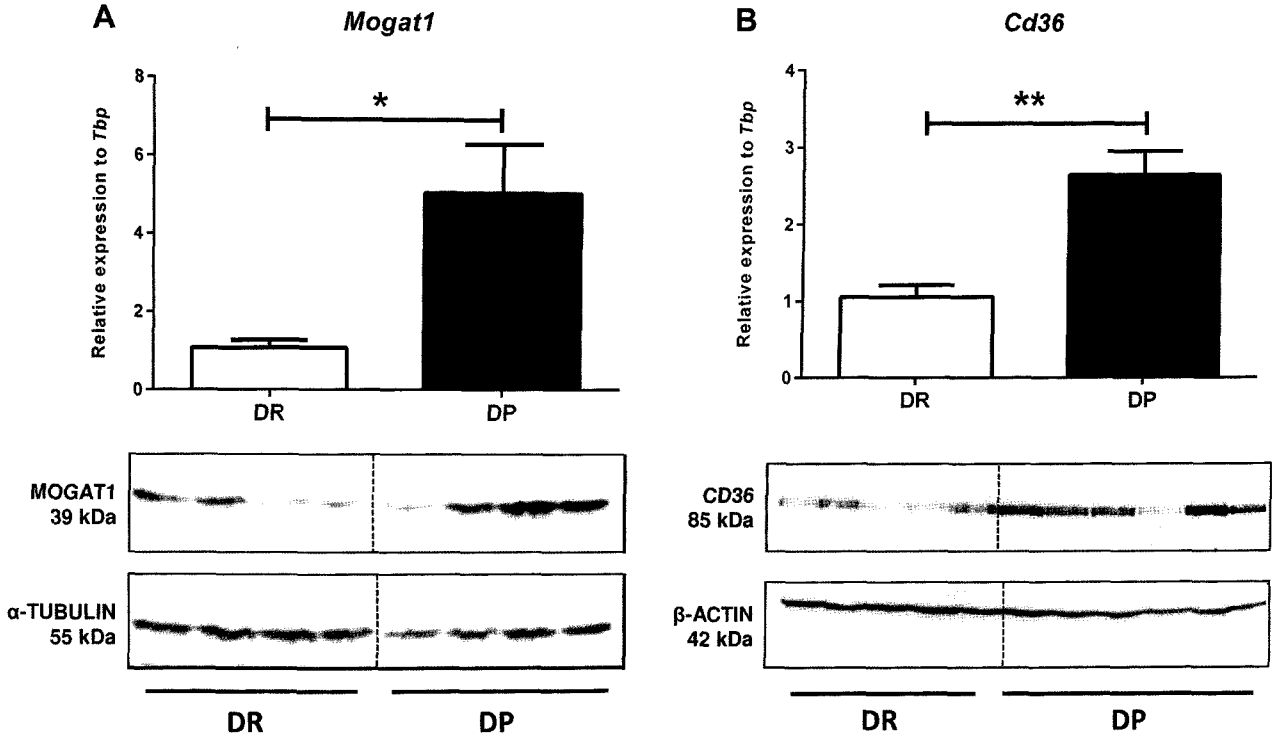


Fig. 5. Expression of monoacylglycerol *O*-acyltransferase 1 (*Mogat1*; *A*) and *Cd36* (*B*) mRNA was increased in livers of DP (n = 5) female NZO mice at *week 11* compared with DR (n = 6) females. MOGAT1 and CD36 protein abundance indicated the same trend, as identified by Western blot analysis. Student's t-test: $^*P < 0.05$; $^{**}P < 0.01$.

firmed the higher abundance of MOGAT1 and CD36 proteins in the livers of DP mice (Fig. 5, *A* and *B*, *bottom*). Furthermore, a closer look at the expression of other genes involved in the generation and degradation of lipid stores revealed no further relevant alterations of mRNA levels between DR and DP mice (Table 2). Besides these alterations in lipogenic transcripts, the expression of *Pck1* and *G6pc* was elevated in the livers of DP mice (Fig. 6), pointing toward an elevated hepatic glucose production in response to insulin resistance. To test whether 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 NAFLD. In contrast to mice, *MOGAT2* but not *MOGAT1* revealed a significantly higher expression ($P = 0.05$) in livers of NAFLD patients. The two other genes, MOGAT1 and MOGAT3, showed only a tendency toward elevated expression (Fig. 7). However, *CD36* expression appeared not to be different between the patients and controls. Subject characteristics are given in Table 3.

Table 2. *Expression of transcripts involved in hepatic lipid metabolism as determined by microarray analysis in livers of 11-wk-old diabetes-prone and diabetes-resistant NZO females*

Gene Symbol	Gene ID	Mean DR	Mean DP	Log2 Fold	Fold Change	*P* Value
			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	3,652.35	4,547.19	0.31615	1.2450	0.0638
Agpat3	NM_053014	10,694.07	9,935.12	−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	6,038.75	6,277.38	0.05591	1.0395	0.5717
Lclat1	NM_001081071	621.42	661.12	0.08934	1.0639	0.1564
Agpat9	NM_172715	1,661.60	1,776.97	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	1,318.62	1,022.74	−0.36660	−1.2893	0.0029
Dgat1	NM_010046	492.49	513.58	0.06051	1.0428	0.3985
Dgat2	NM_026384	46,458.21	43,908.51	−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	1,248.97	1,246.13	−0.0033	−1.0023	0.9811
Scd1	NM_009127	5,984.96	10,108.73	0.7562	1.6890	0.0858
Acacb	NM_133904	891.01	1,185.03	0.4114	1.3300	0.0364
Mcat	NM_001030014	1,704.97	1,702.39	−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	6,677.90	6,498.61	−0.0393	−1.0276	0.6408
Pdk1	NM_172665	172.67	181.83	0.0746	1.0531	0.4737
Pdk2	NM_133667	2,327.40	2,249.41	−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	1,265.16	1,519.55	0.2643	1.2011	0.0017
Elovl6	NM_130450	1,437.22	2,468.09	0.7801	1.7173	0.0589
			TG hydrolysis			
Lipe	NM_010719	37.07	33.49	−0.1465	−1.1069	0.4359
Pnpla2	NM_001163689	1,498.10	1,365.47	−0.1337	−1.0971	0.0948
			Transcription factors			
Srebf1	NM_011480	703.60	718.77	0.0308	1.0216	0.8345
Ppara	NM_011144	4,685.93	4,631.22	−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	46,096.47	41,158.16	−0.1635	−1.0764	0.4849
Slc27a5	NM_009512	70,194.59	68,594.14	−0.0333	−1.0764	0.3783
Cd36	NM_007643	476.48	787.15	0.7242	1.6520	0.0101
Fabp1	NM_017399	135,958.71	143,369.63	0.0766	1.0545	0.1785
Acsl1	NM_007981	15,340.11	16,955.73	0.14446	1.1053	0.0317
Acsl4	NM_207625	1,792.81	1,696.05	−0.0800	−1.0764	0.4999
Acsl5	NM_027976	3,822.18	4,252.40	0.1539	1.1126	0.0777

TG, triglyceride. Genes were regarded as significantly altered according to these criteria: $P < 0.05$, |log2 (fold change)| >0.7; mean signal intensity >90 (in at least 1 group); DR, $n = 6$; DP, $n = 5$. *P* values are according to Student's *t*-test.

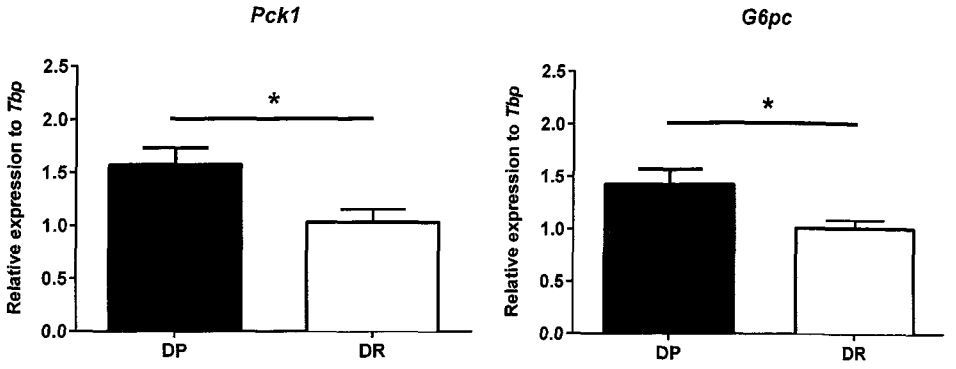


Fig. 6. Expression of genes encoding for the gluconeogenic enzymes phospho*enol*pyruvate (*Pck1*) and glucose-6-phosphatase (*G6pc*) was increased in DP (n = 5) compared with DR (n = 6) females at *week 11*. Student's t-test: *P < 0.05. *Tbp*, TATA box-binding protein.

Treatment with E_2 suppresses the development of T2D and prevents fat accumulation in the liver. To test whether estrogen exhibits protective potential and prevents T2DM, we treated female DP mice with E_2 from the age of 11 wk (Fig. 1*B*). Ten weeks of dietary supplementation with E_2 (800 μg/kg HFD) did not result in any significant differences in body weight compared with control groups (*week 22*: 81.1 ± 1.9 vs. 80.5 ± 1.1 g; not significant). However, E_2-treated mice exhibited significantly lower random blood glucose values than control mice (15.0 ± 2.2 vs. 27.7 ± 1.1 mmol/l, Student's t-test, P < 0.01). As expected, all DP control (DP-C) mice developed T2DM, whereas E_2 treatment reduced diabetes prevalence from 100 to 42% at *week 21* (Fig. 8*A*). Prior to E_2 treatment, the liver fat content was similar in both groups at *week 10* (E_2-treated mice 11.4 ± 0.3%, control mice 11.8 ± 0.7%; not significant). We next tested whether the protective effects of estrogen against T2DM could be mediated by a limited hepatic lipid accumulation. In fact, supplementation of E_2 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; Fig. 8*B*). In addition, E_2-treated mice exhibited significantly lower total diacylglycerol concentrations in the liver (Fig. 8*C*), which was caused by a general reduction in diacylglycerol species (Fig. 8*D*). To clarify a possible mechanism by which E_2 can prevent the impairment of insulin sensitivity in the liver, PKCε activity was assessed by isolation of plasma membranes and its detection by Western blotting. Livers from DP E_2-treated mice showed a reduced plasma membrane localization of PKCε compared with DP-C livers, indicating a reduced PKCε activity (Fig. 8*E*). To test whether E_2 treatment influences the expression of *Mogat1* and *Cd36*, we analyzed their mRNA in E_2-treated and nontreated NZO females. After E_2 treatment, we detected a reduced mRNA expression of both genes (Fig. 8, *F* and *G*), which could contribute to lower hepatic triglyceride and diacylglycerol levels in these livers. These results were also confirmed on protein levels by Western blot analysis (Fig. 8, *F* and *G*, *bottom*).

E_2 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 E_2-treated mice compared with DP-C animals. This was demon-

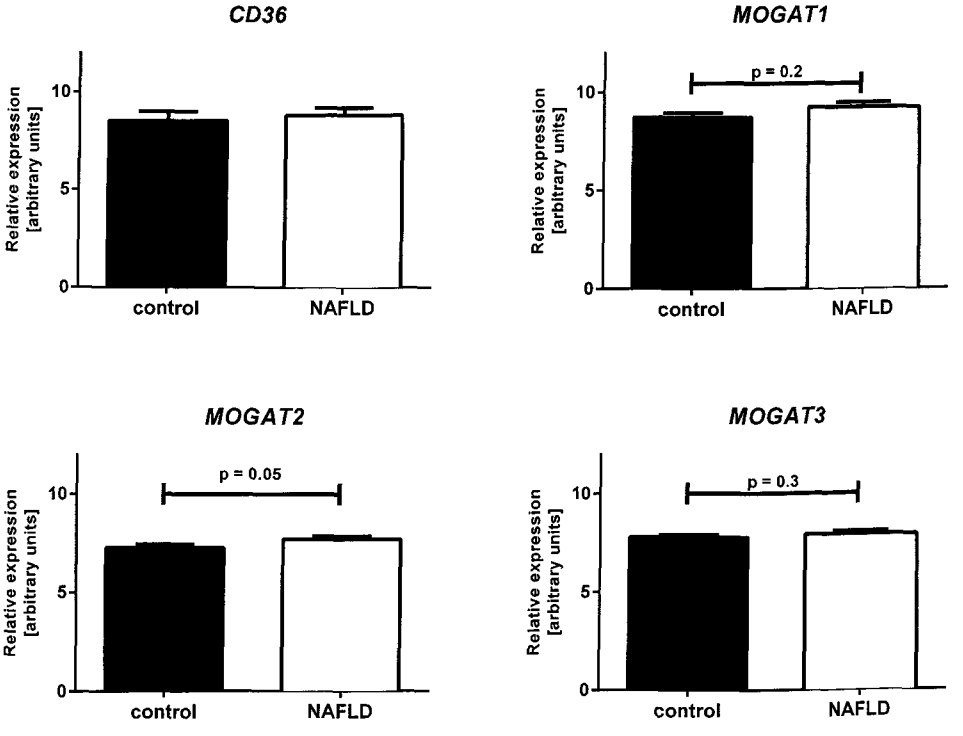


Fig. 7. Microarray expression data for *CD36* and *MOGAT* isoforms of liver biopsies from patients with nonalcoholic fatty liver disease (NAFLD; n = 8) and controls (n = 8). Student's t-test.

Table 3. *Characteristics of human study subjects*

	NAFLD	No NAFLD	*P* Value
n (Males)	8 (3)	8 (5)	
Age, yr	57 ± 5	49 ± 5	0.33
BMI, kg/m^2	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
Hb A_{1c}, %	5.4 ± 0.2	5.6 ± 0.2	0.61
TG, 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

Values are means ± SD. NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; CRP, C-reactive protein. Subjects were paired according to clinical parameters of NAFLD or no NAFLD for microarray data analysis.

strated by reduced blood glucose and increased plasma insulin levels at all time points in the E_2-supplemented group (Fig. 9, *A*, *C*, and *E*). At *week 18*, glucose tolerance had further deteriorated in DP-C mice; their plasma insulin levels during the test dropped markedly compared with the test performed in *week 13*, indicating β-cell loss. In contrast, OGTT results of DP E_2-treated mice were unchanged compared with *week 13* (Fig. 9, *B*, s*D*, and *F*). Furthermore, random plasma insulin levels at *week 22* were markedly reduced in DP-C (3.4 ± 0.8 μg/l) compared with DP E_2-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 E_2 on *Fasn* and *Pparg* expression could not be confirmed in our experiment, whereas *Scd1* was significantly increased in E_2-treated animals (Fig. 10).

DISCUSSION

The present study demonstrates that *1*) early elevated liver fat content is a valuable prediction marker for T2DM, *2*) this parameter was associated with increased hepatic *Mogat1* and *Cd36* levels, and *3*) E_2 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 became diabetic was accompanied by increased diacylglycerol concentrations that have been shown to disrupt the insulin-signaling pathway (2, 47). As a consequence of insulin resistance in hepatocytes, the expression of enzymes involved in gluconeogenesis (*G6pc* and *Pck1*) is 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 *Pck1*, was increased in livers of diabetes-prone mice at *week 11* (Table 1 and Fig. 6).

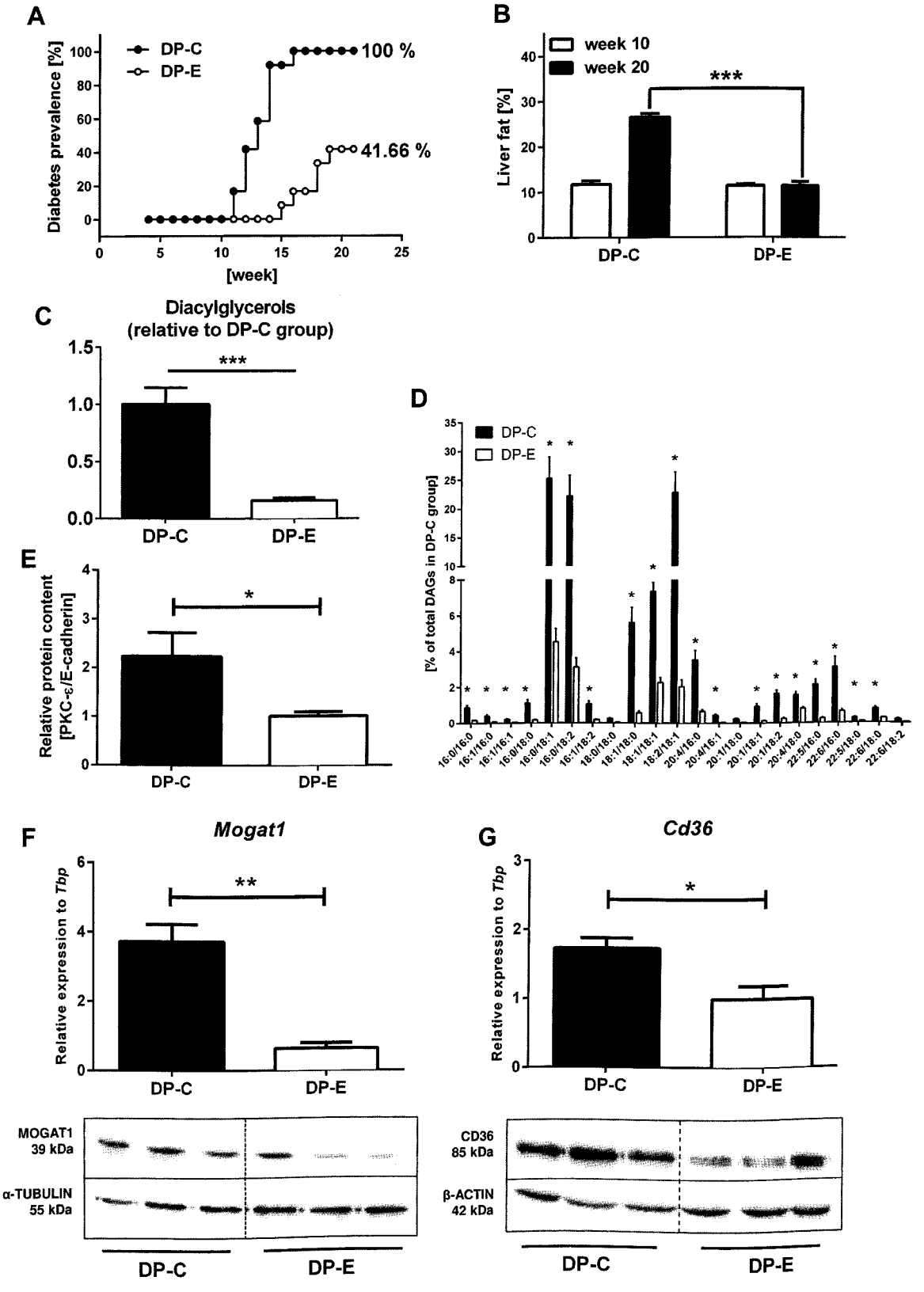
Transcriptome analysis of livers at *week 11* revealed the increased expression of two genes involved in hepatic lipid metabolism (*Mogat1* and *Cd36*). *Mogat1* encodes for a liver-specific monoacylglycerol *O*-acyltransferase 1 (MOGAT1), an enzyme that is involved in the alternative triglyceride synthesis 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) that 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 and body weight (19, 46). Furthermore, in our own human samples and data by Hall et al. (17), the expression of *MOGAT2* was associated with 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 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 PKCε 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, E_2 treatment of diabetes-prone NZO females resulted in a lower expression of *Mogat1* and *Cd36* and lower hepatic diacylglycerols as well as in a reduced PKCε activity that can contribute to the amelioration of T2DM prevalence in this group. In contrast to our data, Hall et al. (18) detected an elevated diacylglycerol concentration in the liver after suppression of *Mogat1* expression by siRNA treatment. This discrepancy might be explained by a compensatory decreased diacylglycerol *O*-acyltransferase (*Dgat*) expression that occurred simultaneously with a decreased *Mogat1* expression in the Hall et al. (18) study.

Interestingly, E_2 treatment of females that exhibit elevated blood glucose and liver fat levels at *weeks 9* and *10*, respec-

Fig. 8. *A*: At *week 22*, all DP control mice (DP-C; $n = 12$) suffered from T2DM, whereas only 41.7% of estrogen-treated DP animals (DP-E; $n = 12$) became diabetic. Both groups received HFD containing 60 kcal% fat from the age of 5 wk; E_2-treated animals received the same diet supplemented with 800 μg/kg diet estradiol from the age of 11 wk. *B*: estrogen treatment averts ectopic accumulation of fat in livers of DP 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-wk-old DP mice by LC-MS/MS indicated reduced diacylglycerol levels after estrogen treatment (DP-E; $n = 11$) compared with control (DP-C; $n = 10$) NZO females. *D*: estrogen supplementation of DP-E mice resulted in a reduction of all hepatic diacylglycerol species compared with DP-C. *E*: PKCε activity was higher in DP-C livers compared with estrogen-treated mice. PKCε activity was determined by localization of PKCε to the plasma membrane in DP-C and DP-E livers ($n = 4$). *F* and *G*: expression of *Mogat1* (*F*) and *Cd36* (*G*) mRNA was lower in livers of DP-E ($n = 6$) female NZO mice at *week 22* compared with DP-C ($n = 6$). MOGAT1 and CD36 protein abundance indicated the same trend, as determined by Western blot analysis. Student's *t*-test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

tively, reduced diabetes prevalence by almost 60%, confirming that estrogen mediates beneficial effects on glucose homeostasis. These results are in accordance with previous studies where E_2 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 asso-



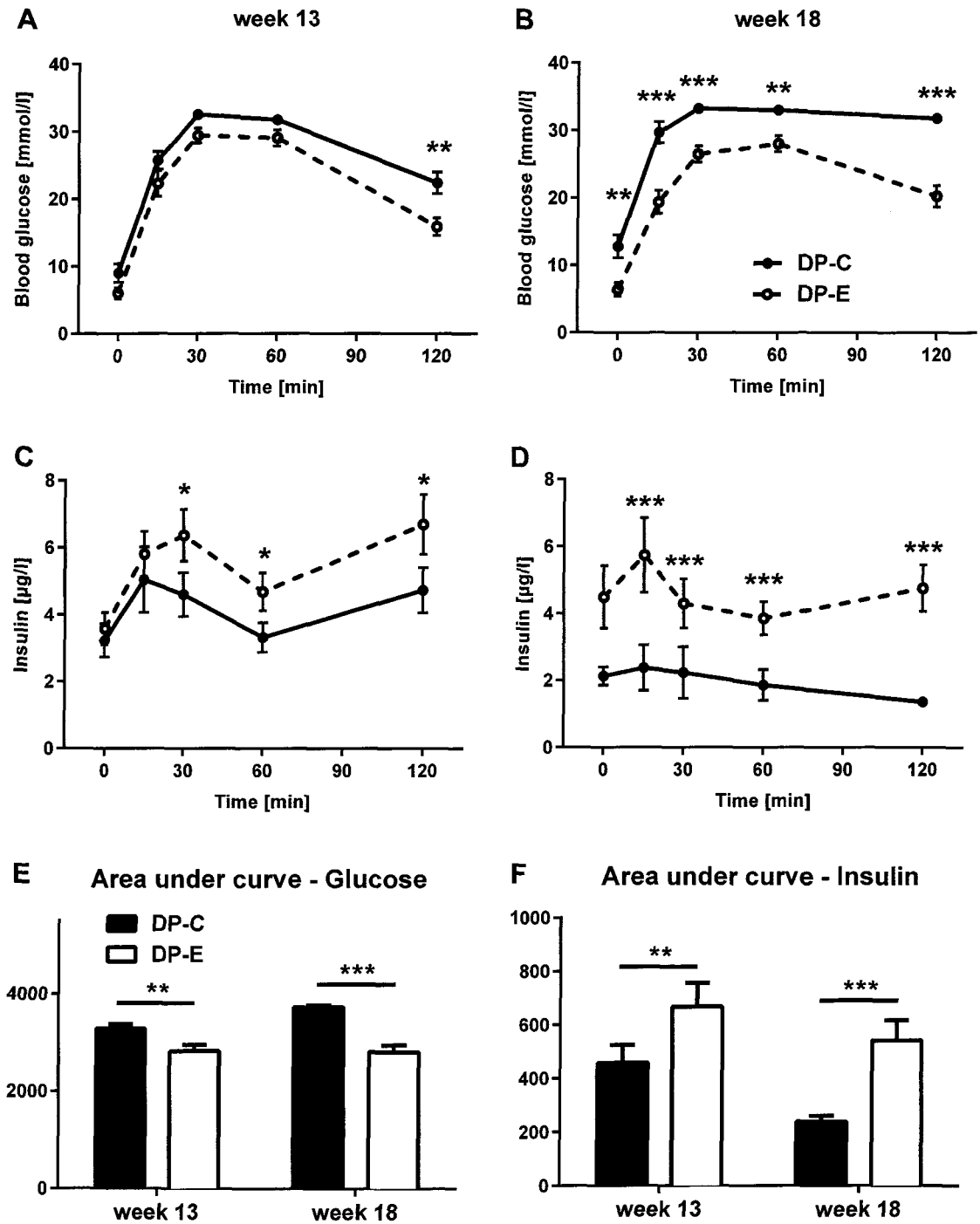


Fig. 9. Estrogen treatment improved glucose tolerance in DP mice at the ages of 13 and 18 wk. *Left*: OGTT at *week 13*. *Right*: OGTT at *week 18*. Blood glucose (*A* and *B*) and plasma insulin (*C* and *D*) concentrations during OGTT; area under the glucose (*E*) and insulin (*F*) curves during OGTT was calculated using the trapezoidal rule. DP-C: $n = 12$; DP-E: $n = 12$. Student's *t*-test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

ciated with a decreased risk of developing insulin resistance and T2DM (7, 42).

Improved glucose homeostasis detected after E_2 treatment might be a consequence of reduced ectopic fat accumulation in the liver (Fig. 8, *B* and *C*). This phenomenon was observed previously in postmenopausal women receiving hormone replacement therapy (11, 32) and in estrogen-treated mice (5, 23).

Because E_2 treatment suppressed the expression of *Mogat1* and *Cd36*, it is possible that E_2 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 two putative

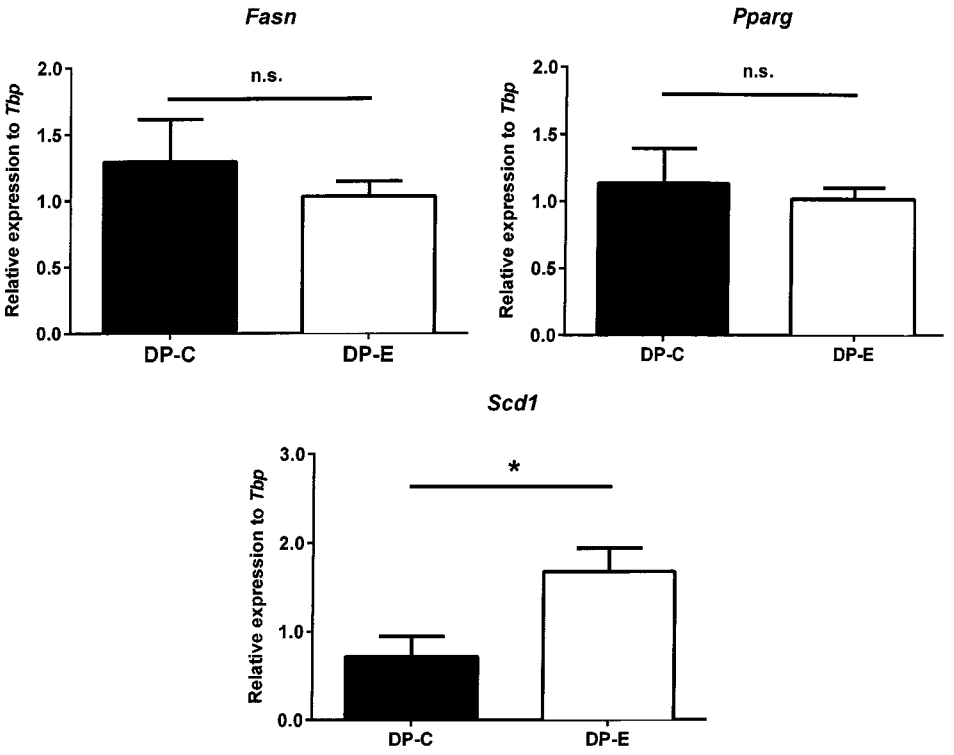


Fig. 10. RT-quantitative PCR analysis could not confirm earlier findings that estrogen reduces hepatic expression of lipogenic genes *Fasn* (*top left*) and *Pparg* (*top right*), whereas the expression of *Scd1* (*bottom*) was slightly increased at *week 22* upon E_2 treatment. DP-C: $n = 6$; DP-E: $n = 6$. Student's *t*-test: $^*P < 0.05$. NS, nonsignificant ($P > 0.05$).

estrogen responsive elements, 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, because 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.

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DISCLOSURES

The authors declare that they have no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

M.L., D.H., and A.S. conception and design of research; M.L., D.H., M.K., and C.J. performed experiments; M.L., D.H., C.v.L., J.K., and L.S. analyzed data; M.L., D.H., M.S., C.v.L., J.K., N.N.R., A.F.P., J.H., C.K., M.R., and A.S. interpreted results of experiments; M.L. and D.H. prepared figures; M.L., D.H., N.H., and A.S. drafted manuscript; M.L., D.H., and A.S. edited and revised manuscript; M.L., D.H., M.K., N.H., M.S., C.v.L., J.K., N.N.R., A.F.P., C.J., L.S., J.H., C.K., M.R., and A.S. approved final version of manuscript.

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