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1 Disturbances in cholesterol, bile acid and glucose metabolism in peroxisomal
2 3-ketoacylCoA thiolase B deficient mice fed diets containing high or low fat
3 contents

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2
3 Abbreviations: ThA: 3-ketoacyl-CoA thiolase A; ThB: 3-ketoacyl-CoA thiolase B;
4 FAO: fatty acid oxidation; ACOX1 : acyl-coA-oxidase 1 ; MFP1: peroxisomal
5 multifunctional enzyme type 1; MFP2: peroxisomal multifunctional enzyme type 2;
6 WAT: white adipose tissue; LFD: Low Fat Diet; HFD: High Fat Diet; GH: growth
7 hormone; IGF-I: insulin Growth Factor-I; IGFBP-3: insulin-like growth factor-binding
8 protein-3; PPAR α : peroxisome proliferator-activated receptor alpha; Wy: Wy14,643;
9 WT: wild-type; KO: knock-out; HDL: high density lipoproteins; SR-BI: scavenger
10 receptor class B member 1; SREBP: sterol regulatory element-binding protein; RXR:
11 retinoid X receptor.

12
13 Short title: Altered lipid and glucose metabolism in *Thb*^{-/-} mice

14
15 Keywords: peroxisomal 3-ketoacyl-CoA thiolase B, hypoglycemia, *de novo*
16 biosynthesis of cholesterol, bile acids, lathosterol.

17 # these authors equally contributed to this work

18
19 Highlights (Nicolas-Francès et al.,)

20
21 “Disturbances in cholesterol, bile acid and glucose metabolism in peroxisomal 3-
22 ketoacylCoA thiolase B deficient mice fed diets containing high or low saturated fat
23 contents”

- 24
25 - Reduced body size and plasma growth hormone levels in *Thb*^{-/-} mice
26 - Higher energy intake but reduced adiposity in *Thb*^{-/-} mice fed a saturated fat diet
27 - A better insulin sensitivity leads to hypoglycemia in *Thb*^{-/-} mice fed synthetic diets
28 - Increased plasma HDL-cholesterol and whole body cholesterol synthesis in *Thb*^{-/-} mice
29 - Increased liver cholesterol content and bile acid synthesis in *Thb*^{-/-} mice
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1

2 **Abstract**

3 The peroxisomal 3-ketoacyl-CoA thiolase B (ThB) catalyzes the thiolytic cleavage of
4 straight chain 3-ketoacyl-CoAs. Up to now, the ability of ThB to interfere with lipid
5 metabolism was studied in mice fed a routinely laboratory chow enriched or not with the
6 synthetic agonist Wy14,643, a pharmacological activator of the nuclear hormone receptor
7 PPAR α . The aim of the present study was therefore to determine whether ThB could play a
8 role in obesity and lipid metabolism when mice are chronically fed a synthetic High Fat Diet
9 (HFD) or a Low Fat Diet (LFD) as a control diet. To investigate this possibility, wild-type
10 (WT) mice and mice deficient for Thb (*Thb*^{-/-}) were subjected to either a synthetic LFD or a
11 HFD for 25 weeks, and their responses were compared. First, when fed a normal regulatory
12 laboratory chow, *Thb*^{-/-} mice displayed growth retardation as well as a severe reduction in
13 the plasma level of growth hormone (GH) and Insulin Growth Factor-I (IGF-I), suggesting
14 alterations in the GH/IGF-1 pathway. When fed the synthetic diets, the corrected energy
15 intake to body mass was significantly higher in *Thb*^{-/-} mice, yet those mice were protected
16 from HFD-induced adiposity. Importantly, *Thb*^{-/-} mice also suffered from hypoglycemia,
17 exhibited reduction in liver glycogen stores and circulating insulin levels under the LFD and
18 the HFD. *Thb* deficiency was also associated with higher levels of plasma HDL (High
19 Density Lipoproteins) cholesterol and increased liver content of cholesterol under both the
20 LFD and the HFD. As shown by the plasma lathosterol to cholesterol ratio, a surrogate
21 marker for cholesterol biosynthesis, whole body cholesterol *de novo* synthesis was increased
22 in *Thb*^{-/-} mice. By comparing liver RNA from WT mice and *Thb*^{-/-} mice using
23 oligonucleotide microarray and RT-qPCR, a coordinated decrease in the expression of
24 critical cholesterol synthesizing genes and an increased expression of genes involved in bile
25 acid synthesis (*Cyp7a* *Cyp17a1*, *Akr1d1*) were observed in *Thb*^{-/-} mice. In parallel, the

1 elevation of the lathosterol to cholesterol ratio as well as the increased expression of
2 cholesterol synthesizing genes were observed in the kidney of *Thb*^{-/-} mice fed the LFD and
3 the HFD. Overall, the data indicate that ThB is not fully interchangeable with the thiolase A
4 isoform. The present study also reveals that modulating the expression of the peroxisomal
5 ThB enzyme can largely reverberate not only throughout fatty acid metabolism but also
6 cholesterol, bile acid and glucose metabolism.

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2 1. Introduction

3 Adipose tissue lipolysis generates free fatty acids that are taken up from the blood plasma by
4 the liver where they are activated into their fatty acyl-CoA derivatives. The activated fatty
5 acyl-CoAs are subsequently imported into mitochondria or peroxisomes for degradation to
6 acetyl-coenzyme A (acetyl-CoA) *via* the β -oxidation process. Whereas the mitochondrion
7 oxidizes short-, medium- and mostly long-chain fatty acids, the peroxisome oxidizes some
8 long-chain but mostly very long chain fatty acids. The peroxisome is also involved in the α -
9 oxidation of very-long-straight-chain or branched-chain acyl-CoAs (reviewed in [1]). At the
10 biochemical level, the peroxisomal β -oxidation of straight-chain acyl-CoAs starts with a
11 reaction catalyzed by the ACOX1 (acyl-CoA oxidase 1) enzyme that is the first and rate-
12 limiting enzyme of the pathway. The step orchestrated by ACOX1 is followed by two
13 enzymatic reactions carried out by the MFP1 or MFP2. The fourth and last step of the
14 process is catalyzed by the peroxisomal 3-ketoacyl-CoA thiolases. In humans, only a single
15 corresponding gene (peroxisomal 3-acetyl-CoA acetyltransferase-1 also known as
16 peroxisomal 3-oxoacyl-CoA thiolase or acyl-CoA:acetyl-CoA-acyltransferase, ACAA1, EC
17 2.3.1.16) has been identified [2, 3] and no isolated peroxisomal deficiency at the level of the
18 peroxisomal 3-ketoacyl-CoA thiolase, has not been reported yet [2, 3]. To date, two closely
19 related but differentially regulated peroxisomal 3-ketoacyl-CoA thiolases isoforms (thiolase
20 A, ThA, Acaa1a and thiolase B, ThB, Acaa1b, EC:2.3.1.16) have been identified in rodents
21 [4, 5]. It is still not clear whether these two proteins stem from a unique and original
22 ancestral gene. In agreement with their very high degree of nucleotide sequence identity
23 (97%), the mature forms of ThA and ThB differ in only nine amino acid in rats [4-6]. Given
24 the large amino acid sequence identity (96%) between ThA and ThB, major overlap in the
25 enzymatic activities of ThA and ThB were found [4, 7]. As a consequence, both enzymes

1 share virtually the same substrate specificity *in vitro* that includes very-long-straight-chain
2 3-oxoacyl-CoAs [7]. The cleavage of 2-methyl-branched as well as straight-chain 3-
3 ketoacyl-CoA esters is under the dependence of a third thiolase isoform (SCP-2/3-ketoacyl-
4 CoA thiolase, SCPx) previously characterized in both humans and rodents [8-10].
5 In addition to fatty acid β -oxidation, the peroxisome is involved in other aspects of lipid
6 metabolism ranging from synthesis of bile acids, plasmalogens, cholesterol and isoprenoids.
7 Consistent with this notion, different studies have shed light on the existence of an
8 alternative pathway for cholesterol synthesis in the peroxisomal matrix, with a specific 3-
9 hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that displays functional and
10 structural properties different from the endoplasmic reticulum HMG-CoA reductase [11-13].
11 In order to investigate the physiological role of the *3-ketoacylCoA thiolase B* (ThB) *in vivo*,
12 a mouse model deficient for *Thb* (*Thb*^{-/-}) has been partly characterized in our laboratory [14].
13 At the molecular level, *Thb* was identified as a direct target gene of the peroxisome
14 proliferator-activated receptor alpha (PPAR α , NR1C1) in the liver [15]. PPAR α forms a
15 heterodimer with the Retinoid X Receptor (RXR) and following the physical binding of
16 PPAR α /RXR α by the synthetic agonist Wy14,643 (Wy), the mRNA expression of a large
17 arrays of genes ranging from lipid, amino-acid, glycerol and glycogen metabolism as well as
18 inflammation control is upregulated ([16-23]). A crucial piece of evidence that liver *Thb* is
19 functionally regulated by the nuclear receptor PPAR α is the observation that some hepatic
20 fatty acid contents are different between WT and *Thb*^{-/-} mice exposed to the PPAR α agonist
21 Wy [21, 24]. Follow-up investigations also revealed that ThB could play an indirect role in
22 the control of PPAR α mediated upregulation of Sterol Regulatory-Element-Binding-Protein-
23 2 (SREBP-2) target genes in the liver of mice fed with Wy [25]. This result further
24 demonstrates that the deletion of a single peroxisomal activity such as ThB is sufficient to
25 impact the transcription of biosynthetic cholesterol genes in the liver. Combined, these

1 previous data support the notion that despite the similarities between ThA and ThB, ThB
2 displays a unique biochemical function that deserves further characterization.

3 In addition to be the molecular target of the fibrate drug Wy, PPAR α also mediates the
4 effects of a High Fat Diet (HFD) on hepatic gene expression [26]. As *Thb* is a PPAR α target
5 gene, we expected that chronic HFD could shed light on the putative consequences of the
6 deletion of *Thb* in mouse in a more physiological context, *i.e.* HFD-induced obesity and
7 insulin-resistance.

8 The recent demonstration that resistance to diet-induced obesity is accompanied by a
9 marked increase in peroxisomal β -oxidation has been instrumental in advancing our
10 thoughts about the origin of oxidative changes during obesity [27, 28]. Using two strains of
11 mice resistant (A/J) or sensitive (C57Bl/6) to diet-induced hepatosteatosis and obesity, it
12 was found that 10 peroxisomal oxidative genes were specifically upregulated in A/J mice
13 leading to a significant increase in peroxisomal β -oxidation [27]. It was thereby
14 hypothesized that this peroxisomal fatty acid β -oxidation could partly prevent diet-induced
15 hepatosteatosis and obesity.

16 The aim of the present study was therefore to evaluate the role of ThB and its ability to
17 potentially interfere with HFD-induced lipid metabolism disorders. To explore the function
18 of ThB in this aspect, WT mice and *Thb*^{-/-} mice were chronically fed a synthetic High-Fat
19 Diet (HFD) and a Low-Fat Diet (LFD) as control. Our results indicate that *Thb* deletion in
20 mouse induces alterations not only in fatty acid metabolism but also in carbohydrate,
21 cholesterol and bile acid metabolism, extending the function of ThB to other unexpected
22 metabolic pathways.

23

24 **2. Materials and methods**

25

26 *2.1. Animal experiments and ethical considerations*

27

1 Only male mice on a pure-bred *Sv129* genetic background have been used and previously
2 described [14]. Male mice were kept in normal cages with food and water *ad libitum*, unless
3 clearly indicated. In absence of dietary challenge, mice were routinely fed a standard
4 commercial pellet diet (UAR A03-10 pellets from Usine d'Alimentation Rationnelle, Epinay
5 sur Orge, SAFE, France, 3.2 kcal/g) consisting (by mass) of about 5.1% fat (C16:0 ± 0.89%;
6 C16:1 n-7 ± 0.09%; C18:0 ± 0.45%; C18:1 n-9 ± 1.06%, C18:2 n-9 ± 1.53% and traces of
7 C18:3 n-9), similar to previous studies [24, 25]. At the time of sacrifice, animals were only
8 7-weeks old. The animal experiments were approved by the animal experimentation
9 committee of the University of Burgundy (protocol number n°1904) and were performed
10 according to the European Union guidelines for animal care.

11

12 2.2. Nutritional intervention

13

14 Two-month-old male mice were fed with a LFD or a HFD for 25 weeks, as previously
15 described elsewhere [26, 29]. The diets provided either 10% or 45% energy (kcal) percent in
16 the form of lard (D12450B or D12451, Research Diets Inc., New Brunswick, NJ, USA).
17 Content in cholesterol was 0,00136% (w/w) for D12450B and 0,01489% (w/w) for D12451.
18 Table I shows the composition of the diet. At the time of sacrifice, animals were around 7
19 months of age. Tissues were excised, weighted and immediately frozen in liquid nitrogen
20 before being stocked at -80°C. The animal experiments were approved by the animal
21 experimentation committee of the University of Burgundy (protocol n° 0107) and were
22 performed according to the European Union guidelines for animal care.

23

24

25 2.3. Food intake and body size

26 Food intake was measured over 3 to 4 days for 25 weeks to the nearest 0.1 g. The
27 experiment was conducted in three replications of 5 WT and 6 *Thb*^{-/-} mice each. For

1 measurement of body size, animals were lightly anesthetized with 3% of isoflurane and
2 extended to their maximal length to determine the nose-to-anus distance.

3

4 *2.4. Histochemistry*

5 Hematoxylin and Eosin staining of adipose sections were done using standard protocols. We
6 kindly acknowledge Amandine Bataille (Plateforme d'Imagerie Cellulaire CellImaP, IFR
7 100 Santé- STIC, Université de Bourgogne, Dijon, France) for expert technical assistance.

8

9 *2.5. Plasma metabolite levels*

10

11 Plasma was initially collected into EDTA tubes *via* caudal or retroorbital puncture and
12 centrifuged at + 4°C (10 min, 6000 rpm). Plasma glycerol concentration was determined
13 using a kit from Instruchemie (Delfzijl, the Netherlands). Plasma free fatty acids were
14 determined using a kit from WAKO Chemicals (WAKO Chemicals, Germany). Plasma
15 glucose concentration was obtained by the enzymatic method employing glucose-oxidase
16 (Glucose GOD FS, Diasys, Diagnostic Systems International, Condom, France). Plasma β -
17 hydroxybutyrate, cholesterol and triglycerides levels were determined using a kit from
18 Diasys. For lathosterol measurements, plasma or tissue (about 10 to 20 mg of kidney) was
19 mixed with epicoprostanol, which was used as a control standard. Potassium hydroxide
20 saponification was followed by lipid extraction with hexane. Cholesterol and lathosterol
21 were analyzed in the trimethylsilyl ether state by GC-MS using a Hewlett Packard HP6890
22 Gas Chromatograph equipped with an HP7683 Injector and an HP5973 Mass Selective
23 Detector, as previously described [25].

24

25 *2.6. Plasma hormone levels*

26 Plasma growth hormone level was determined using the rat/mouse Growth Hormone ELISA
27 kit from Linco Research (USA). Plasma mouse IGF-1 level was determined using the

1 Mouse Insulin-like Growth Factor 1 (IGF-1) ELISA Kit from ASSAYPRO (USA). IGFBP-3
2 serum concentration was measured using the mouse/rat IGFBP-3 ELISA kit provided by
3 BioVendor GmbH (Heidelberg, Germany). Plasma insulin level was evaluated using a
4 mouse insulin enzyme-linked immunosorbent assay kit (Merckodia SA, Sweden).

5

6 *2.7. Liver fatty acid profile, hepatic triglyceride and bile acid content in the liver*

7 Liver fatty acid profile as well as quantification of hepatic triglyceride content were
8 performed as previously described [24]. Levels of bile acids in the liver were determined by
9 capillary gas chromatography/mass spectrometry as previously reported [30].

10

11 *2.8. Liver glycogen content*

12

13 Hepatic glycogen content was measured using a method that derives from that of Passoneau
14 and Landerdale [31]. Briefly, 100 mg of liver or muscle sample was homogenized in 700 µl
15 of 0.4 M HClO₄ and was centrifuge for 5 min at 13 000 g. 650 µl of the supernatant was
16 then neutralized with 184 µl of 0.75 M K₂CO₃ and was incubated 10 minutes at 4°C. After
17 centrifugation, the supernatant fraction (50 µl) was diluted in 200 µl sodium acetate 0.3 M
18 (pH 4.8) and incubated in the presence or not of 10 mg/ml of amyloglucosidase (Sigma-
19 Aldrich) for 2 h at 37°C. The glucose concentration with or without amyloglucosidase
20 treatment was measured using the Glucose-DOD from Diasys. Oyster glycogen type II
21 (Sigma-Aldrich) was similarly treated and used as standard.

22

23 *2.9. RNA isolation and Real-time quantitative PCR (RTqPCR)*

24 Total RNA was extracted from liver with TRIzol (Invitrogen) using the supplier's
25 instructions. RNA was then further purified (from free nucleotides and contaminating
26 genomic DNA) using RNeasy columns (Qiagen) with DNase treatment. A 260 nm/280 nm
27 ratio of ~ 2 indicated that samples were essentially free from contaminants such as protein.

1 The reverse-transcription step was subsequently performed with 1 µg of RNA using iScript
2 Reverse Transcriptase (Bio-Rad). PCR reactions were performed using the qPCR
3 MasterMix Plus for SYBR Green I with fluorescein (Eurogentec) using an iCycler PCR
4 machine (Bio-Rad). Primers were designated to generate a PCR amplification product of
5 100-200 bp and were selected according to indications provided by the Primer 3 software
6 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequences are available from
7 S.M. on request. Specificity of the amplification, evaluation of primer dimers formation and
8 efficiency of PCR amplification were verified by melt curve analysis. The “delta-delta Ct”
9 quantification method was used and the expression of each tested gene was related to the
10 control gene 36B4, which did not change under any of the experimental conditions studied.

11

12 *2.10. Preparation of nuclear extracts of kidney and immunoblot analysis*

13 Nuclear extracts of kidney were prepared following established protocols [32]. 10 µg of
14 nuclear protein was separated on a 10% (w/v) polyacrylamide gel in the presence of 0.1%
15 (w/v) SDS and transferred on to PVDF membranes. A broad range pre-stained SDS-PAGE
16 standard (Bio-Rad, 161-0318) was used as a protein ladder. After membrane saturation at
17 room temperature for 90 min with TBS (0.1 M Tris/HCl, pH 8.0, 0.15 M NaCl) containing
18 0.1% (v/v) Tween 20 and 5% (w/v) fat-free milk, blots were incubated overnight at 4°C with
19 a polyclonal anti-SREBP-2 rabbit antibody (Ab28482, 1:200, Abcam) or a polyclonal sheep
20 antibody to histone H1 (Ab1938, 1:200, Abcam), respectively. After three washes in TBS
21 containing 0.1% (v/v) Tween 20, primary antibodies were detected using a peroxidase-
22 conjugated IgG antibody, the choice of which depends on the primary antibody of interest: a
23 goat anti-rabbit IgG Antibody, (1:30000, sc-2004, Santa Cruz Biotechnology) or a rabbit
24 anti-sheep IgG antibody (Ab6747, 1:2000, Abcam). The protein bands labeled with the
25 antibodies were visualized using a Western-blotting chemiluminescence luminol reagent

1 (Santa Cruz Biotechnology) by exposure to X-ray films (Amersham). Densitometry of
2 proteins on Western blots was performed using the Scion Image software.

3

4

5 *2.12. Lipoprotein profiling*

6 Individual mouse plasma from WT (n=12) or *Thb*^{-/-} (n=8) mice fed the LFD or the HFD
7 (WT, n=11 and *Thb*^{-/-}, n=10) was injected onto a Superose 6HR 10/30 column (Amersham
8 Biosciences) connected to a fast protein liquid chromatography (FPLC) system (Amersham
9 Biosciences). Lipoproteins were eluted at a constant 0.3 ml/min flow rate with Tris-buffer
10 saline containing 0.074% EDTA and 0.02% sodium azide. Total cholesterol and triglyceride
11 levels were determined in the different fractions using commercially available kits from
12 Diasys (Diagnostic Systems International, Condom, France).

13

14 *2.13. DNA microarray/transcriptional profiling*

15 The transcriptional profiles in liver from 7-month-old WT mice and *Thb*^{-/-} mice fed either
16 the LFD or the HFD for 5 months (mice were fasted 6h before sacrifice) were probed using
17 the whole genome Affymetrix GeneChip® Mouse Genome 430 2.0 Array. For each
18 condition, livers from 4 different mice were used, and the liver RNAs were further isolated
19 but importantly not pooled. Hybridizations (4 per condition), washing and scanning of the
20 chips were carried out at the MicroArray Facility in Leuven (MAF, Leuven, Belgium). The
21 rest of the procedure was performed as previously described [33].

22

23 *2.14. Statistical analyses*

24 Data are presented as means ± standard errors. The effect of the genotype (KO vs WT) was
25 tested using the Student's t-test. The cut-off for statistical significance was set at a p-value of

1 0.05. Area under Curve was calculated with Prism (GraphPad Software Inc., Version 3.0,
2 San Diego, CA, USA).

3

4

5 **3. Results**

6 **3.1 Post-natal growth restriction and alteration of the GH/IGF-1 axis in *Thb*^{-/-} mice**

7 The generation of mice deficient for *Thb* has been previously described elsewhere [14]. In
8 absence of any dietary challenge (normal laboratory chow), the most apparent phenotype of
9 *Thb*^{-/-} mice was their reduced body mass (Fig. 1a) and reduced body length (Fig. 1b) as soon
10 as 4 weeks of age onwards, suggesting post-natal growth restriction. Growth hormone (GH)
11 as released by the pituitary is essential for normal growth and development. Of note,
12 deletion of *Thb* severely decreased GH circulating levels in 7-weeks-old mice supporting the
13 hypothesis of alterations in the hypothalamo-pituitary axis (Fig. 1c). Most of the effects of
14 GH are exerted by IGF-I, (Insulin-like Growth Factor-I also known as somatomedin C) a
15 peptide hormone produced mainly by the liver but also locally by specific tissues [34, 35].
16 Systemic levels of IGF-I were significantly reduced in 7-weeks-old *Thb*^{-/-} mice compared to
17 age-matched controls (Fig. 1d). The bioavailability of IGF-I is regulated by specific
18 binding proteins (IGFBP), insulin-like growth factor-binding protein-3 (IGFBP-3) being the
19 most abundant IGFBP in circulation that binds the majority ($\approx 90\%$) of circulating IGF-I
20 [36]. Plasma IGFBP-3 concentrations were similar in 7-weeks-old WT mice and *Thb*^{-/-} mice
21 indicating that reduced IGF-I content by change in plasma IGFBP-3 concentrations is
22 unlikely and cannot account for the small-body phenotype observed in *Thb*^{-/-} mice (Fig. 1e).
23 Liver is responsible for at least 75% of the circulating IGF-I levels [34]. Therefore, we
24 checked for hepatic *Igf-I* mRNA levels in 7-weeks-old WT mice and *Thb*^{-/-} mice. Our results

1 indicated that *Igf-1* mRNA in liver showed a similar pattern to plasma IGF-I in *Thb*^{-/-} mice,
2 as a probable consequence of the reduced stimulation by GH (Fig. 1f). GH-dependent
3 stimulation of *Igf-1* expression also takes place in peripheral tissues among which adipose
4 tissue and skeletal muscle. Similar to the liver, it was found that brown adipose tissue and
5 white adipose tissue as well as muscle *Igf-1* mRNA levels were severely reduced in 7-
6 weeks-old *Thb*^{-/-} mice, suggesting that the peripheral production of IGF-1 was globally
7 impaired by the deletion of *Thb* (Fig. 1f). Together, it was concluded that *Thb*^{-/-} mice exhibit
8 early growth retardation due to a probable alteration of the GH/IGF-I axis.

9

10 **3.2 *Thb*^{-/-} mice are protected from HFD-induced adipocyte hypertrophy**

11 Chronic administration of a HFD is known to induce obesity and obesity-associated insulin
12 resistance in mice [37]. We therefore decided to study the role that ThB may play in diet-
13 induced obesity by placing wild-type (WT) and *Thb*^{-/-} males, at 2 months of age, on either a
14 synthetic control LFD (10% fat, D12450B, Research Diets, Inc.) or a synthetic high
15 (saturated-lard) fat diet (45% fat, D12451, Research Diets, Inc.) for 25 weeks (see Table I
16 for the composition). D12450B and D12451 diets have been extensively used with success
17 in previous metabolic studies [29, 38, 39]. First, detailed analysis of food intake was
18 assessed throughout the period of the diet intervention. Surprisingly, food intake (g) and
19 consequently energy intake (Kcal) were significantly reduced in *Thb*^{-/-} mice compared to WT
20 mice after the HFD (supplemental Fig. 1a and supplemental Fig. 1b). However, when energy
21 consumption was corrected for body mass, it becomes significantly higher in *Thb*^{-/-} mice fed
22 the LFD or the HFD, as compared to appropriate WT control mice, suggesting that the
23 differences observed in body mass between the 2 sets of mice may not be attributable to
24 decreased energy consumption (supplemental Fig. 1c). Furthermore, while feeding the HFD
25 caused a significant mass gain in WT mice, the effect was less evident in *Thb*^{-/-} mice (Fig.

1 2a). Area under the curve (for the evolution of the body mass throughout the diet
2 intervention) confirmed that body mass of *Thb*^{-/-} mice was smaller compared to WT control
3 mice, whatever the quality of the food (Fig. 2b). We also assessed body mass at the end of
4 the diet intervention (Fig. 2c). It was confirmed that *Thb*^{-/-} mice did not recover from their
5 initial reduced body mass. Then, we turned to the study of the overall body mass change
6 (expressed in % of the initial body mass) (Fig. 2d). HFD feeding caused significantly more
7 body mass gain compared with LFD feeding in WT mice and while not significant (p=0,07),
8 *Thb*^{-/-} mice tend to be protected from HFD-induced mass gain.

9 After 25 weeks of diet intervention, significant effects for the diet but also of the genotype
10 were observed for the weight of subcutaneous (Fig. 3a; expressed as a percentage of total
11 body weight) and epididymal fat pads (Fig. 3b), two fat depots that are assumed to reflect
12 the overall adiposity of the animals [40]. Of note, deletion of *Thb* had no impact on the
13 relative brown adipose tissue weight (Fig. 3b). A histological analysis of the reproductive
14 white adipose tissue (WAT) between WT mice and *Thb*^{-/-} mice revealed a reduction in
15 adipocyte size in *Thb*^{-/-} mice, fed the LFD or the HFD (Fig. 3c). Further analysis of the
16 adipose tissue morphology using image analysis confirmed this reduction in adipocyte size
17 in *Thb*^{-/-} mice and consistently, demonstrates the presence of hyperplasia (Fig. 3d). To
18 uncover the potential systemic manifestations of reduced adiposity in *Thb*^{-/-} mice, we
19 measured two different plasma lipid parameters (free fatty acids and glycerol are good
20 indicators of adipose spontaneous lipolysis) in WT mice and *Thb*^{-/-} mice. No significant
21 changes in plasma free fatty acids and glycerol content were observed between WT mice
22 and *Thb*^{-/-} mice indicating that spontaneous lipolysis is likely not exacerbated by the deletion
23 of *Thb* and cannot account for by the reduced adipose tissue mass (Fig. 3e and 3f). Together,
24 we concluded that *Thb*^{-/-} mice are smaller than WT counterparts, while they ingest more
25 calories and display adipose hyperplasia.

1

2 **3.3 *Thb*^{-/-} mice display better insulin sensitivity and hypoglycemia**

3 It is known that small adipocytes are more insulin-sensitive than large adipocytes and
4 therefore, they may augment glucose transport and whole body sensitivity [41, 42]. Hence,
5 reduction in the adipocyte size, as observed in *Thb*^{-/-} mice fed the LFD or the HFD, could
6 limit the HFD-induced insulin resistance, as demonstrated in several animal models of diet-
7 induced obesity [43]. To test whether insulin sensitivity was indeed better in *Thb*^{-/-} mice, we
8 performed an insulin tolerance test (ITT) in WT mice and *Thb*^{-/-} mice fed the LFD or the
9 HFD. In mice fed the LFD, an i.p. insulin challenge (0.75 unit per kilogram of body weight)
10 resulted in a similar blood glucose decrease (Fig. 4a). However, in mice fed the HFD,
11 insulin injection resulted in a more prominent blood glucose decrease in *Thb*^{-/-} mice
12 compared to WT mice, indicating that *Thb*^{-/-} mice are more sensitive to exogenous insulin.
13 Area-under-the-curve (AUC) values confirmed a significantly better preserved insulin
14 sensitivity of *Thb*^{-/-} mice on the HFD compared to WT mice (Fig. 4b).

15 We next determined whether glucose metabolism could be altered along with fat deposition
16 in *Thb*^{-/-} mice. To do so, we first evaluated circulating blood glucose levels in *Thb*^{-/-} mice.
17 Surprisingly and despite their higher relative energy intake compared to bodyweight, a
18 significant lower plasma glucose concentration was observed in fed or in short-term (5h)
19 fasted *Thb*^{-/-} mice, independently of the quality of the food (Fig. 4c and Fig. 4d). Following
20 18h and 24h of fasting, no differences were observed in plasma glucose levels between WT
21 mice and *Thb*^{-/-} mice suggesting that neoglucogenesis (a biological process that mainly
22 operates in the liver under fasting conditions and that describes the conversion of non-
23 carbohydrates such as glycerol, propionate, lactate and pyruvate into glucose) becomes
24 similarly active in both genotypes (Fig. 4d).

1 Taking into account the massive hypoglycaemia observed in *Thb*^{-/-} mice, it can be
2 hypothesized that *Thb*^{-/-} mice are more insulin sensitive, as also suggested by the data of Fig.
3 4a. In agreement with this notion, endogenous plasma insulin levels were consistently lower
4 in *Thb*^{-/-} mice compared with WT mice, under both the LFD and the HFD (Fig. 4e). Finally,
5 the insulin-mediated glucose uptake by the muscle is probably more efficient in *Thb*^{-/-} mice
6 than in WT mice because glycogen content of muscle was higher in *Thb*^{-/-} mice than in WT
7 mice (Fig. 4f). Overall, *Thb*^{-/-} mice appeared to be more insulin sensitive because they
8 maintained lower glucose concentrations with reduced amounts of insulin.

9 Hepatic glycogenolysis normally sustains blood glucose levels after short-term fasting.
10 Defective glycogenolysis and/or depletion of the initial glycogen stores in the liver of *Thb*^{-/-}
11 mice could lead to hypoglycemia. To determine if the absence of *Thb* also modify glycogen
12 metabolism in the liver, we evaluated the concentration of hepatic glycogen in WT mice and
13 *Thb*^{-/-} mice. It is of note that hepatic glycogen content was significantly reduced following
14 the disabling of *Thb* (Fig. 5a). Reduced concentration of glycogen in the liver of *Thb*^{-/-} mice
15 could reflect a marked reduction in the glycogen synthesis rate by the liver. A defect in the
16 mRNA expression level of Glycogen synthase 2 (*Gys2*), the rate-limiting enzyme for
17 glycogen synthesis in liver, is unlikely because RT-qPCR and DNA arrays demonstrated
18 that *Gys2* expression tend to be higher in *Thb*^{-/-} mice, although it did not reach statistical
19 significance (Fig. 5b). On the other hand, glycogen utilization could be theoretically
20 enhanced by *Thb* deletion and account for by the reduced glycogen content. Yet, the mRNA
21 expression of genes encoding critical enzymes for glycogen utilisation (*Gbe1*, *Gaa*) was
22 lower in *Thb*^{-/-} mice and therefore does not support this hypothesis (supplemental Table).

23 Overall, it was concluded that peroxisomal ThB is involved in carbohydrate metabolism
24 because circulating glucose levels as well as liver/muscle glycogen content are affected
25 when *Thb* is deleted.

1

2 3.4 *Thb*^{-/-} mice do not develop HFD-induced liver steatosis

3 Consistent with the notion that the peroxisomal ThB is primarily an oxidative enzyme,
4 recent evidence suggests that ThB plays a functional role in fatty acid pattern of total liver
5 lipids, especially when mice are metabolically challenged with the potent PPAR α agonist
6 Wy14,643 [24]. Therefore, it can be expected that the effects of *Thb* deletion become much
7 more severe under conditions of fat overload such as chronic high fat feeding, a
8 physiological condition that activates PPAR α and PPAR α signaling in liver [26]. The liver
9 is a critical organ that may indirectly contribute to the reduced adiposity observed in *Thb*^{-/-}
10 mice *via* increased peroxisomal and/or mitochondrial fatty acid β -oxidation (FAO).
11 Quantitative assays were therefore conducted with liver samples of WT mice and *Thb*^{-/-} mice
12 in order to evaluate the impact of *Thb* deletion on triglyceride content. As expected, HFD
13 significantly increased hepatic lipid storage in WT mice (supplemental Fig. 1a). Regarding
14 *Thb*^{-/-} mice, liver triglyceride content was also increased by the HFD to a further extent
15 compared to WT mice, yet the effect was not significantly different (p=0.07). The definitive
16 evidence that ThB was required for the maximal rate of peroxisomal fatty acid β -oxidation
17 came with the finding that the content of docosanoic acid (C22:0), tetracosanoic acid
18 (C24:0) and possibly hexacosanoic acid (C26:0) was higher in liver samples of *Thb*^{-/-} mice
19 (supplemental Figure 1b). Together, these data fit with the notion that ThB plays a
20 functional role in lipid handling.

21

**22 3.5 *Thb*^{-/-} mice display increased plasma HDL-cholesterol (HDL-c) levels together with
23 higher liver cholesterol content**

24 To go further into the analysis of the *Thb* deficient mouse model, other plasma lipid
25 parameters were assessed. Whatever the genotype and the feeding status, no significant

1 changes in circulating TG were found (Fig. 6a). In contrast, total cholesterol content was
2 markedly elevated in the plasma of *Thb*^{-/-} mice, fed the LFD or the HFD (Fig. 6d). Profiling
3 of lipoproteins using Fast Protein Liquid Chromatography analysis confirmed that plasma
4 TG was only modestly affected by the deletion of *Thb* (Fig. 6b and 6c). Lipoprotein profiles
5 obtained from animal fed the LFD or the HFD demonstrated that the plasma cholesterol was
6 found almost entirely as HDL (High Density Lipoproteins) cholesterol (HDL-c) in both
7 genotypes (Fig. 6d). In agreement, the increase in total plasma cholesterol previously
8 observed in *Thb*^{-/-} mice was exclusively attributable to the HDL fractions (Fig. 6e and 6f).
9 In mammals, the liver plays a critical role in lipoprotein cholesterol metabolism and
10 hepatocytes can acquire cholesterol *via* the scavenger receptor class B, type I (SR-BI), that
11 mediates the selective cholesterol uptake from HDL. Consistent with cholesterol enrichment
12 of HDL particles that transport excess cholesterol from peripheral tissues back to the liver,
13 the hepatic cholesterol content was significantly elevated in the liver of *Thb*^{-/-} mice,
14 especially under the HFD (Fig. 7a). These findings support the notion that ThB may play an
15 unexpected and indirect role in reverse cholesterol transport, and overall in cholesterol
16 homeostasis.

17

18 **3.6 Whole body cholesterol *de novo* biosynthesis is increased in *Thb*^{-/-} mice**

19 It is worth noting that some authors have reported that plasma HDL-cholesterol would be
20 related to cholesterol synthesis markers [44]. Furthermore, cumulative evidence in the
21 literature points to a functional role of the peroxisome in cholesterol metabolism, which is
22 consistent with the fact that part of the isoprenoid pathway is localized within the
23 peroxisomal matrix [11, 25, 33, 45-49]. Therefore, to further establish without any
24 ambiguity that ThB plays a functional in cholesterol homeostasis in mice, we evaluated
25 plasma lathosterol to cholesterol ratio, a parameter that often serves as a surrogate marker

1 for global cholesterol synthesis, because it correlates well with the cholesterol balance [50-
2 52]. Unlike cholesterol, the pool of lathosterol is small and turns over rapidly, making
3 lathosterol an analyte of choice [53]. As shown in Fig. 7b, lathosterol concentration and
4 lathosterol to cholesterol ratio were significantly elevated in plasma samples of *Thb*^{-/-} mice
5 fed either the LFD or the HFD, indicating enhanced whole body cholesterol biosynthesis.

6

7 **3.7 *De novo* cholesterol biosynthesis in liver and intestine cannot account for by the** 8 **elevated cholesterol concentration in the plasma of *Thb*^{-/-} mice.**

9 Liver and small intestine are major organs for cholesterol homeostasis and HDL-cholesterol
10 metabolism. In the mouse, *Thb* is expressed at a much higher level in liver than in intestine
11 where mRNAs for *Thb* are barely detectable [4, 15]. We therefore first examined hepatic
12 cholesterol metabolism in WT mice and *Thb*^{-/-} mice, fed either the LFD or the HFD.
13 Increased hepatic content of cholesterol in *Thb*^{-/-} mice could be normally associated with
14 reduced expression of cholesterol metabolism associated genes, as a common negative
15 feedback process. To verify whether this was the case, we used RT-qPCR to quantify the
16 steady-state expression level of two highly critical genes for cholesterol biosynthesis, i.e.
17 *Hmg-CoA synthase* and *Hmg-CoA reductase* (the rate-limiting enzyme in cholesterol
18 biosynthesis) in the liver of WT mice and *Thb*^{-/-} mice fed the LFD or the HFD. Even if it did
19 not reach statistical significance, the absence of *Thb* tend to decrease the mRNA levels of
20 *Hmg-CoA synthase* (Fig. 7c). In parallel, the deletion of *Thb* had no consequence on the
21 mRNA levels of *Hmg-CoA reductase* and HFD induced a similar decrease in hepatic *Hmg-*
22 *CoA reductase* mRNA levels (Fig. 7c). Western blotting experiment confirmed this
23 observation at the level of HMG-CoA reductase protein (data not shown). Importantly, the
24 mRNA levels of downstream genes of *Hmg-CoA synthase* and *Hmg-CoA reductase* such as
25 *Pmvk*, *Mvd*, *Idi1*, *Fdps*, *Fdft1* (mevalonate pathway) or *Sqle* (squalene pathway) and *Cyp51*,

1 *Sc5d*, *Dhcr7*, *Nsdhl* (lanosterol pathway) were all coordinately decreased in the liver of *Thb*^{-/-}
2 ^{-/-} mice fed the LFD (supplemental table and data not shown). Because the HFD *per se*
3 strongly decreases the mRNA levels of the abovementioned genes, we failed to observe
4 exacerbated decrease of this array of genes in *Thb*^{-/-} mice fed this particular regimen,
5 compared to WT control mice (supplemental table and data not shown). Last, the hepatic
6 mRNA expression level of *Srebp2*, the transcription factor regulating the cholesterol
7 biosynthesis pathway, was also decreased in *Thb*^{-/-} mice fed the LFD.

8 While this is not a definitive proof, the gene expression analysis observed here globally
9 supports the hypothesis that cholesterol biosynthesis in the liver probably does not
10 massively account for by the elevated cholesterol concentration in the plasma of *Thb*^{-/-} mice.
11 As previously recall, the small intestine is an important site for the synthesis and secretion of
12 the Apo-AI protein, the principal apoprotein of HDL. In agreement, about 50% of total
13 plasma Apo-AI protein comes from the intestine [54]. Therefore, in view of the elevated
14 plasma HDL cholesterol levels observed in *Thb*^{-/-} mice, it could be reasoned that this
15 metabolism is modulated in the small intestine. However, RT-qPCR experiments do not
16 support this hypothesis because none of the following genes (*Abca1*, *Apo-aI*, *Apo-aII*, *Sr-bI*)
17 were dysregulated by the deletion of *Thb* (data not shown). The same was also true for the
18 jejunal expression of *Hmg-CoA synthase*, *Hmg-CoA reductase*, *Abcg5* and *Abcg8* genes
19 suggesting that elevated jejunal *de novo* biosynthesis of cholesterol cannot account for by
20 the elevated whole body cholesterol metabolism observed in *Thb*^{-/-} mice (data not shown).

21

22 **3.8 *Thb*^{-/-} mice display increased mRNA levels of some bile acid synthesizing genes as** 23 **well as elevated content of some bile acids**

24 Excess cholesterol can be toxic in the liver where it may contribute to non-alcoholic fatty
25 liver disease. As part of the physiologic response to the relative cholesterol enrichment

1 observed in the liver of *Thb*^{-/-} mice, mRNA expression levels of *Cyp7a1* (*cytochrome P450,*
2 *family 7, subfamily A, polypeptide 1*; CYP7A1 is the rate limiting step in the classical
3 pathway of bile acid biosynthesis), *Cyp39a1* (*cytochrome P450, family 39, subfamily A,*
4 *polypeptide 1*; CYP39A1 is involved in bile acid hydroxylation) and *Akr1d1* (*aldo-keto*
5 *reductase, family 1, member d1*; AKR1D1 has a crucial role in synthesis of primary bile
6 acids) were significantly higher in the livers of *Thb*^{-/-} mice fed the LFD or the HFD (Fig.
7 7d). Hence, synthesis and perhaps secretion of bile acids, which constitutes a major route for
8 elimination of excess cholesterol from the liver, might be increased in the liver of *Thb*^{-/-}
9 mice. To verify whether this up-regulation of mRNA levels of bile acid synthesizing genes
10 was functionally translated, the content of some bile acid species in mice was evaluated in
11 WT and *Thb*^{-/-} mice. As illustrated Fig.7e, deletion of *Thb* was associated with a significant
12 increase in cholic acid (CA, 3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid) and deoxycholic acid
13 ((DCA, (3 α , 5 β , 12 α , 20R)- 3, 12- dihydroxycholan- 24- oic acid)) when mice are fed the
14 LFD, suggesting the increased conversion of cholesterol into bile acids. The same picture
15 was also present under the HFD but did not reach statistical significance. The gene
16 expression level of *Abcg5* and *Abcg8* (*Abcg*: *ATP-binding cassette, subfamily G*; two ABC
17 half-transporters that work cooperatively in the biliary excretion of sterols into the
18 canalculus) was significantly increased in hepatic samples of *Thb*^{-/-} mice compared to WT
19 control mice (Fig. 7f and supplemental table).

20 Together, these findings support the concept of a compensatory hepatic up-regulation of bile
21 acid synthesizing enzymes as well as of transporters (that mediate active efflux of
22 cholesterol) in the liver of *Thb*^{-/-} mice.

23

24 **3.9 Cholesterol *de novo* biosynthesis is increased in the kidney of *Thb*^{-/-} mice**

1 Given that in addition to the liver, peroxisomes are abundantly present in the kidney where
2 both the *Thb* gene is well expressed and the ThB protein localized, we sought for evidence
3 that cholesterol *de novo* biosynthesis could be increased in the kidney of *Thb*^{-/-} mice [4, 55].
4 Importantly, lathosterol content and lathosterol to cholesterol ratio were significantly
5 elevated in kidney samples of *Thb*^{-/-} mice, indicating enhanced *in situ* cholesterol
6 biosynthesis (Fig. 8a and Fig. 8b). However, renal cholesterol content was not different
7 between the two genotypes (Fig. 8c). This last observation suggests that excess production
8 of cholesterol by the kidney in *Thb*^{-/-} mice may be directly delivered into the bloodstream,
9 where it could perhaps be further associated with circulating HDL lipoproteins. The increase
10 in renal lathosterol to cholesterol ratio observed in *Thb*^{-/-} mice was supported by the parallel
11 increase expression of *Hmg-CoA synthase*, *Fdpps* and *Srebp2* in the kidney (Fig. 8d).
12 Finally, western-blotting experiment confirmed the enrichment in the nuclear and active
13 form of the transcription factor SREBP-2, a master transcriptional regulator of cholesterol
14 biosynthetic genes (Fig. 8e).

15

16

17 **4. Discussion**

18

19 It was previously reported that peroxisomal β -oxidation of fat was enhanced during the
20 course of diet-induced obesity [27, 28]. The authors have proposed that elevated
21 peroxisomal β -oxidation would contribute to the resistance of diet-induced hepatic steatosis
22 and obesity. In light of this finding, the initial goal of this study was to evaluate the
23 consequences of *Thb* deletion in mice chronically fed a HFD. Unexpectedly, our data first
24 establish a critical role for ThB in the control of body growth, under baseline conditions.
25 The reduced body mass and size probably account for by the marked reduction in the
26 circulating levels of GH and IGF-I in 7-weeks-old *Thb*^{-/-} mice, because circulating levels of
27 IGF-1 regulate bone growth and density [56]. Further studies using dual-emission X-ray

1 absorptiometry (DEXA)-scan analysis of whole-body are deserved to assess for potential
2 changes in bone mineral content, bone density and bone size in *Thb*^{-/-} mice. Altered
3 biodisponibility of the remaining 66% IGF-I in 7-weeks-old *Thb*^{-/-} mice is unlikely because
4 circulating IGFBP-3 levels and liver *Igfbp-3* mRNA levels were unaltered (data not shown).
5 Here, it should be acknowledged that the deletion of the closely related gene *Scpx* thiolase
6 did not reveal any body growth impairment [8]. However, post-natal growth retardation has
7 been previously reported for other mouse models deficient for the peroxisomal enzymes
8 such as ACOX1 and MFP2 [57, 58]. The mechanism of body growth retardation in *Acox1*^{-/-}
9 and *Mfp2*^{-/-} mutant mice remains largely speculative and was not elucidated at the moment.
10 Therefore, one of the future challenges will be to delineate the exact cause(s) responsible for
11 the body growth restriction observed in *Thb*^{-/-} mice. Also more extensive analyses that aim
12 at determining if the systemic infusion of recombinant GH or IGF-I could correct the
13 defective growth of *Thb*^{-/-} mice have to be envisioned. It should be acknowledged here that
14 the reduction of global body weight in *Thb*^{-/-} mice is probably not due to a reduction in
15 energy intake because corrected energy intake to body mass is higher in *Thb*^{-/-} mice fed the
16 LFD or the HFD. However, we cannot rule out at this point that growth retardation is caused
17 by intestine malabsorption.

18 Strikingly, our studies indicate that *Thb*^{-/-} mice are protected from HFD-induced expansion
19 of epididymal adipose tissue, the most commonly used fat depot in mouse studies, which
20 likely reflects differences in overall adiposity [40]. An important question is why there is an
21 apparent limit to the expansion of adipose tissue in *Thb*^{-/-} mice fed the HFD, despite
22 increased (corrected) energy intake. Total body composition analysis by quantitative nuclear
23 magnetic resonance and determination of whole body metabolic activity as well as fuel
24 preference should provide the first clues. It is also conceivable that the reduced adiposity in
25 *Thb*^{-/-} mice stems from increased energy expenditure arising from physical activity or

1 changes in the ratio between lipogenesis and lipolysis/β-oxidation. It is also possible that the
2 mobilization of fat stores, which occurs predominantly through the hydrolysis of
3 triglycerides into glycerol and fatty acids, is increased in *Thb*^{-/-} mice. However, this latter
4 hypothesis is unlikely because plasma free fatty acids and glycerol levels were not
5 differently affected by the deletion of *Thb* (figure 9).

6 Our results also support the notion that ThB is involved in glucose homeostasis because *Thb*^{-/-}
7 mice fed either the LFD or the HFD display hypoglycaemia in fed and after a short-term
8 (5h) fasting period. This finding is exciting because i) this is the first report showing that a
9 peroxisomal defect in mice is associated with a marked hypoglycemia that takes place not
10 only after a short-term food deprivation but also in the fed state, ii) it definitively provides
11 evidence that despite their high amino acid sequence homology and high overlap in substrate
12 specificity, ThA and ThB isoforms do not drive the same *in vivo* functions in mice [7]. Of
13 note, only a moderate diminution in glucose plasma level (that did not reach statistical
14 significance) was observed in *Scp2/Scpx*^{-/-} mice, when kept under standard laboratory
15 conditions [8]. Whether similar conclusions would have been drawn with *Scp2*^{-/-} mice fed
16 the HFD remains an unresolved issue at present. Regarding the *Acox1*^{-/-} and the *Mfp2*^{-/-}
17 mouse models, it was reported that insulin and glucose levels were normal, both in fed and
18 fasted conditions [33, 59]. The question arises how the lack of the peroxisomal ThB enzyme
19 leads to hypoglycemia? At present, the underlying molecular mechanisms are far to be
20 understood but it may be hypothesized that the hypoglycemia observed in *Thb*^{-/-} mice is the
21 consequence of the hormonal and metabolic perturbations that include reduced circulating
22 levels of insulin and hepatic glycogen stores. One possibility is that *Thb* deficiency leads to
23 enhanced hepatic insulin signaling resulting in the suppression of gluconeogenesis and
24 enhanced glycolysis. However, this scenario is rather unlikely because glucose production
25 by the liver (gluconeogenesis) in the fed state is relatively minor and plasma glucose is

1 primarily derived from ingestion of nutrients. If we adhere to the notion that glycolysis
2 would be enhanced in *Thb*^{-/-} mice, it also implies that glucose oxidation, and in turn the
3 derived pool of pyruvate/acetyl-CoA, are higher providing more substrates for the
4 subsequent enzymatic reactions of the Krebs's cycle. However, the experimental backing of
5 this claim is weak because the mitochondrial succinate dehydrogenase activity and the
6 mitochondrial oxidation rate of palmitoyl-CoA were similar in WT mice and in *Thb*^{-/-} mice
7 (data not shown). Despite this, we cannot rule out that the absence of ThB causes a higher
8 flux of pyruvate towards the Krebs's cycle, explaining at least in part, the hypoglycemia
9 observed in *Thb*^{-/-} mice. Intriguingly, compared to WT mice, *Thb*^{-/-} mice fed the LFD display
10 similar fat pads mass supporting the notion that ThB may have some functions in glucose
11 metabolism that could be independent of the degree of fat expansion.

12 During the first hours of fasting, hepatic glycogenolysis normally provides most of the
13 circulating blood glucose. Therefore, the steeper drop in blood glucose observed in *Thb*^{-/-}
14 mice during early (5h) fasting probably reflects the reduced glycogen reserve and/or an
15 impaired liberation of glucose. After 18h and 24h of fasting, blood glucose concentrations
16 were similar in WT mice and in *Thb*^{-/-} mice reinforcing the notion that gluconeogenesis is
17 likely not responsible for the hypoglycemia observed in fed and short-term fasted *Thb*^{-/-}
18 mice.

19 *Thb*^{-/-} mice display heightened response to exogenous insulin administration when placed on
20 the HFD, which might explain at least in part, their concomitant hypoglycaemia. *Thb*^{-/-} mice
21 seems to be more sensitive to insulin than WT mice but the definitive evidence would imply
22 further *in vivo* experiments that aim at calculating both the percentage stimulation of whole-
23 body glucose utilization and the percentage inhibition of hepatic glucose output by insulin.
24 Future investigations that aim at characterizing the metabolic phenotype of the *Thb*^{-/-} mice
25 under a synthetic diet will also require the determination of the relative contribution of each

1 insulin target tissue in the observed hypoglycaemia.

2 Higher glucose utilization due to an impaired mitochondrial FAO in the skeletal muscle
3 could theoretically explain part of the reduction in circulating blood glucose. It was recently
4 reported by Derks and colleagues that inhibition of mitochondrial FAO *in vivo* slightly
5 suppresses gluconeogenesis but instead enhances clearance of glucose in mice [60]. In our
6 mouse model of global deletion of *Thb*, liver mitochondrial oxidation of palmitate (C16:0)
7 and plasma levels of β -hydroxybutyrate were similar as compared to WT mice (data not
8 shown). These results suggest that *Thb* deletion does not massively reverberate on
9 mitochondrial oxidative functions at least in the liver, but whether the same may also apply
10 to the muscle remains to be established. Therefore, increased glucose consumption by the
11 muscle remains a plausible explanation for the hypoglycemia observed in *Thb*^{-/-} mice.

12 Similar to previous studies performed by others with mice carrying a single deletion of a
13 peroxisomal enzyme, our data point to the notion that cholesterol metabolism is altered in
14 *Thb*^{-/-} mice [11, 25, 33, 45-49]. However, it was also recently reported that cholesterol
15 synthesis is not altered in peroxisome deficient hepatocytes of liver *Pex5*^{-/-} mice, casting
16 doubts on the real impact of the peroxisome in hepatic cholesterologenesis. Our results
17 strengthen the notion that peroxisome is functionally involved in *de novo* biosynthesis of
18 cholesterol, as well as in plasma HDL cholesterol metabolism. Interestingly, and in contrast
19 to what we observed for the deletion of *Thb*, the deficiency of peroxisomes as the result of
20 the *Pex2* gene knockout was shown to lead to decreased HMG-CoA reductase activity and
21 cholesterol biosynthesis in the kidney [47]. It demonstrates that the cholesterol homeostatic
22 response within a tissue differs according to the identity and function of the peroxisomal
23 gene targeted. At the moment, it is unclear to us why HDL cholesterol was increased in the
24 plasma of *Thb*^{-/-} mice. It should be stressed that we found increased Apo-AI protein content
25 in the plasma HDL fraction in *Thb*^{-/-} mice, pointing toward an increase in the number of

1 HDL particles (unpublished data). In addition to a larger number of circulating HDL
2 particles, it is also possible that *Thb*^{-/-} mice display higher content of cholesterol in these
3 HDL particles. From a mechanistic view, it can be expected some changes in hepatic
4 lipoprotein synthesis and/or receptor-mediated lipoprotein cholesterol clearance following
5 the deletion of *Thb*. However, this aspect remains to be firmly established because *Thb*
6 deficiency did not change the steady-state mRNA expression level of liver *Sr-bI*, a cognate
7 receptor for HDL in the liver, or that of *Apo-aI*, the major ligand for SR-BI (supplemental
8 Table).

9 Even if we cannot rule out differences in intestinal absorption rates between WT mice and
10 *Thb*^{-/-} mice, it is likely that the increase in cholesterol synthesis is the larger factor
11 contributing to elevated plasma total cholesterol levels given the small amount of cholesterol
12 especially in the LFD (< 0,002%), and in the HFD (to some extent, ≈ 0,015%) used in the
13 present study. Supporting this notion, others have previously reported that in mice fed a
14 standard rodent chow containing relatively little cholesterol, the requirement in cholesterol
15 is achieved primarily through *de novo* biosynthesis [61]. Future studies should be directed at
16 investigating the consequence of *Thb* deficiency when mice are fed chow alone or
17 supplemented with 2% cholesterol.

18 Lathosterol content is higher in plasma and kidney in *Thb*^{-/-} mice, a phenotype reminiscent
19 of what is observed in mice deficient for the *Sc5d* (lathosterol-5-desaturase) gene [62].
20 Consistent with this observation, the hypothesis by which SC5D activity (the enzyme that
21 catalyzes the conversion of lathosterol to 7-hydroxycholesterol in the next to last step of
22 cholesterol synthesis) could be reduced in *Thb*^{-/-} mice becomes plausible. However, although
23 mice deficient in the *Sc5d* gene displayed elevated lathosterol levels compared to WT
24 control mice, a parallel decrease in plasma and liver cholesterol levels is observed, which is
25 not the case for *Thb*^{-/-} mice [62]. Therefore, it is unlikely that this postulated mechanism

1 could explain the hypercholesterolemia present in *Thb*^{-/-} mice.

2 Importantly, active synthesis of cholesterol by the kidney has been previously noted by
3 others especially under a cholesterol-free diet which is close to the LFD used in the present
4 study, leading to a level accounting for over 50% of the total renal cholesterol [63].
5 However, because the synthesis by the kidney of the Apo-AI protein is unlikely, it
6 reasonably raises the question to what extent the local increased synthesis of cholesterol is
7 involved in the elevated HDL-cholesterol plasma content observed in *Thb*^{-/-} mice [64].

8 What is also puzzling is that in a previous study using *Thb*^{-/-} mice (on the same genetic
9 background, Sv129) routinely fed the standard commercial pellet diet UAR A03-10
10 (SAFE), we did not find any changes in plasma lathosterol to cholesterol ratio between WT
11 mice and *Thb*^{-/-} mice [25]. While the exact reason for this discrepancy remains odd at the
12 moment, the quality of the long-chain fatty acids present in the diet, which is presumably
13 different from the synthetic LFD and HFD used in the present study, together with the
14 cholesterol/oxysterol content that likely differs, may play a prominent role in the observed
15 phenotype.

16 From a biochemical point of view, ThB catalyzes the last step of the β -oxidation pathway,
17 i.e., the conversion of 3-ketoacyl-CoA into shorter acyl-CoA (by two carbon atoms) together
18 with acetyl-CoA. Only limited information concerning the substrate specificity of ThB is
19 available at present. Mature form of ThA and ThB differing only in several amino acids, it
20 came with no surprise that both enzymes equally react with medium and long-straight chain
21 3-oxoacyl-CoAs [7, 65]. However, given the different traits of the phenotype observed in
22 *Thb*^{-/-} mice, it becomes clear that *ACCA1a* (ThA) and *ACCA1b* (ThB) isoforms are not fully
23 interchangeable. Others have previously reported that acetyl-CoA derived from the
24 peroxisomal β -oxidation of very long-chain fatty acids and medium-chain dicarboxylic acids
25 would be preferentially channeled to cholesterol synthesis inside the peroxisomes, without

1 mixing with the cytosolic acetyl-CoA pool [11, 46, 66]. However, the picture is far from
2 complete because it was also argued that peroxisomal β -oxidation of (very) long-chain fatty
3 acids supplies acetyl-CoA to the cytosol [67]. Here, we found that the single deletion of *Thb*
4 was leading to higher C22:0, C24:0 and possibly C26:0 content in the liver of *Thb*^{-/-} mice, as
5 a consequence of the probable reduced peroxisomal β -oxidation pathway. Whatever is the
6 metabolic fate of the peroxisomal pool of acetyl-CoA, the single deletion of *Thb* may
7 concurrently lead to its lower availability. One would thereby expect reduced *de novo*
8 cholesterol synthesis in the peroxisome and/or in the cytosol of (kidney) cells in *Thb*^{-/-} mice.
9 However, our results do not support this hypothesis because whole body synthesis of
10 cholesterol was enhanced in *Thb*^{-/-} mice fed the LFD or the HFD. Future investigations
11 employing mice with targeted disruption of the *Thb* gene in the liver or in the kidney should
12 help to precise the respective role of *Thb* in local cholesterol homeostasis *in vivo*.
13 Besides conversion of cholesterol into bile salts, the liver also disposes of plasma cholesterol
14 by secretion into bile unmodified. Consistent with the upregulation of the *Abcg5/Abcg8*
15 canalicular cholesterol transporters in the liver of *Thb*^{-/-} mice, the rate of biliary cholesterol
16 secretion might be increased in response to the accumulation of liver cholesterol. However,
17 proof of this concept will require future investigations that are beyond the scope of the
18 present study.
19 Collectively, our data support the concept that ThB plays a role in hepatic cholesterol and
20 lipoprotein metabolism in mice and opens the road to future investigations regarding bile
21 acid and glucose metabolism.
22 In conclusion, the *Thb*^{-/-} mouse model used in this study demonstrates that the peroxisome
23 could play a more important role than solely regulating very-long chain fatty acid β -
24 oxidation. In particular, our data suggest that regulating the activity of ThB *in vivo* in mice,

1 may provide an additional route for the therapeutic correction of disorders affecting plasma
2 cholesterol and glucose levels.

3

4

5 **Figure 1. Growth restriction and reduced plasma IGF-I and GH levels in *Thb*^{-/-} mice**

6 (a) Body mass and (b) body length of WT mice (white bars, n=27) and *Thb*^{-/-} mice (gray
7 bars, n=19) throughout week 4 till week 50 of life. (c) Plasma GH concentration in WT mice
8 (n=18) and *Thb*^{-/-} mice (n=16) aged of 1 month and half. (d) Plasma IGF-I concentration in
9 WT mice (n=16) and *Thb*^{-/-} mice (n=18) aged of 1 month and half. (e) Plasma IGFBP-3
10 concentration in WT mice (n=18) and *Thb*^{-/-} mice (n=16) aged of 1 month and half. (f) *Igf-1*
11 mRNA expression in the liver, brown adipose tissue (BAT), white adipose tissue (WAT)
12 and gastrocnemius of WT mice (n=9) and *Thb*^{-/-} mice (n=11) as determined by RT-qPCR.
13 Error bars represent ± SEM. Differences between WT mice and *Thb*^{-/-} mice were evaluated
14 using the Student's t-test (GraphPad Prism 3 software, San Diego, CA). * p<0.05, ** p<0.01;
15 *** p<0.001.

16

17 **Figure 2. LFD and HFD feeding of WT mice and *Thb*^{-/-} mice**

18 a) Evolution of body weight during the experimental feeding. Mice were weighed twice a
19 week. Data were expressed as mean ± SEM (n=9 to n=12 per group) b) Area Under the
20 Curve calculated from the evolution in body mass presented in a) c) Graph shows mean
21 body mass of WT mice and *Thb*^{-/-} mice fed the HFD or the LFD for 150-days. d) Body mass
22 changes (expressed in %) of mice are determined by comparing body mass values at the
23 beginning of the diet intervention and after the LFD or the HFD intervention. The Student's
24 t-test was used to compare the results obtained between two different groups of mice with

1 GraphPad Prism 3 software (San Diego, CA). * p<0.05, ** p<0.01; *** p<0.001, LFD: low-
2 fat diet; HFD: high-fat diet.

3

4 **Figure 3. *Thb*^{-/-} mice are protected from HFD-induced adipocyte hypertrophy**

5 a) Subcutaneous and b) epididymal and brown adipose tissues weights as a percentage of
6 body weight c) representative light micrographs (magnification 20x) of epididymal fat pads
7 d) adipocytes were counted from eight-to-ten randomly selected fields for each fat
8 epididymal pads (left part); quantification of adipocyte size using software analysis (right
9 part) e) free fatty acids and f) plasma glycerol in the four experimental groups of mice (n=8
10 to n=11 per group) that were fasted for 5h (starting at 5 am). Data were expressed as mean ±
11 SEM. The Student's t-test was used to compare the results obtained between two different
12 groups of mice (GraphPad Prism 3 software, San Diego, CA). * p<0.05, ** p<0.01; ***
13 p<0.001, LFD: low-fat diet; HFD: high-fat diet.

14

15 **Figure 4. *Thb*^{-/-} mice display hypoglycemia as the consequence of better insulin
16 sensitivity**

17 a) Insulin sensitivity test was performed in 5-h-fasted WT mice and *Thb*^{-/-} mice previously
18 fed the LFD or the HFD; note that plasma glucose was significantly less in HFD-KO mice
19 compared to HFD-WT mice at time T=20 min and T=40 min b) AUC, area under curves of
20 glucose response expressed as arbitrary units (a.u), c) Plasma glucose in WT and KO mice
21 fed the LFD or the HFD d) plasma glucose in WT and KO mice fed the LFD or the HFD
22 fasted for 0, 5, 18, and 24h ; note that plasma glucose level was significantly less in KO
23 mice compared to WT mice (both LFD and HFD) at time T=0 and T=5h of fasting e)
24 Endogenous plasma insulin levels in (fed) WT and *Thb*^{-/-} mice, f) Glycogen content in
25 muscle of WT mice and *Thb*^{-/-} mice, (WT LFD, n= 12; WT HFD, n=10; KO LFD, n= 8; KO

1 HFD, n= 8). Values are means ± SEM. The Student's t-test was used to compare the results
2 obtained between two different groups of mice (GraphPad Prism 3 software, San Diego,
3 CA). * p<0.05, ** p<0.01; *** p<0.001, LFD: low-fat diet; HFD: high-fat diet. KO = *Thb*^{-/-}.

4

5

6

7 **Figure 5. Hepatic glycogen content in WT mice and *Thb*^{-/-} mice previously fed the LFD**
8 **or the HFD.**

9 Hepatic glycogen content was determined as described under “Materials and Methods”. a)
10 liver glycogen content in WT mice and *Thb*^{-/-} mice normally fed (*ad libitum*) the LFD or the
11 HFD. Sacrifice was performed between 10 and 11 am b) Relative liver glycogen synthase-2
12 (*Gys2*) mRNA levels in WT mice and *Thb*^{-/-} mice, as measured by RT-qPCR. Values were
13 expressed as fold-change as compared to WT mice fed LFD arbitrarily set at 1.0. (WT LFD,
14 n= 10; WT HFD, n=9; KO LFD, n= 8; KO HFD, n= 9). Values are means ± SEM. The
15 Student's t-test was used to compare the results obtained between two different groups of
16 mice (GraphPad Prism 3 software, San Diego, CA). * p<0.05, ** p<0.01; *** p<0.001,
17 LFD: low-fat diet; HFD: high-fat diet. KO = *Thb*^{-/-}.

18

19 **Figure 6. Plasma HDL-cholesterol levels is elevated in *Thb*^{-/-} mice.**

20 a) Plasma triglyceride and d) plasma cholesterol content was determined in EDTA plasma of
21 WT mice and *Thb*^{-/-} mice fed the LFD or the HFD. b-c) Fast-protein liquid chromatography
22 analysis of plasma triglyceride or e-f) cholesterol of WT mice and *Thb*^{-/-} mice fed the LFD
23 or the HFD. 0,2 ml of individual mouse plasma sample was passed through a Superose 6-HR
24 column and total triglyceride and cholesterol content of individual fractions was determined

1 by an enzymatic method, as described in Materials and Methods. Each point is the mean ±
2 SEM. (WT LFD, n=12; KO LFD, n=8; WT HFD, n=11; KO HFD, n=10). The
3 corresponding legend for the different circles is indicated in the figure. KO = *Thb*^{-/-}. LFD:
4 low-fat diet; HFD: high-fat diet.

5

6

7

8 **Figure 7. Whole body cholesterol synthesis as well as hepatic cholesterol efflux and bile**
9 **acid synthesis are increased in *Thb*^{-/-} mice.**

10 a) Hepatic cholesterol concentration, b) Plasma lathosterol concentration and plasma
11 lathosterol to cholesterol ratio, c) mRNA expression of 3-hydroxy-3-methylglutaryl-
12 Coenzyme A synthase-1 (Hmg-Coa synthase) and 3-hydroxy-3-methylglutaryl-Coenzyme A
13 reductase (Hmg-Coa reductase), d) mRNA expression of cholesterol-7-alpha hydroxylase
14 (Cyp7a1), cholesterol-17-alpha hydroxylase (CYP17a1) and aldo-keto reductase family 1,
15 member D1 (Akr1d1) in the liver of WT mice and *Thb*^{-/-} mice fed the LFD or the HFD, e)
16 Hepatic content of cholic acid (CA) and deoxycholic acid (DCA), e) mRNA expression of
17 the ATP-binding cassette sub-family G member 5 (Abcg5) and ATP-binding cassette sub-
18 family G member 8 (Abcg8) in the liver of WT mice and *Thb*^{-/-} mice fed the LFD or the
19 HFD. (WT LFD, n= 12; WT HFD, n=12; KO LFD, n= 9; KO HFD, n= 8). Values were
20 expressed as fold-change as compared to WT mice fed LFD arbitrarily set at 1.0. The
21 Student's t-test was used to compare the results obtained between two different groups of
22 mice (GraphPad Prism 3 software, San Diego, CA). * p<0.05, ** p<0.01; *** p<0.001,
23 LFD: low-fat diet; HFD: high-fat diet. KO = *Thb*^{-/-}.

24

1 **Figure 8. The higher lathosterol to cholesterol ratio is accompanied by the**
2 **upregulation of cholesterol synthesizing genes in the kidney of *Thb*^{-/-} mice.**

3 a) Kidney lathosterol concentration, b) Kidney lathosterol to cholesterol ratio, c) Kidney
4 cholesterol content, d) mRNA expression of 3-hydroxy-3-methylglutaryl-Coenzyme A
5 synthase-1 (Hmg-Coa synthase), farnesyl di-phosphate synthase (Fdpps) and Sterol
6 Regulatory Element-Binding Protein-2 (Srebp-2) in the kidney, e) Nuclear protein extracts
7 pooled from kidneys of WT mice and *Thb*^{-/-} mice fed the LFD or the HFD, were analyzed by
8 Western blotting for the abundance of the mature form of the transcription factor SREBP-2.
9 Histone H1 was used for normalization of nuclear proteins. Quantification of bands relative
10 to histone H1 is given under the picture corresponding to the blot of SREBP-2, nSREBP-2:
11 nuclear SREBP-2. Ten to twelve mice were used per group. Values were expressed as fold-
12 change as compared to WT mice fed LFD arbitrarily set at 1.0. The Student's t-test was used
13 to compare the results obtained between two different groups of mice (GraphPad Prism 3
14 software, San Diego, CA). * p<0.05, ** p<0.01; *** p<0.001, LFD: low-fat diet; HFD:
15 high-fat diet. KO = *Thb*^{-/-}.

16

17 **Figure 9: ThB is involved in cholesterol and bile acid metabolism and plays a**
18 **prominent role in glucose homeostasis in mouse.** The important metabolic alterations
19 observed in mice deficient for *Thb* fed a LFD or a HFD for 5 months are summarized and
20 depicted. ThB: Thiolase B; SREBP2: sterol regulatory element-binding protein-2; *Abcg*:
21 ATP-binding cassette, subfamily G; *Cyp7a1*: cytochrome P450, family 7, subfamily A,
22 polypeptide 1; *Cyp39a1*: cytochrome P450, family 39, subfamily A, polypeptide 1; *Akr1d1*:
23 aldo-keto reductase, family 1, member d1; CA: cholic acid, DCA: deoxycholic acid; ↑:
24 increased; ↓: decreased; → : unchanged. Note that body weight and growth are also affected
25 by the deletion of *Thb* in mice fed a normal laboratory chow.

1

2 **Table 1:** Composition of diets used in the present study.

3

4 **Supplemental Table:**

5 Microarray analysis was performed on individual liver mRNA samples (n = 4 per group)
6 comparing the gene expression signals induced by the deletion of *Thb* in mice fed the LFD
7 or the HFD. Column C-F: Expression in WT mice fed the LFD was arbitrarily set at 1.0.
8 Changes in gene expression are expressed as fold-changes in comparison with WT mice fed
9 the LFD. LFD: low-fat diet; HFD: high-fat diet.

10

11

12 **Supplemental Figure 1:**

13 a) Food intake (expressed as g/week/mouse) of the four experimental groups (n=9 to n=12
14 per group) b) Energy intake (expressed as kcal/week/mouse) of the four experimental groups
15 (n=9 to n=12 per group) c) Energy intake corrected for body weight (expressed as
16 kcal/week/g of body weight). The Student's t-test was used to compare the results obtained
17 between two different groups of mice with GraphPad Prism 3 software (San Diego, CA). *
18 p<0.05, ** p<0.01; *** p<0.001, LFD: low-fat diet; HFD: high-fat diet.

19

20 **Supplemental Figure 2:**

21 a) Liver triglyceride content was evaluated using gas-chromatography in WT mice and *Thb*^{-/-}
22 mice at the end of the intervention diet (n=7 for each condition). b) Liver content of
23 docosanoic acid (C22:0), tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) in WT
24 mice and *Thb*^{-/-} mice fed the LFD or the HFD, (WT LFD, n= 11; WT HFD, n=4; KO LFD,

1 n= 7; KO HFD, n= 6). Errors bars represent \pm S.E.M. Significant effects were observed
2 using the Student's t-test. * $p < 0.05$, ** $p < 0.01$; LFD: low-fat diet; HFD: high-fat diet.

3

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15

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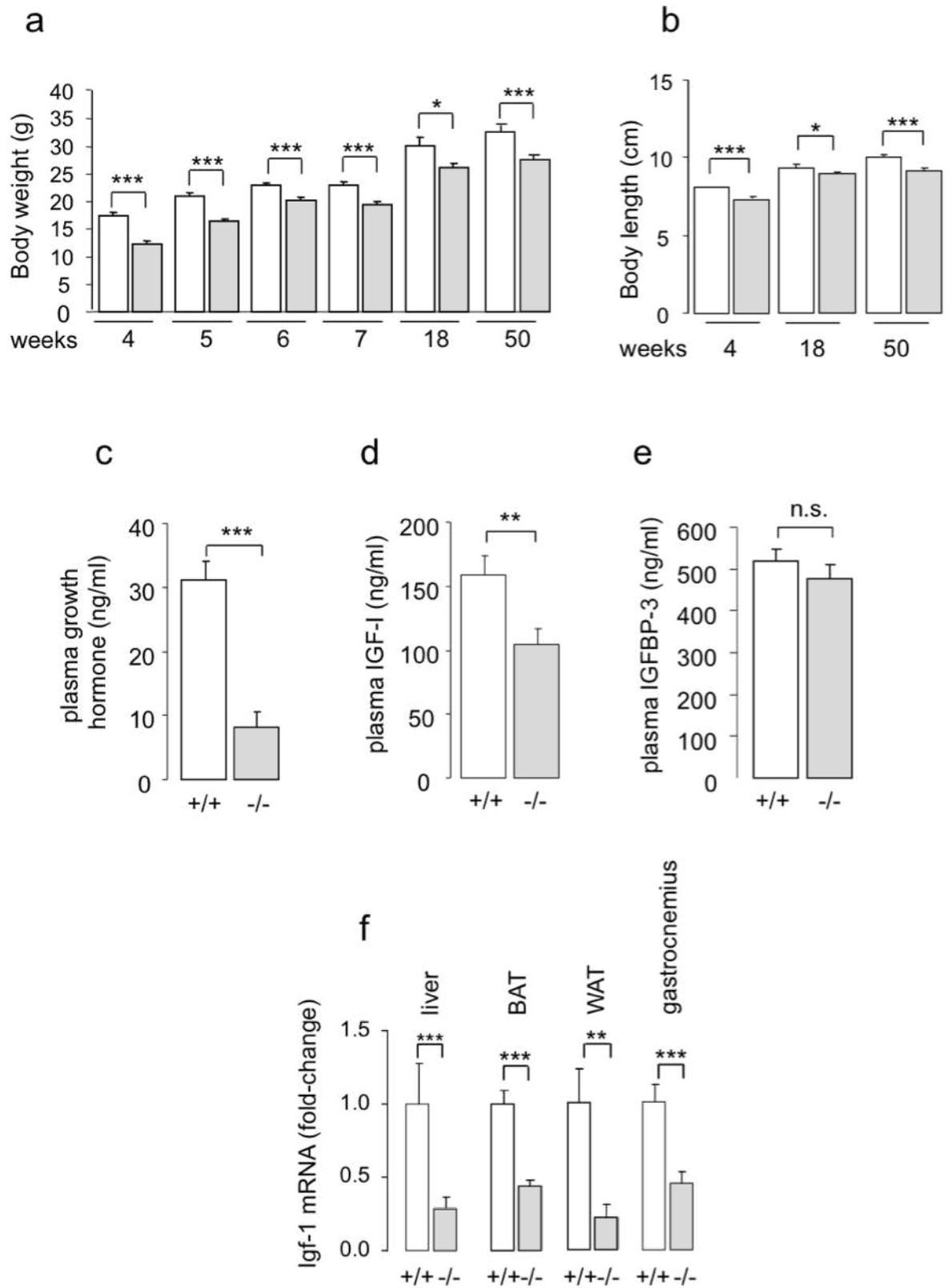
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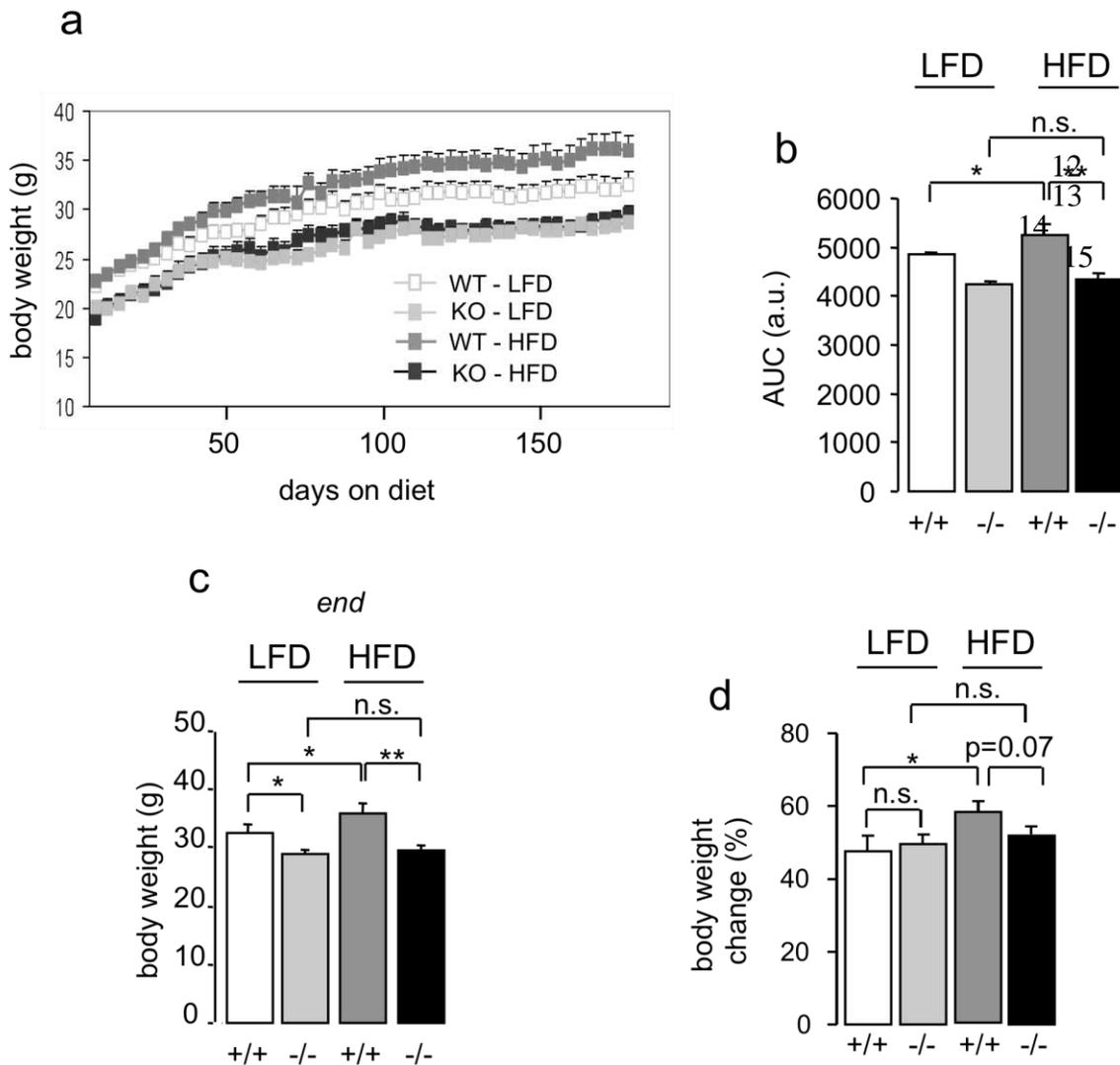


NICOLAS-FRANCES *et al.*, Fig. 1

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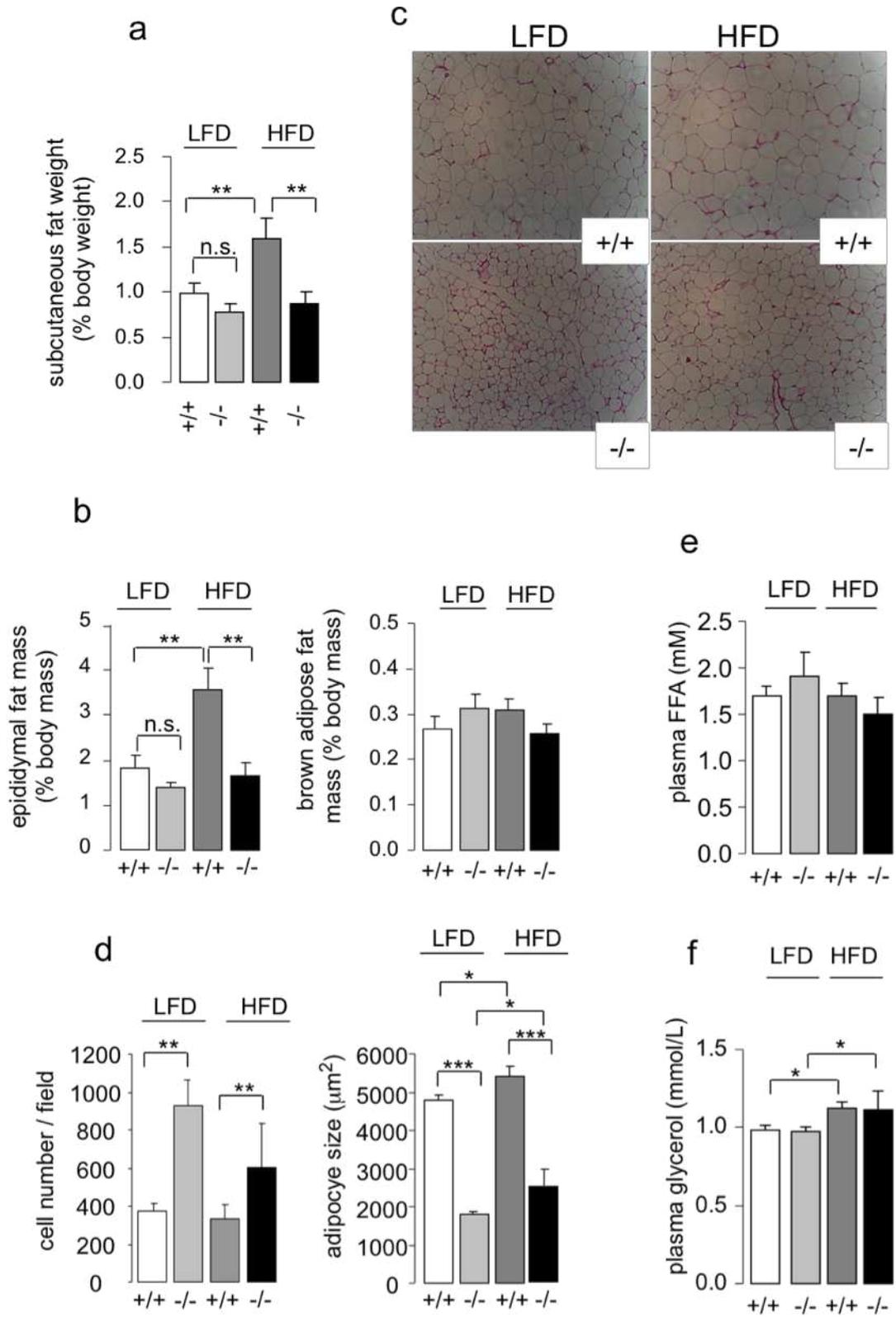
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NICOLAS-FRANCES *et al.*, Fig. 2

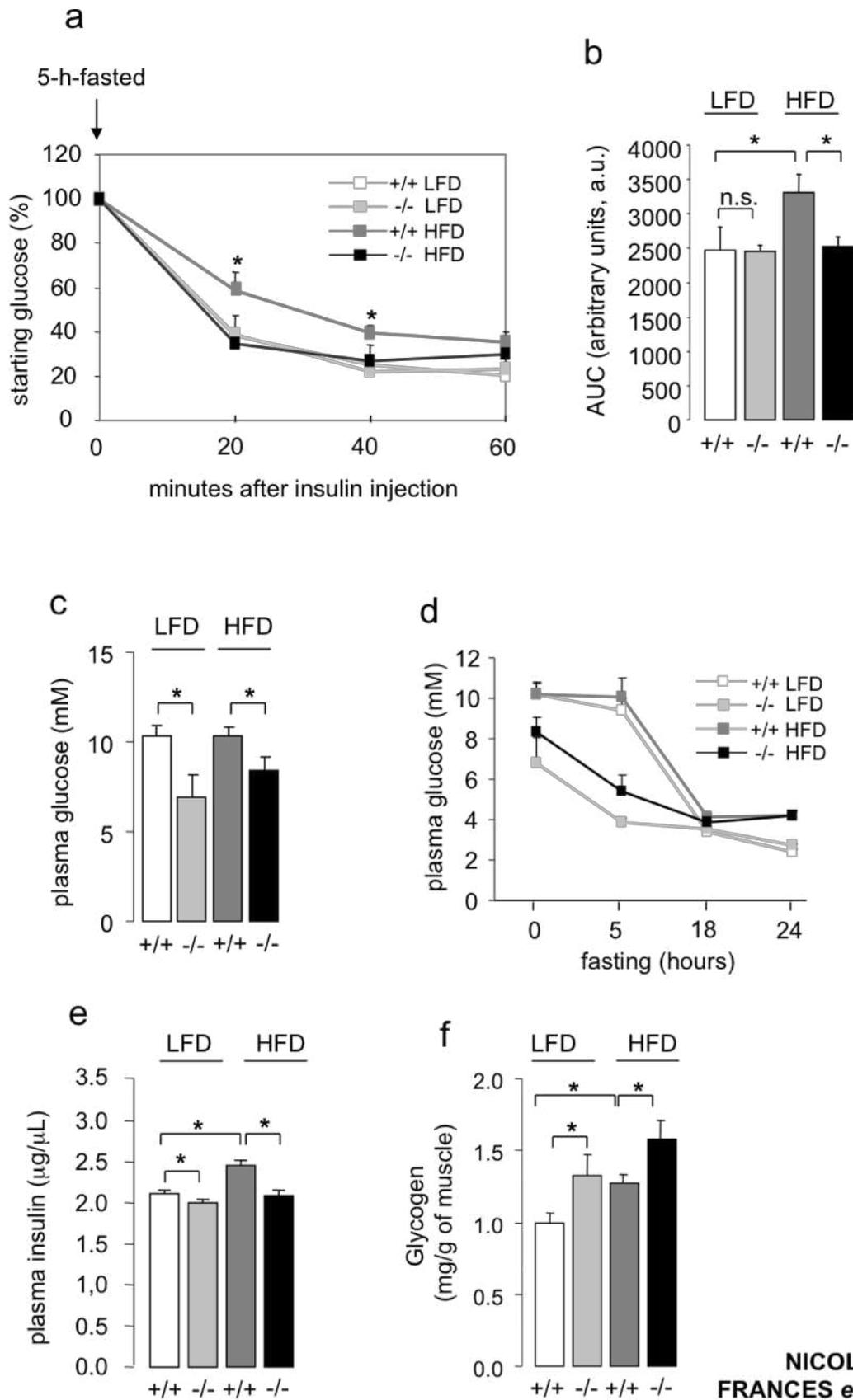
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NICOLAS-FRANCES *et al.*, Fig. 3

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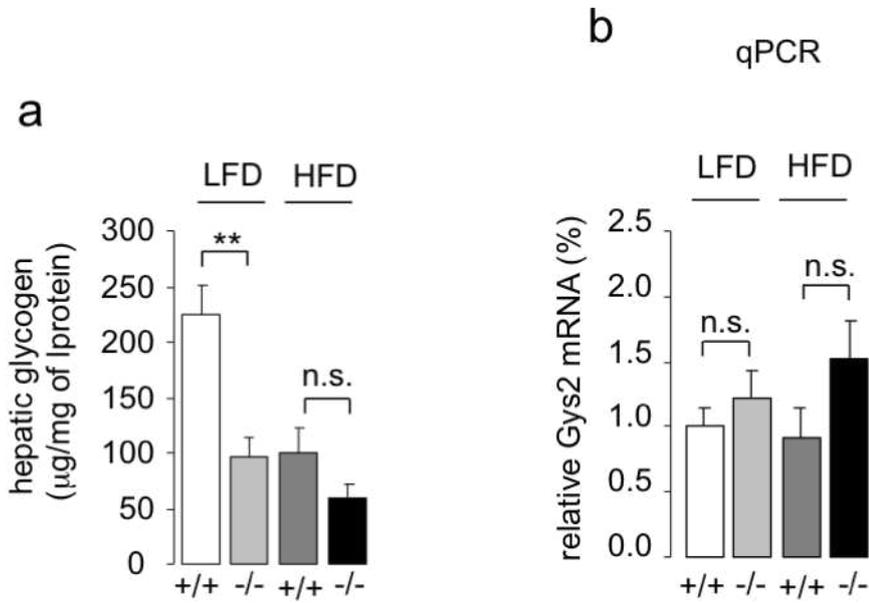
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NICOLAS-FRANCES *et al.*, Fig. 4

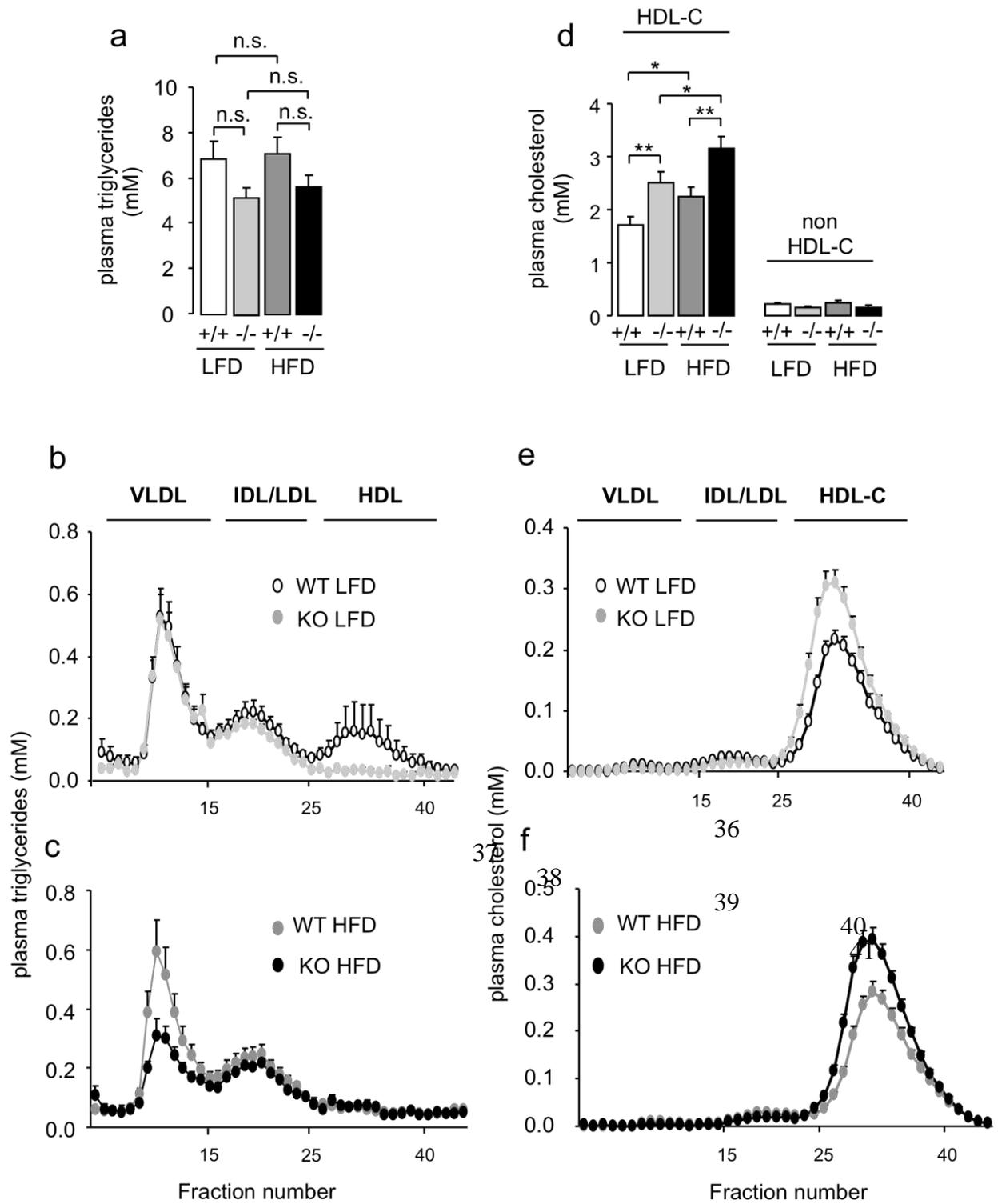
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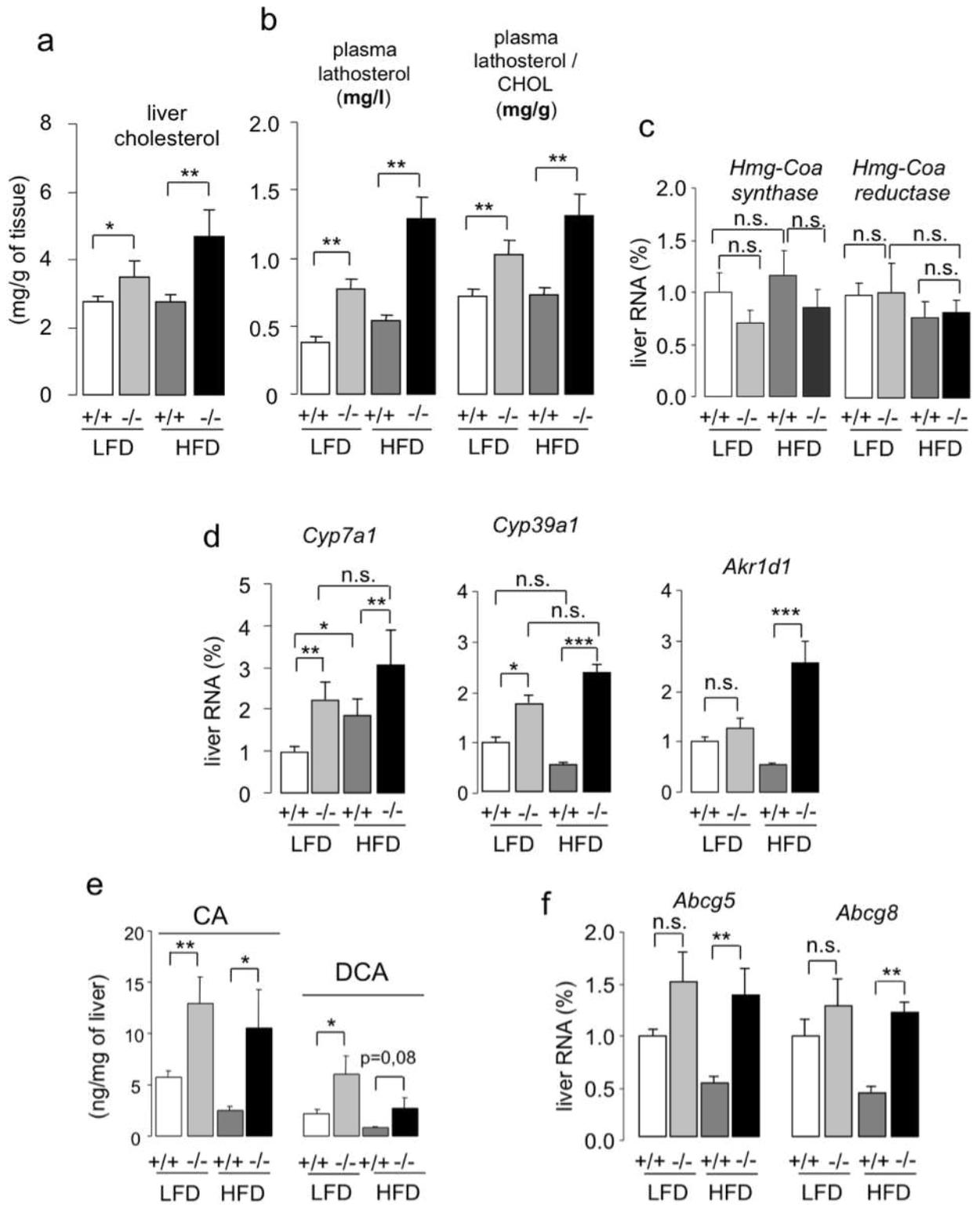
NICOLAS-FRANCES *et al.*, Fig. 5

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NICOLAS-FRANCES *et al.*, Fig. 6

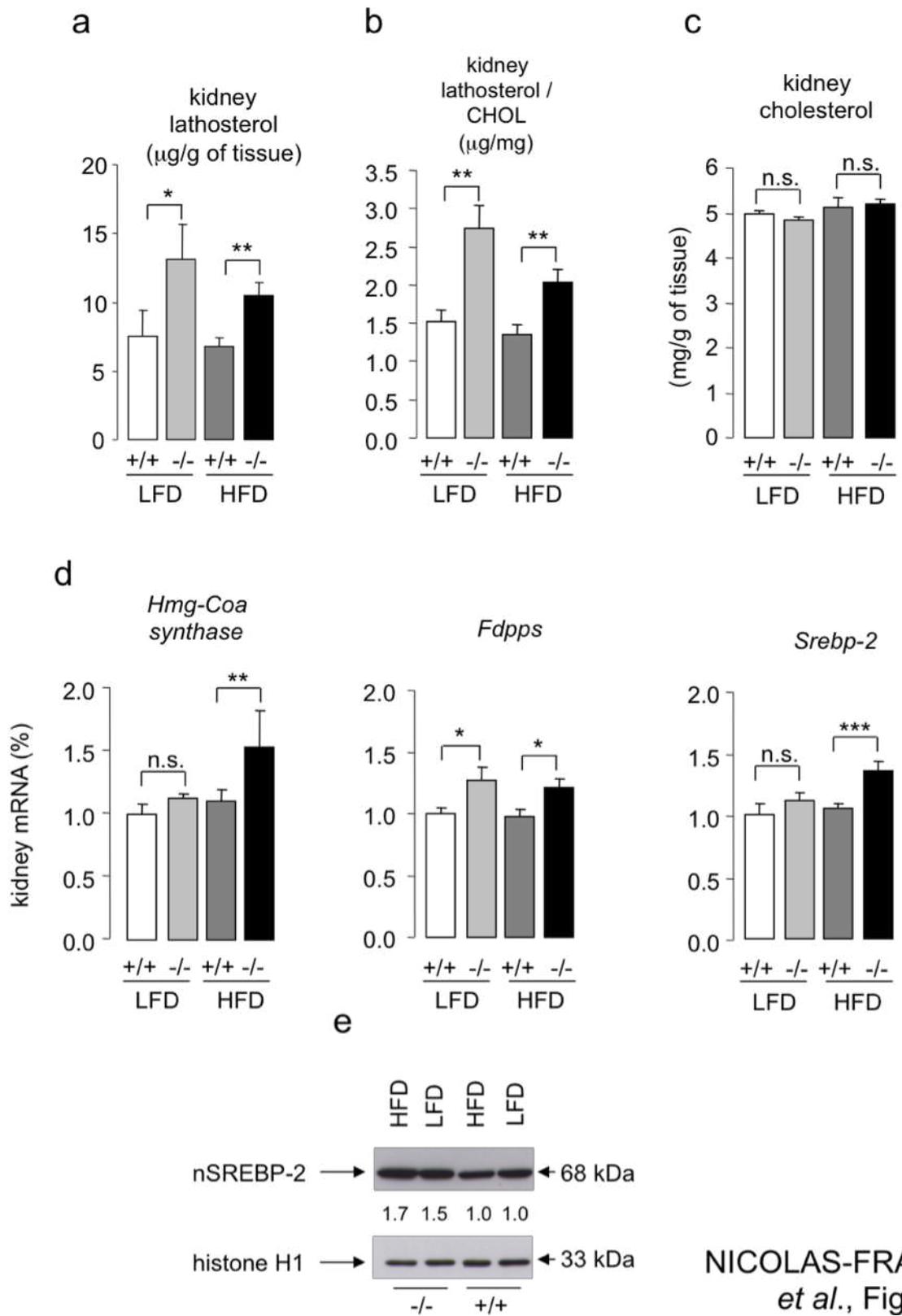
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NICOLAS-FRANCES *et al.*, Fig.7

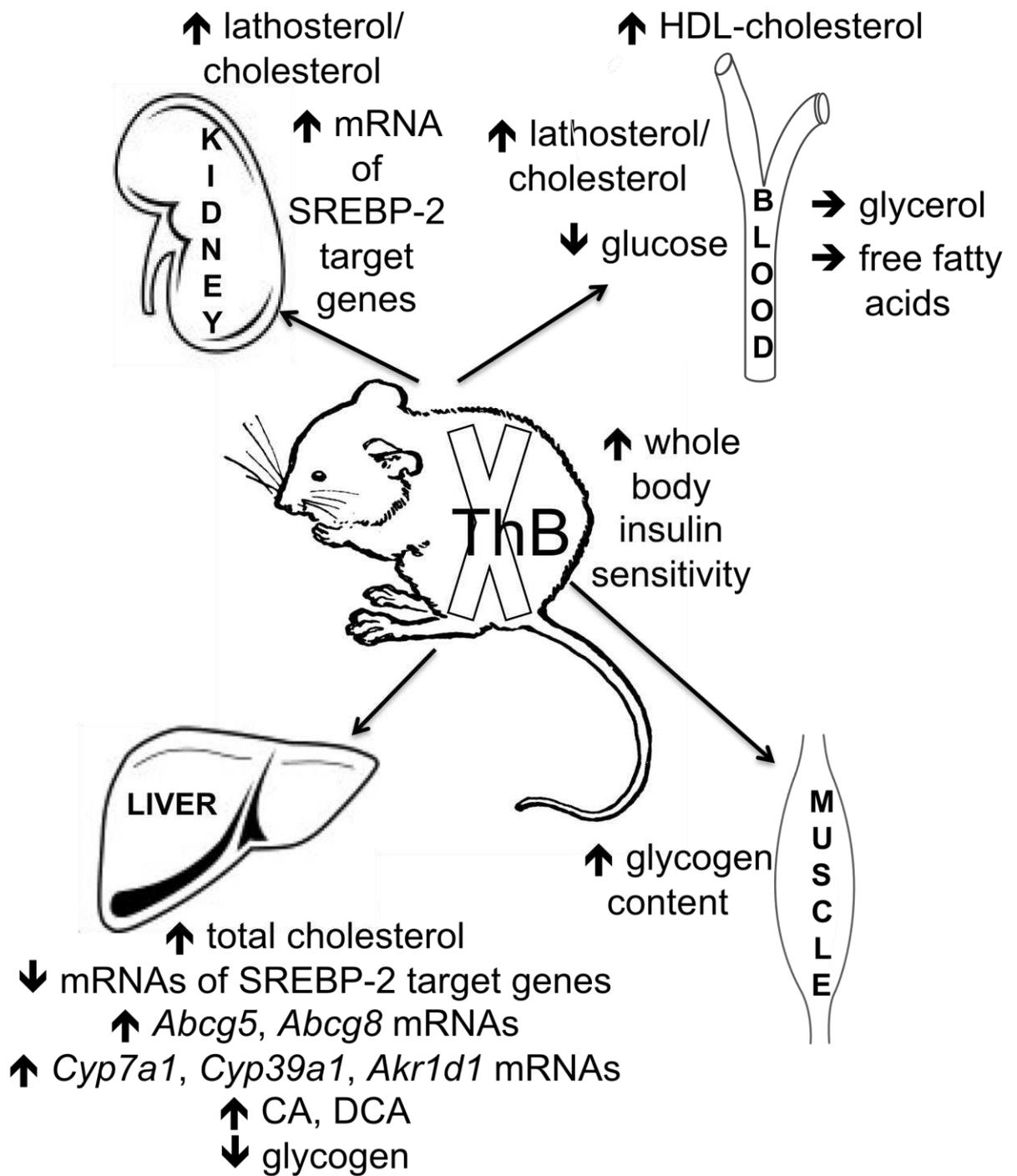
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NICOLAS-FRANCES *et al.*, Fig.8

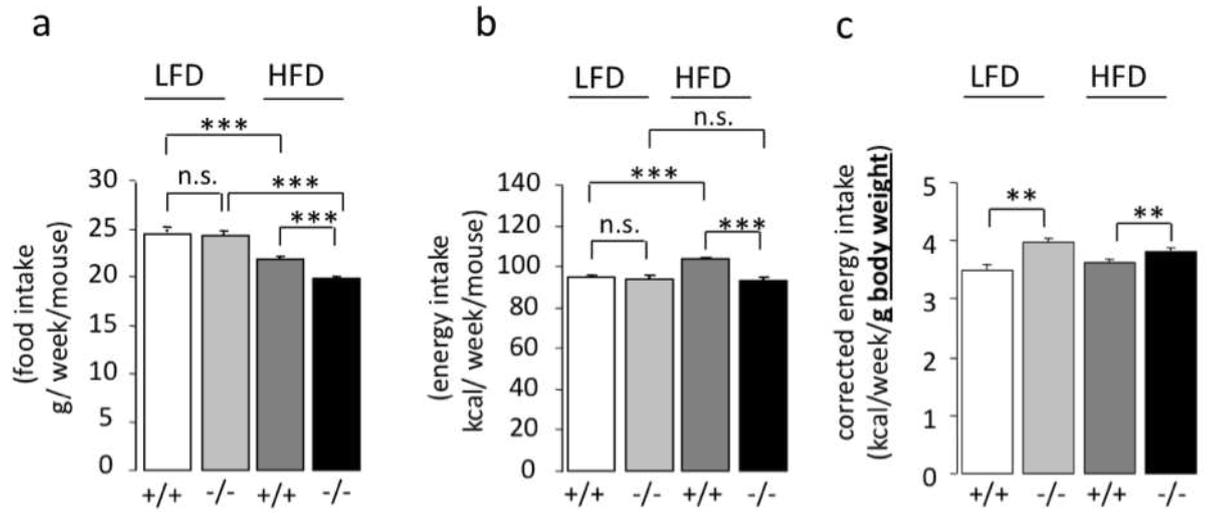
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NICOLAS-FRANCES *et al.*, Fig.9

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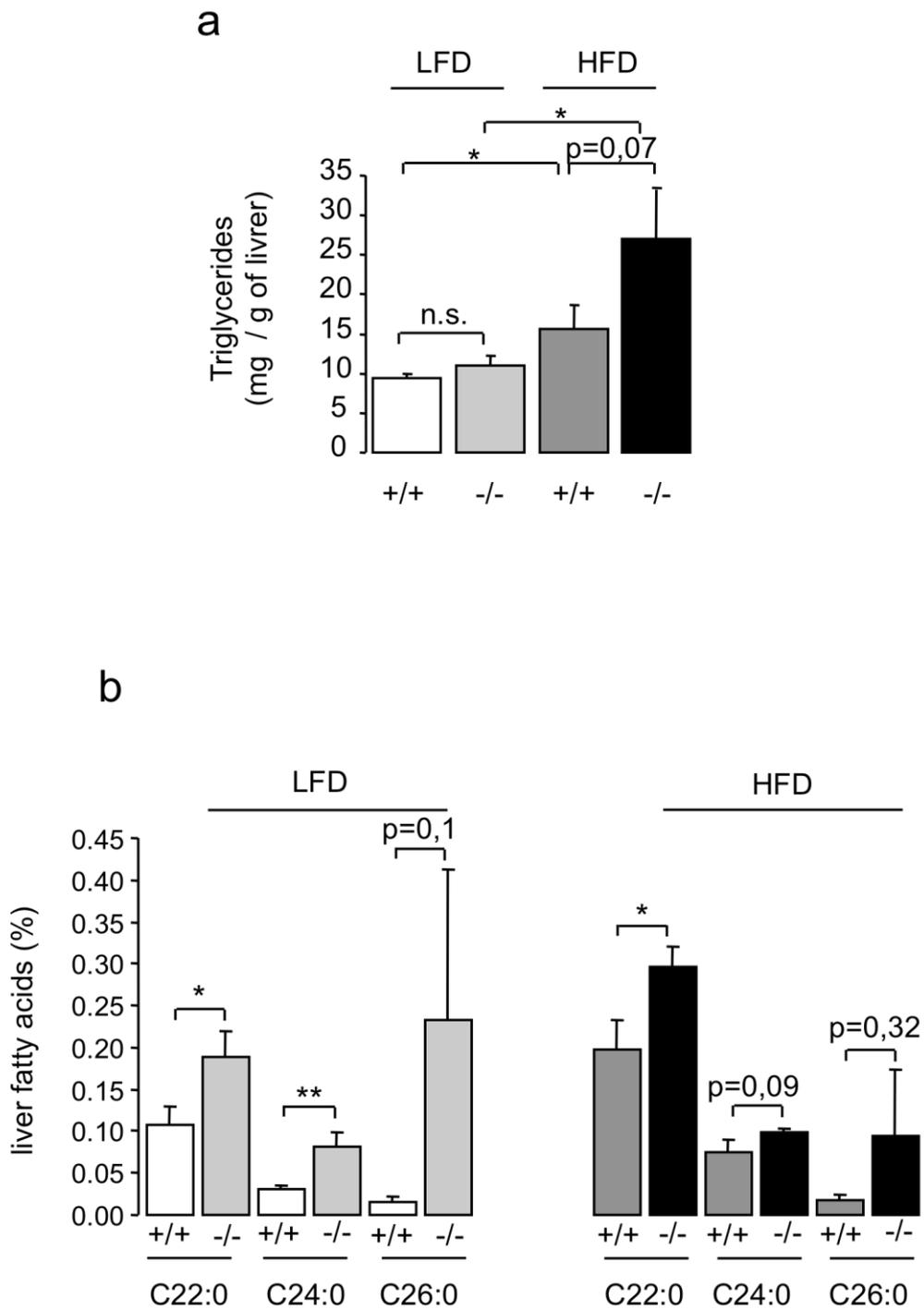
	D12450B (LFD)	D12451 (HFD)
Protein (g/100g)	19.2	24.0
Carbohydrate (g/100g)	67.3	41.0
Starch	29.9	8.5
Sucrose	33.2	20.1
Fat (g/100g)	4.3	24.0
ingredient (g)		
Lard	20.0	177.5
C14, Myristic	0.2	1.6
C14:1, Myristoleic	0.1	0.9
C16, Palmitic	7.2	43.2
C16:1, Palmitoleic	0.8	6.7
C18, Stearic	3.6	24.6
C18:1, Oleic	14.3	79.0
C18:2, Linoleic	15.1	28.6
C18:3, Linolenic	2.2	3.7
C20:4, Arachidonic	0.3	3.0
Total (g)		
saturated	11.0	69.4
monounsaturated	15.2	86.7
polyunsaturated	17.6	35.4
Total (%)		
saturated	25.1	36.3
monounsaturated	34.7	45.3
polyunsaturated	42.0	18.5
Cholesterol (%; w/w)	0.00136	0.01489
Cholesterol (mg/kg)	13.6	148.9



Nicolas-Francès *et al.*, supplemental Fig. 1

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Nicolas-Frances *et al.*, supplemental Fig. 2