Modulation of the hepatic fatty acid pool in peroxisomal 3-ketoacyl-CoA thiolase B-null mice exposed to the selective PPARalpha agonist Wy14,643.

Ségolène Arnauld, Marco Fidaleo, Marie-Claude Clémencet, Grégory Chevillard, Anne Athias, Joseph Gresti, Ronald J Wanders, Norbert Latruffe, Valérie Nicolas-Francès, Stéphane Mandard

Modulation of the hepatic fatty acid pool in peroxisomal 3-ketoacyl-CoA thiolase B-null mice exposed to the selective PPARalpha agonist Wy14,643

Ségolène Arnauld[1,2¶], Marco Fidaleo[1,2,6¶], Marie-Claude Clémencet[1,2], Grégory Chevillard[1,7], Anne Athias[3], Joseph Gresti[1,4], Ronald J. Wanders[5], Norbert Latruffe[1,2], Valérie Nicolas-Francès[1,2] and Stéphane Mandard[1,2,*]

[1]Centre de recherche INSERM U866, Dijon, F-21000, France; [2]Université de Bourgogne, Faculté des Sciences Gabriel, Equipe Biochimie Métabolique et Nutritionnelle, Dijon, F-21000, France; [3]Université de Bourgogne, IFR100, Plateau Technique de Lipidomique - Dijon, France. [4]INSERM U866, Equipe Physiopathologie des dyslipidémies, Faculté des Sciences Gabriel, 21000 Dijon, France; [5]Laboratory Genetic Metabolic Diseases, Academic Medical Center at the University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands. [6]Present address: Department of Cellular and Developmental Biology, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy. [7]Present address: Lady Davis Institute for Medical Research, McGill University, 3755 Côte Ste. Catherine Road, Montreal, QC H3T 1E2, Canada.

*Address correspondence to:

Stéphane MANDARD (PhD)

LBMN, centre de recherche INSERM U866, 6, Boulevard Gabriel, 21000 Dijon, France.

Phone: (+33) 3 80 39 62 02, Fax: (+33) 3 80 39 62 50

E-mail: stephane.mandard@u-bourgogne.fr

Short title: Elevation of MUFA n-7 and n-9 fatty acids in *Thb* $^{-/-}$ mice fed Wy

¶ S.A. and M.F. contributed equally to this work

ABSTRACT

The peroxisomal 3-ketoacyl-CoA thiolase B (Thb) gene was previously identified as a direct target gene of PPARalpha, a nuclear hormone receptor activated by hypolipidemic fibrate drugs. To better understand the role of ThB in hepatic lipid metabolism in mice, Sv129 wild-type and Thb null mice were fed or not the selective PPARalpha agonist Wy14,643 (Wy).

Here, it is shown that in contrast to some other mouse models deficient for peroxisomal enzymes, the hepatic PPARalpha signalling cascade in Thb null mice was normal under regular conditions. It is of interest that the hypotriglyceridemic action of Wy was reduced in Thb null mice underlining the conclusion that neither thiolase A nor SCPx/SCP2 thiolase can fully substitute for ThB *in vivo*. Moreover, a significant increased in the expression of lipogenic genes such as Stearoyl CoA Desaturase-1 (SCD1) was observed in Thb null mice fed Wy. Elevation of Scd1 mRNA and protein levels led to higher SCD1 activity, through a molecular mechanism that is probably SREBP1 independent. In agreement with higher SCD1, enrichment of liver mono-unsaturated fatty acids of the n-7 and n-9 series was found in Thb null mice fed Wy.

Overall, we show that the reduced peroxisomal β-oxidation of fat observed in Thb null mice fed Wy is associated with enhanced hepatic lipogenesis, through the combined elevation of microsomal SCD1 protein and activity. Ultimately, not only the amount but also the quality of the hepatic fatty acid pool is modulated upon the deletion of Thb.

Keywords: Peroxisomal 3-ketoacyl-CoA thiolase B, PPARα, Mono-Unsaturated Fatty Acids n-7 and n-9, stearoyl-CoA desaturase-1, Wy14,643.

1. Introduction

Under normal physiologic conditions, mitochondrial β-oxidation is quantitatively the most important pathway for fatty acid catabolism. Besides mitochondrial β-oxidation ((80% of long-chain fatty acids (LCFA) up to 22 carbons)), β-oxidation of long-chain (for about 20%) and that of very-long chain fatty acids (VLCFA, > 22 atoms of carbon) occurs within the peroxisome [1]. Similar to mitochondrial β-oxidation, peroxisomal β-oxidation can be divided into different steps, involving multiple enzymes that sequentially act. Some of these enzymes (fatty acyl-CoA oxidase, peroxisomal D-3-hydroxyacyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase ((D-PBE)) and peroxisomal 3-ketoacyl-CoA thiolase) are considered to be much more prone to oxidize straight-chain fatty acyl-CoAs. In rodents, two closely related genes, namely Thiolase a (*Tha*) and Thiolase b (*Thb*) have been cloned and characterized, while in humans only a single gene encoding for a peroxisomal 3-oxoacyl-CoA thiolase has been reported so far [2-4]. An infant girl with a deficiency in peroxisomal 3-ketoacyl-CoA thiolase has been initially reported [5]. However, close reinvestigation of this patient identified the true defect at the level of d-bifunctional protein [6]. Thus, whether human patients with alterations at the level of the peroxisomal 3-ketoacyl-CoA thiolase exist still remains an open question.

Other and/or similar peroxisomal enzymes such as branched-chain fatty acyl-CoA oxidase, D-PBE and sterol carrier protein2/3-ketoacyl-CoA thiolase (SCP2/SCPx) are rather devoted to the oxidation of branched-chain fatty acyl-CoAs.

Besides LCFAs and VLCFAs, 2-methyl-branched-chain, dicarboxylic fatty acids, eicosanoids and bile acids derivatives have been shown to be also metabolized within the peroxisomal matrix, extending the importance of the peroxisome. In agreement with the critical role of the peroxisome in lipid metabolism, absence of a single enzyme of the peroxisomal β-oxidation system has been shown to be associated with serious plasma lipid disorders, as shown by the

marked elevation of plasma VLCFAs levels in patients suffering from *Acox-1* deficiency [7]. Ablation of the *Acox-1* gene in mice also revealed the critical role of this enzyme in lipid metabolism, since *Acox-1* null mice suffered from a pronounced fatty liver which was associated with the constitutive activation of prototypical PPARα target genes and a spontaneous peroxisome proliferation [8]. Besides *Acox-1*, other mouse models deficient for a single peroxisomal enzyme have been established over the past few years. It was recently found that mice deficient for *Mfp-2* displayed a coordinated induction of the transcription factors PPARα and SREBP2 [9]. In turn, the expression of typical SREBP2 and PPARα target genes was markedly induced in the liver of *Mfp-2*$^{-/-}$ mice, leading to decreased levels of plasma triglycerides (TG) and free fatty acids (FFA) in adult mice. Others studies indicate that lack of *Scp-x thiolase* gene (Sterol Carrier Protein) only was enough to decrease ability of the mutant mice to metabolize branched-chain lipids [10]. With respect to the double loss of Sterol Carrier Protein X/ Sterol Carrier Protein-2, null mice displayed alteration in the peroxisomal β-oxidation of 2-methyl-branched chain fatty acids [11]. Further investigations also led to the finding that SCPx/SCP2 thiolase was involved in the conversion of cholesterol into bile acids [12].

A deficient mouse model for ThB was previously generated and partially characterized, yet the *in viv*o role of ThB still remains ill defined [13]. In order to close in on the potential *in vivo* relevance of ThB, we studied WT and *Thb*$^{-/-}$ mice fed or not the potent PPARα agonist Wy14,643 (Wy). The nuclear hormone receptor PPARα plays a pivotal role in fatty acid handling by up-regulating the expression of numerous genes involved in mitochondrial and peroxisomal oxidation [14]. Here, exposure of mice to Wy is expected to markedly stimulate peroxisomal FAO and *Thb* mRNA levels in WT mice and not or less in *Thb*$^{-/-}$ mice, thus helping in the identification and characterization of the roles of ThB *in vivo*.

Our main finding is that exposure of *Thb*$^{-/-}$ mice to Wy leads to a significant elevation of the hepatic content of MUFA n-7 and n-9 fatty acids, secondary to the induction of the microsomal SCD1 enzyme, through a likely SREBP1 independent manner.

2. Materials and methods

2.1 Animal experiments

All mice were on a pure-bred Sv129 genetic background. Male animals were kept in normal cages with food and water *ad libitum.* Mice were routinely fed a commercial and standard pellet diet (UAR A03-10 pellets from Usine d'Alimentation Rationnelle, Epinay sur Orge, France, 3.2 kcal/g) consisting (by mass) about 5.1% of fat (± 0.89% of C16:0, ± 0.09% of C16:1n-7, ± 0.45% of C18:0, ±1.06% of C18:1n-9, ±1.53% of C18:2n-9 and traces of C18:3n-9). At the time of sacrifice, animals were around 4-5 months of age. Male mice in the fasted state were deprived of food for 6h starting at 4:0 pm. Blood was collected *via* cardiac or orbital puncture into EDTA tubes. Tissues were excised, weighted and immediately frozen in liquid nitrogen before being stocked at -80°C. The animal experiments were approved by the animal experimentation committee of the University of Burgundy (protocol number n°1904) and were performed according to the European Union guidelines for animal care.

2.2 Chemicals

Wy14,643 (Wy) was obtained from ChemSyn Laboratories (Lenexa, Kanasa). SYBR green was from Eurogentec. Standards for fatty acids measurement by HPLC were from Sigma or Larodan. Pentafluorobenzyl bromide, N,N'-diisopropylethylamine, potassium hydroxide and BHT were from Sigma. All solvents were HPLC grade.

2.3 Histology and neutral fat staining

Haematoxylin and Eosin staining of liver sections were done using standard protocols. Histology was examined on frozen sections after Oil Red O staining for neutral lipid using standard procedures.

2.4 Liver triglycerides

Total lipids from liver were extracted with chloroform/methanol (2/1 v/v) according to the method of Folch. Glyceryl triheptadecanoate as an internal standard was added for quantification. Triglycerides were separated by thin layer chromatography (TLC) using hexane/ethylique ether/acetic acid (90/30/1 v/v/v) as solvent. The triglycerides were then scaped and extracted from silica gel with chloroform/methanol (9/1 v/v). The total fatty acids of triglycerides were methylated and quantified by Gas Chromatography (GC) using a Chrompack CP 9002 Gas Chromatograph equipped with a Varian FactorFour VF-23ms capillary column (30mx0,32mm).

2.5 Liver and plasma Fatty Acid Profile

10 mg of liver (or 50 µl of plasma) was saponified with 1 ml of ethanolic potassium hydroxide (final concentration 0.6N) containing 10 µg of heptadecanoic acid and 250 ng of tricosanoic acid as internal standards and 50 mg/L of butylated hydroxytoluene. The tubes were incubated at 56°C for 45 min. After incubation, fatty acids were extracted by adding 1 ml of 1.2 M HCl and 2 ml of hexane to each tube. The tubes were shaken at room temperature for 5 mn, centrifuged and the organic phase was evaporated to dryness under nitrogen and derivatized to pentafluorobenzyl esters with 100 µl of acetonitrile, 5 µl of pentafluorobenzyl bromide and 5 µl of diisopropylethylamine at room temperature for 30 min. One ml of water was added and derivatives were extracted with 2 ml of hexane. After centrifugation, the organic phase was evaporated and 100 µL of hexane were added. One µl was further injected

in the split mode. Quantification of liver and plasma fatty acid esters was performed using a HP7890A Gas Chromatograph equipped with an HP7683 Injector and a HP5975C Mass Selective Detector (Agilent Technologies). Chromatography was performed using a HP-5MS fused silica capillary column (30m x 0.25 mm inner diameter, 0.25 μm film thickness, Agilent Technologies). The GC-MS conditions were as follows: carrier gas, helium at a flow-rate of 1.1 ml/min; injector temperature, 250°C, split mode; oven temperature 140°C, increased at 5 °C/min to 300°C, and held for 10 min. The mass spectrometer was operated under negative chemical ionization mode with methane as reactant gaz. The ion source temperature and the quadrupole temperature were 150 °C and 106 °C respectively. A SIM program was used for mass spectrometry with [M-181] (-) ions as quantification.

The desaturation index was determined by calculating product:substrate ratios (16:1/16:0 and 18:1/18:0) using the quantitated values for palmitoleate, palmitate, oleate and stearate.

2.6 Lipoprotein profiling

Lipoproteins were separated using fast protein liquid chromatography (FPLC) on a Superose 6 HR 10/30 column (Amersham Biosciences) as described previously [15].

2.7 Preparation of nuclear and microsomal fractions of liver

Nuclear and cytosolic fractions of liver were prepared as previously published [16]. Concerning microsomal fractions, pooled excised livers (100mg for each animal) were thawed, finely chopped and homogenized in ice cold 10 mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose and 1 mM EGTA, in a Potter glass homogenizer equipped with a Teflon pestle. Pooled homogenates were centrifuged at 4350 g for 3 min at 4°C. The precipitate was discarded and supernatant was again centrifuged at 22 000g for 5 min (4°C). Supernatant was collected and further centrifuged at 100 000g for 5 min in a Beckman airfuge

ultracentrifuge. Pellet was suspended in 100 µl of the upper pre-cited buffer. Protein estimation of each preparation was done using Bradford's method.

2.8 Plasma metabolites

Plasma was initially collected into EDTA tubes *via* retro-orbital punctures and then centrifuged at 4°C (10 min, 6000 rpm). Plasma cholesterol and triglyceride concentration were determined using kits from Diasys (Diagnostic Systems International, France).

2.9 RNA Isolation and RT-PCR

RNA from liver was extracted with Trizol reagent (Invitrogen) using the supplier's instructions. RNA was then further purified (from free nucleotides and contaminating genomic DNA) using RNeasy columns (Qiagen) with DNAse treatment. 1 µg of RNA was used for reverse transcription with iScript Reverse Transcriptase (Biorad).

2.10 Real Time Quantitative PCR

PCR reactions were performed using the qPCR MasterMix Plus for SYBR Green I with fluorescein (Eurogentec). All PCR reactions were performed with MultiGuard Barrier Tips (Sorenson BioScience, Inc.) and an iCycler PCR machine (Bio-Rad Laboratories). Primers were designated to generate a PCR amplification product of 100-200 bp and were selected according to indication provided by the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequences are available from S.M. on request. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The "delta-delta Ct" quantification method was used and expression was related to the control gene 36B4, which did not change under any of the experimental conditions studied.

2.11 Immunoblot

Immunoblotting on liver samples was carried out as described previously [17,18]. Scd1 (sc-14720, 1:200), Srebp1 (sc-366, 1:200), histone H4 (sc-10810, 1:2000) antibodies were all purchased from Santa Cruz Biotechnology.

2.12 Statistical analyses

The ANOVA one way test was used to calculate statistically significant differences.

3.0 Results

3.1 Hepatic mRNA levels of PPARα target genes are normal in Thb$^{-/-}$ mice.

It was previously reported that the isolated deletion of peroxisomal enzymes such as ACOX-1, MFP-2 or SCPx was associated with a constitutive expression of typical PPARα target genes in the liver, supporting the hypothesis that substrates of these enzymes are putative natural ligands for PPARα [9,19-21]. Hence, using quantitative RT-qPCR, we checked whether the ablation of *Thb* would impact on the hepatic expression of PPARα target genes as well. Under basal conditions, hepatic mRNA levels of the PPARα target genes tested so far (involved in different aspects of fat handling such as mitochondrial, peroxisomal, microsomal oxidation as well as fatty acid transport) were similar between both genotypes, under basal conditions (Fig. 1). Considering that *Cyp4a10* is a PPARα target gene that is extremely sensitive to the presence and activation of PPARα in mouse liver, *Cyp4a10* is usually used as a marker of PPARα activity. Of interest, *Cyp4a10* mRNA levels were higher in *Thb*$^{-/-}$ mice fed Wy than in corresponding WT mice. Hence, deletion of *Thb* may have been associated with a sensitizing effect towards Wy, which would be secondary to the elevated levels of *Pparα*. The mRNA levels of the other PPARα regulated genes were similarly induced by Wy, suggesting that PPARα signaling cascade was not blunted in *Thb*$^{-/-}$ mice. Overall, our data

suggest that the deletion of *Thb* was not associated with a significant impairment of the hepatic PPARα signaling cascade.

3.2 Hepatic and plasma cholesterol are normal in Thb$^{-/-}$ mice.

Under a normal laboratory chow, mice deficient for other peroxisomal enzymes such as *Mfp-2* or *Scp2* genes displayed a steady-state level of hepatic and plasma cholesterol [9]. Here, we first wondered what could be the consequence of *Thb* deletion on these parameters. It was found that 6h-fasted *Thb$^{-/-}$* mice (previously fed or not with Wy) displayed normal plasma cholesterol contents, suggesting that *Thb* is dispensable for cholesterol homeostasis in mice (Fig. 2A). Profiling of lipoproteins using FPLC analysis confirm the lack of differences in VLDL and HDL cholesterol fractions (Fig. 2B). Quantification of liver cholesterol content by gas chromatography also revealed that ablation of *Thb* has no strong consequences on hepatic cholesterol load (Fig. 2C).

3.3 Wy reverses the reduced hepatic TG content observed in Thb$^{-/-}$ mice

To check whether ablation of *Thb* may increase plasma triglycerides (TG) content through increase of lipoprotein secretion, plasma TG levels were evaluated, in 6h fasted WT and *Thb$^{-/-}$* mice, previously fed or not with Wy (Fig. 3A). Plasma TG levels were similar for both genotypes. Profiling of lipoproteins using FPLC analysis confirm the lack of differences in VLDL and LDL TG fractions (Fig. 3B). Together, our data indicate that ThB is not critical for the maintenance of normal circulating TG levels under a normal regulatory laboratory chow.

Because it was previously reported that mice deficient for the peroxisomal *Acox-I* gene displayed a pronounced hepatic fatty liver under a normal laboratory chow, a survey of liver TG contents was performed in WT and *Thb$^{-/-}$* mice, fed or not Wy [9]. It was found that hepatic TG contents were lower in *Thb$^{-/-}$* mice, indicating that ThB can not be fully compensated by other peroxisomal thiolases (Fig. 3C). Wy is known to activate PPARα that

in turn promotes FAO and shifts away part of fatty acids from esterification and storage. In agreement, it was found that Wy feeding lowered TG contents in liver of WT mice (Fig. 3C). Of interest, when combining *Thb* deletion and Wy treatment, hepatic TG content was not reduced suggesting that the hypotriglyceridemic effect of Wy was blunted. This was further confirmed by Oil red O staining of liver sections (Fig. 4A). In line with a similar increase of the relative liver mass upon Wy treatment in WT and *Thb*$^{-/-}$ mice (data not shown), the size of hepatocytes from both genotypes appeared to be equally enlarged by Wy, indicating similar hepatomegaly (Fig. 4B).

3.4 Elevated liver TG observed in Thb$^{-/-}$ *mice fed Wy is likely secondary to increased mRNA levels of lipogenic genes*

Reduced peroxisomal (but not mitochondrial) β-oxidation of palmitate by 30% in *Thb*$^{-/-}$ mice fed Wy was found (Fidaleo *et al.*, in preparation). This piece of data suggests that the lack of the hypotriglyceridemic effect of Wy observed in *Thb*$^{-/-}$ mice might be secondary, at least in part, to altered fat oxidation. However, besides oxidation of fat, lipogenesis and triacylglycerol build-up also take place in liver and are important pathways for the management of hepatic TG content. We then wondered whether in addition to fat oxidation, these pathways could be affected by the lack of *Thb*. To do so, the expression level of a subset of critical lipogenic genes such as *Acly*, *Acc*α, *Fas*, *Scd1*, *Scd2* and *Srebp1c* was evaluated in WT and *Thb*$^{-/-}$ mice fed or not Wy. Of interest, *Acc*α and *Fas* mRNA levels were up-regulated following *Thb* deletion only (Fig. 5A). Furthermore, as the result of Wy activation, mRNA levels of *Acc*α, *Fas*, *Scd1*, *Scd2*, *Mgat1* were coordinately elevated in WT mice. However, despite up-regulation of these lipogenic genes by Wy in WT mice, liver TG contents were lower indicating that oxidation prevailed on TG build-up. Of note, a comparative increase in *Srebp1c* mRNA levels and those of its target genes *Acc*α (≈2-fold)

and *Fas* (2-fold) was found in *Thb*$^{-/-}$ mice fed Wy (Fig. 5A). *Srebp1c* mRNA levels in liver have been shown to be under the control of *Lxr*α, yet *Lxr*α mRNA levels remained unchanged (Fig. 5A) [22]. *Chrebp* mRNA levels that codes for the carbohydrate response element binding protein (ChREBP) were also unaltered following *Thb* deletion (Fig. 5A). Combined, these observations suggest that lipogenesis may be induced in *Thb*$^{-/-}$ mice fed Wy that displayed reduced peroxisomal FAO. In line with a higher TG content in liver of *Thb*$^{-/-}$ mice fed Wy, genes encoding for enzymes involved in TG synthesis tended also to increase (*Gyk*, *Dgat1*, *Gpam*) (Fig. 5A). Given that *Srebp1c* is a master gene for *Acc*α and *Fas* expression and because its activity is under the control of post-translational maturation, we evaluated whether maturation of SREBP1 might be altered in *Thb*$^{-/-}$ mice (fed or not Wy) by western-blotting performed with nuclear samples. Although the antibody we used can not discriminate between SREBP1a and SREBP1c isoforms, our data support the idea that SREBP1 maturation (into a mature nuclear and cleaved form of 68 kDa) is not drastically impaired in *Thb*$^{-/-}$ mice (Fig. 5B). Given that SREBP1c is the predominant isoform of SREBP1 in the liver, we believed that the amount of nuclear SREBP1c was likely not altered by *Thb* deletion and Wy. Interestingly, although not significant, *Scd1* mRNA levels also tended to be higher in the liver of *Thb*$^{-/-}$ mice fed Wy (Fig. 5A). Under normal conditions, protein content of liver SCD1 (microsomal fraction only) was below the detection level of the antibody used for western-blotting (Fig. 5C). Yet, following Wy treatment, SCD1 protein content was comparatively much elevated in *Thb*$^{-/-}$ mice, indicating that ThB is likely indirectly involved in the control of SCD1 expression (Fig. 5C).

3.5 Lack of Thb modulates hepatic and plasma MUFA n-7 and n-9 concentrations as well as PUFA n-3

Given its critical role in VLCFA oxidation, peroxisome appears to be essential for lipid homeostasis as shown by the high plasma and/or hepatic concentrations of straight-chain VLCFAs (C24:0, C26:0, C26:1) observed in patients/mice suffering from isolated defects in peroxisomal enzymes [7,8,23]. We then decided to perform a complete analysis of medium-to long-chain fatty acid species (C:12-C:20) as well as of VLCFA (C:22-C:26) present in liver and plasma of WT and *Thb*$^{-/-}$ mice (fed or not Wy). Noteworthy, it was found that upon Wy activation, *Thb*$^{-/-}$ mice displayed a significant enrichment of liver MUFA n-7 and n-9, among which C16:1n-7, C18:1n-7, C16:1n-9 and C18:1n-9 (Table 1). In mammalian liver, SCD1 is the main enzyme responsible for the conversion of palmitoyl-CoA (C16:0, the saturated fatty acid products of FAS) and stearoyl-CoA (C18:0) to palmitoleyl-CoA (C16:1n-7) and oleyl-CoA (C18:1n-9), respectively. Supporting the hypothesis of enhanced lipogenesis partially by SCD1 in *Thb*$^{-/-}$ mice fed Wy, the desaturation index of total liver lipids, which is considered to reflect *SCD1* activity, was significantly increased (Fig. 6A) [24]. Besides C16:1 and C18:1 as well as hepatic MUFA (n-7 and n-9) contents such as C20:1n-7, C20:1n-9, C22:1n-7, C22:1n-9 and C24:1n-7 were more elevated in *Thb*$^{-/-}$ mice fed Wy compared to corresponding control mice, it suggests that lack of *Thb* favored the emergence of MUFAs (Table 1).

With respect to PUFA, it was found that deletion of *Thb* alone was sufficient to increase C18:3n-3, C20:3n-3, C20:4n-3, C20:5n-3 and C22:5n-3 fatty acids in liver and partially for some of them in plasma (Table 2 and supplementary data, Table 4). Increased levels of these fatty acids can reflect reduced peroxisomal β-oxidation, which consecutively may provide more substrates for the microsomal fatty acid elongation system. Alternatively, enhanced activity of elongases might be secondary to increased gene expression. To check for this, mRNA levels of several elongases/desaturases were quantified using hepatic samples of WT

and *Thb*$^{-/-}$ mice, fed or not Wy (Fig. 6B). ELOVL1 and ELOVL3 have been shown to display chain length specificity towards VLCFAs. Of interest, it was found that *Elovl3* (*Cig30*) but not *Elovl1* (elongation of C24:0 to C26:0) mRNA levels were significantly higher in *Thb*$^{-/-}$ mice fed Wy. Note that we failed to detect any corresponding signal for ELOVL3 protein in hepatic samples from WT or *Thb*$^{-/-}$ mice fed or not Wy, rendering only speculative an increase at the protein level too (data not shown). Similar to *Elovl3*, hepatic *Kar* mRNA levels (another component of the microsomal membrane bound fatty acid elongation system which produces the 26-C VLCFA from palmitate) were also higher in *Thb*$^{-/-}$ mice fed Wy. However, *Elovl1*, *Elovl2, Elovl5*, *Elovl6*, *Fads1*, *Fads2, Ter* mRNA levels remained unaffected suggesting that lack of *Thb* was not associated with a general mechanism leading to the increase of all elongases. Summarizing, deletion of *Thb* favors hepatic lipogenesis when β-oxidation of lipids is challenged by the potent PPARα agonist Wy.

3.6 Ablation of Thb is associated with decrease levels of hepatic C26:0 contents

Supporting a defect in peroxisomal β-oxidation of fatty acids in *Thb*$^{-/-}$ mice fed Wy, hepatic C22:6n-3/C24:6n-3 ratio was strongly reduced (-50%) (Table 2). This point is important because production of C22:6n-3 from C24:6n-3 necessarily implies a reduction of 2C by an obligatory process that involves peroxisomal β-oxidation. We concluded that deletion of *Thb* functionally impacts on peroxisomal β-oxidation and then can not be fully compensated by ThA or SCPx/SCP2 thiolase *in vivo*. In agreement with a similar oxidation rate of palmitate in WT and *Thb*$^{-/-}$ mice at baseline, C22:6n-3/C24:6n-3 ratio was unaffected by *Thb* deletion alone, pointing out that the effects of *Thb* deletion need to be elicited by activators of peroxisomal FAO (such as Wy).

With regard to VLCFAs such as C22:0 and C24:0, both hepatic and plasma contents were similar in WT and *Thb*$^{-/-}$ mice fed or not Wy (Table 3 and supplementary data in Table 4).

Intriguingly, decreased C26:0 levels were observed in hepatic samples of *Thb*$^{-/-}$ mice fed Wy, leading to decreased hepatic C26:0/C22:0 and C26:0/C24:0 ratios.

Severe peroxisomal disorders have also been associated with the elevated plasma and/or tissue levels of branched-chain phytanic/pristanic acids and bile acids precursors [11,25]. One prominent theory also suggests that ThB and ThA would preferentially oxidize long-straight chain fatty acids while branched-chain very long chain fatty acids would rather be substrates for SCPx/SCP2 thiolase. To verify whether lack of *Thb* could modulate plasma branched-chain fatty acids levels, plasma pristanic and phytanic acids levels were then assessed in WT and *Thb*$^{-/-}$ mice fed or not Wy (supplementary data in Table 5). None of the branched-chain fatty acids levels were significantly different between the four groups of mice. While other additional investigations are needed to reach a firm conclusion, these results suggest that ThB is not critical for maintaining branched-chain fatty acids levels in a normal range. Together, our data support the hypothesis that ThB is somehow involved in hepatic C26:0 homeostasis in mice, through a biochemical route that remains ill defined.

4. Discussion

Little is known about the roles of *Thb* in mice, hence a mouse model deficient for the *Thb* gene has been previously established in the laboratory [13]. However, the first characterization of the phenotype was rather incomplete. We now provide a more detailed analysis of the *Thb* deficient mouse model by showing that at baseline, hepatic peroxisomal FAO was not altered by *Thb* deletion as shown by the ratio of C22:6n-3 / C24:6n-3 (liver and plasma) that remained similar. Hence, ThA and/or SCPx/SCP2 thiolase may act on behalf of ThB under basal conditions. After Wy feeding, the situation is somewhat different as shown by the amplitude of the peroxisomal FAO activation which is comparatively reduced (- 30%) in *Thb* $^{-/-}$ mice (Fidaleo *et al,* in preparation).

Although seemingly paradoxical, *Thb*$^{-/-}$ mice displayed reduced hepatic TG content which is associated with increased levels of C20:3n-3, C20:5n-3 and C22:5n-3, both in liver and plasma. Hence, the deletion of *Thb* alone leads to alterations in lipid metabolism in liver through a likely PPARα-independent manner. Supporting this, the mRNA levels of hepatic typical PPARα regulated genes was not altered by *Thb* deletion alone. Further, no spontaneous hepatomegaly or peroxisome proliferation, two biological processes that are largely under the positive control of PPARα, could be observed in *Thb*$^{-/-}$ mice [26]. Decreased lipogenesis and hepatic TG content have been shown to be possibly secondary to the action of some dietary PUFAs n-3 such as C20:5n-3, through the modulation of the proteolytic maturation of SREBP1c. However, in spite of the elevation of C20:5n-3 in the liver of *Thb*$^{-/-}$ mice, we have provided some evidence that the SREBP1 maturation was likely not impaired. Hence, the precise molecular mechanism that governs the decrease of TG in liver of *Thb*$^{-/-}$ mice still remains mysterious.

Up-regulation of *Thb* mRNA in liver of WT mice by Wy was associated with decreased hepatic TG levels, while deletion of *Thb* hampered the hypotriglyceridemic action of Wy. The combination of decreased peroxisomal FAO and increased fatty acid synthesis likely accounts for the higher concentration of TG in the liver of *Thb*$^{-/-}$ mice fed Wy. In support of an indirect role of ThB in hepatic lipogenesis, an increased content of MUFA n-7 and n-9 fatty acids in the liver of *Thb*$^{-/-}$ mice fed Wy was found, probably because of higher hepatic SCD1 protein content and activity. MUFA have been described as the preferred substrates for TG synthesis [27]. Considering that esterification of MUFA n-7 and n-9 to the glycerol backbone is not compromised in *Thb*$^{-/-}$ mice, elevation of TG in *Thb*$^{-/-}$ mice fed Wy is reasonably expected. An important question which is still not resolved is how a peroxisomal enzyme such as ThB can impact on SCD1 content and activity. Different studies in mice in which *Scd1* gene has been mutated (knock-out, siRNA, antisense oligonucleotides) revealed the critical role of

SCD1 in hepatic neutral lipid synthesis [28-30]. Endogenous rather than dietary palmitoleate (C16:1) and oleate (C18:1) have been proposed to be preferred substrates for TG production [31]. In line with this observation, *Thb*$^{-/-}$ mice fed Wy displayed significant hepatic accumulation of C16:1/18:1 compared to age-matched WT mice. *Scd1* gene expression is tightly controlled, being under the control of nutritional and hormonal changes as well as transcription factors such as PPARα [32]. However, no differences in the nuclear content of PPARα in the liver of WT and *Thb*$^{-/-}$ mice (fed or not Wy) were observed (data not shown). Furthermore, since typical PPARα target genes are equally expressed in WT and *Thb*$^{-/-}$ mice fed Wy, it is difficult to ascribe to PPARα the increase in SCD1 mRNA and protein content observed in *Thb*$^{-/-}$ mice fed Wy.

In addition to *Pparα* and *Scd1*, *Elovl3* mRNA levels were comparatively elevated in *Thb*$^{-/-}$ mice fed Wy, suggesting that *Pparα*, *Scd1* and *Elovl3* gene expression might be under the positive control of a common denominator. Since *Elovl3* gene expression has been shown not to be under the control of PPARα in liver, it is tempting to speculate that Wy-mediated increased of *Elovl3* mRNA in *Thb*$^{-/-}$ mice is PPARα-disconnected, as it is probably also the case for *Scd1* [33]. Illustrative for this hypothesis is that glucocorticoids have been shown to activate *Pparα*, *Elovl3* and *Scd1* mRNA levels in liver [34,35]. It can then be reasonably hypothesized that the hormonal status may be changed by *Thb* deletion and further aggravated by Wy feeding [33]. Up till now, *Pparα* is not known to be auto-regulated in liver, hence the hypothesis of increased stress metabolites in the plasma of *Thb*$^{-/-}$ mice fed Wy, that in turn would up-regulate *Pparα* mRNA levels in liver, is strengthened [34,35].

X-linked adrenoleukodystrophy (X-ALD), the most common peroxisomal disorder reported to date, is biochemically characterized by elevated plasma and tissue levels of straight-chain saturated fatty acids and VLCFAs such as C24:0, C26:0 and C26:1 [36]. In agreement with a functional peroxisomal β-oxidation under basal condition, *Thb*$^{-/-}$ mice displayed normal liver

and plasma concentrations of these three metabolites. Following Wy activation, liver C26:0 levels were comparatively reduced in *Thb*$^{-/-}$ mice while those of C24:0 remained unaltered. Hepatic reduction of C26:0 was accompanied by a significant accumulation of n-7 and n-9 MUFA. Others have previously found that cholesterol-deprivation increases MUFA in skin fibroblasts from patients with X-ALD, yet liver as well as plasma cholesterol concentrations were unaltered by *Thb* deletion and Wy feeding, ruling out at first, this hypothesis [37].

Of interest, Rizzo *et al* have previously demonstrated that incubation of cultured skin fibroblasts (X-ALD) with oleic acid (C18:1) and erucic acid (C22:1) normalized C26:0 levels, paving novel roads for therapies based on the oral administration of a dietary mixture composed of C22:1 and C18:1 fatty acids (commonly referred as “Lorenzo’s oil”) [38]. Hence, we propose that enrichment of MUFA (particularly that of C18:1n-7, C18:1n-9, C22:1n-7, C22:1n-9) observed in *Thb*$^{-/-}$ mice mice fed Wy might protect from aberrant increase of hepatic C26:0 contents. In conclusion, in the normal fed state deletion of *Thb* favors the emergence of some PUFA n-3. Following activation of FAO by the peroxisome proliferator Wy, deletion of *Thb* promotes accumulation of MUFA n-7 and n-9 *via* the activation of SCD1. One of the most pressing challenges is to check whether this finding can be translated from rodents to humans, in which only a single gene has been identified so far [4]. Further studies that aim at delineate the molecular connection between ThB and SCD1 are also awaited to better define the impact of *Thb* deletion in the regulation of cell growth and membrane fluidity signal transduction which are all pathways known to be modulated by MUFA n-7 and n-9.

Acknowledgments: This work was supported by grants from the European Union project “Peroxisomes” LSHG-CT-2004-512018, the Regional Council of Burgundy and the INSERM U866 center (Dijon). M.F. was supported by the Italian “Ministero della Ricerca Scientifica e Tecnologica”. We thank members of the staff from the Centre de Zootechnie (Dijon, France)

for their help in mice housing and breeding. We are also indebted to Amandine Chlémaire (IFR100 Imagerie Cellulaire – Histologie) for her excellent technical support and to Dr Laurent Lagrost for critical reading of the manuscript.

Abbreviations: ThB: peroxisomal 3-ketoacyl-CoA thiolase B; ThA: peroxisomal 3-ketoacyl-CoA thiolase A; ACOX-I: peroxisomal acyl-CoA oxidase-I; PPARα: Peroxisome Proliferator-Activated Receptor alpha; LCFAs: Long-Chain Fatty Acids; VLCFAs: Very-Long-Chain Fatty Acids; SFAs: Saturated Fatty Acids; MUFAs: Mono-Unsaturated Fatty Acids; PUFAs: Poly-Unsaturated Fatty Acids; SREBP: Sterol Response Element Binding Protein; TG: Triglycerides; Wy: Wy14,643; FAO: Fatty acid oxidation.

References

[1] J.K. Reddy, G.P. Mannaerts, Peroxisomal lipid metabolism, Annu. Rev. Nutr. 14 (1994) 343-370.
[2] G. Chevillard, M.C. Clemencet, P. Etienne, P. Martin, T. Pineau, N. Latruffe, V. Nicolas-Frances, Molecular cloning, gene structure and expression profile of two mouse peroxisomal 3-ketoacyl-CoA thiolase genes, BMC Biochem. 5 (2004) 3.
[3] M. Hijikata, J.K. Wen, T. Osumi, T. Hashimoto, Rat peroxisomal 3-ketoacyl-CoA thiolase gene. Occurrence of two closely related but differentially regulated genes, J. Biol. Chem. 265 (1990) 4600-4606.
[4] A. Bout, M.M. Franse, J. Collins, L. Blonden, J.M. Tager, R. Benne, Characterization of the gene encoding human peroxisomal 3-oxoacyl-CoA thiolase (ACAA). No large DNA rearrangement in a thiolase-deficient patient, Biochim Biophys Acta 1090 (1991) 43-51.
[5] S. Goldfischer, J. Collins, I. Rapin, P. Neumann, W. Neglia, A.J. Spiro, T. Ishii, F. Roels, J. Vamecq, F. Van Hoof, Pseudo-Zellweger syndrome: deficiencies in several peroxisomal oxidative activities, J. Pediatr. 108 (1986) 25-32.
[6] S. Ferdinandusse, E.G. van Grunsven, W. Oostheim, S. Denis, E.M. Hogenhout, I.J. L, C.W. van Roermund, H.R. Waterham, S. Goldfischer, R.J. Wanders, Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency: identification of the true defect at the level of d-bifunctional protein, Am. J. Hum. Genet. 70 (2002) 1589-1593.
[7] S. Ferdinandusse, S. Denis, E.M. Hogenhout, J. Koster, C.W. van Roermund, I.J. L, A.B. Moser, R.J. Wanders, H.R. Waterham, Clinical, biochemical, and mutational spectrum of peroxisomal acyl-coenzyme A oxidase deficiency, Hum. Mutat. 28 (2007) 904-912.
[8] C.Y. Fan, J. Pan, R. Chu, D. Lee, K.D. Kluckman, N. Usuda, I. Singh, A.V. Yeldandi, M.S. Rao, N. Maeda, J.K. Reddy, Hepatocellular and hepatic peroxisomal alterations in mice with a disrupted peroxisomal fatty acyl-coenzyme A oxidase gene, J. Biol. Chem. 271 (1996) 24698-24710.
[9] K. Martens, E. Ver Loren van Themaat, M.F. van Batenburg, M. Heinaniemi, S. Huyghe, P. Van Hummelen, C. Carlberg, P.P. Van Veldhoven, A. Van Kampen, M. Baes, Coordinate induction of PPARalpha and SREBP2 in multifunctional protein 2 deficient mice, Biochim. Biophys. Acta (2008).
[10] B.P. Atshaves, A.L. McIntosh, D. Landrock, H.R. Payne, J.T. Mackie, N. Maeda, J. Ball, F. Schroeder, A.B. Kier, Effect of SCP-x gene ablation on branched-chain fatty acid metabolism, Am. J. Physiol. Gastrointest. Liver Physiol. 292 (2007) G939-951.
[11] U. Seedorf, M. Raabe, P. Ellinghaus, F. Kannenberg, M. Fobker, T. Engel, S. Denis, F. Wouters, K.W. Wirtz, R.J. Wanders, N. Maeda, G. Assmann, Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-x gene function, Genes Dev. 12 (1998) 1189-1201.
[12] F. Kannenberg, P. Ellinghaus, G. Assmann, U. Seedorf, Aberrant oxidation of the cholesterol side chain in bile acid synthesis of sterol carrier protein-2/sterol carrier protein-x knockout mice, J. Biol. Chem. 274 (1999) 35455-35460.
[13] G. Chevillard, M.C. Clemencet, N. Latruffe, V. Nicolas-Frances, Targeted disruption of the peroxisomal thiolase B gene in mouse: a new model to study disorders related to peroxisomal lipid metabolism, Biochimie 86 (2004) 849-856.
[14] S. Mandard, M. Muller, S. Kersten, Peroxisome proliferator-activated receptor alpha target genes, Cell. Mol. Life Sci. 61 (2004) 393-416.
[15] P.J. Voshol, R. Havinga, H. Wolters, R. Ottenhoff, H.M. Princen, R.P. Oude Elferink, A.K. Groen, F. Kuipers, Reduced plasma cholesterol and increased fecal sterol loss in multidrug resistance gene 2 P-glycoprotein-deficient mice, Gastroenterology 114 (1998) 1024-1034.

[16] G. Denis, S. Mandard, C. Humblet, M. Verlaet, J. Boniver, D. Stehelin, M.P. Defresne, D. Regnier, Nuclear localization of a new c-cbl related protein, CARP 90, during in vivo thymic apoptosis in mice, Cell. Death Differ. 6 (1999) 689-697.
[17] S. Kersten, S. Mandard, N.S. Tan, P. Escher, D. Metzger, P. Chambon, F.J. Gonzalez, B. Desvergne, W. Wahli, Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene, J. Biol. Chem. 275 (2000) 28488-28493.
[18] S. Mandard, F. Zandbergen, N.S. Tan, P. Escher, D. Patsouris, W. Koenig, R. Kleemann, A. Bakker, F. Veenman, W. Wahli, M. Muller, S. Kersten, The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment, J. Biol. Chem. 279 (2004) 34411-34420.
[19] P. Ellinghaus, C. Wolfrum, G. Assmann, F. Spener, U. Seedorf, Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient mice, J. Biol. Chem. 274 (1999) 2766-2772.
[20] C.Y. Fan, J. Pan, N. Usuda, A.V. Yeldandi, M.S. Rao, J.K. Reddy, Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism, J. Biol. Chem. 273 (1998) 15639-15645.
[21] M. Baes, S. Huyghe, P. Carmeliet, P.E. Declercq, D. Collen, G.P. Mannaerts, P.P. Van Veldhoven, Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids, J. Biol. Chem. 275 (2000) 16329-16336.
[22] J.J. Repa, G. Liang, J. Ou, Y. Bashmakov, J.M. Lobaccaro, I. Shimomura, B. Shan, M.S. Brown, J.L. Goldstein, D.J. Mangelsdorf, Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta, Genes Dev. 14 (2000) 2819-2830.
[23] M. Maxwell, J. Bjorkman, T. Nguyen, P. Sharp, J. Finnie, C. Paterson, I. Tonks, B.C. Paton, G.F. Kay, D.I. Crane, Pex13 inactivation in the mouse disrupts peroxisome biogenesis and leads to a Zellweger syndrome phenotype, Mol. Cell. Biol. 23 (2003) 5947-5957.
[24] A.D. Attie, R.M. Krauss, M.P. Gray-Keller, A. Brownlie, M. Miyazaki, J.J. Kastelein, A.J. Lusis, A.F. Stalenhoef, J.P. Stoehr, M.R. Hayden, J.M. Ntambi, Relationship between stearoyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia, J. Lipid Res. 43 (2002) 1899-1907.
[25] S. Kemp, F. Valianpour, S. Denis, R. Ofman, R.J. Sanders, P. Mooyer, P.G. Barth, R.J. Wanders, Elongation of very long-chain fatty acids is enhanced in X-linked adrenoleukodystrophy, Mol. Genet. Metab. 84 (2005) 144-151.
[26] S.S. Lee, T. Pineau, J. Drago, E.J. Lee, J.W. Owens, D.L. Kroetz, P.M. Fernandez-Salguero, H. Westphal, F.J. Gonzalez, Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators, Mol. Cell. Biol. 15 (1995) 3012-3022.
[27] S. Kajikawa, T. Harada, A. Kawashima, K. Imada, K. Mizuguchi, Highly purified eicosapentaenoic acid prevents the progression of hepatic steatosis by repressing monounsaturated fatty acid synthesis in high-fat/high-sucrose diet-fed mice, Prostaglandins Leukot. Essent. Fatty Acids 80 (2009) 229-238.
[28] A. Dobrzyn, J.M. Ntambi, The role of stearoyl-CoA desaturase in the control of metabolism, Prostaglandins Leukot. Essent. Fatty Acids 73 (2005) 35-41.
[29] P. Dobrzyn, A. Dobrzyn, M. Miyazaki, P. Cohen, E. Asilmaz, D.G. Hardie, J.M. Friedman, J.M. Ntambi, Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver, Proc. Natl. Acad. Sci. U S A 101 (2004) 6409-6414.

[30] G. Jiang, Z. Li, F. Liu, K. Ellsworth, Q. Dallas-Yang, M. Wu, J. Ronan, C. Esau, C. Murphy, D. Szalkowski, R. Bergeron, T. Doebber, B.B. Zhang, Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearoyl-CoA desaturase-1, J. Clin. Invest. 115 (2005) 1030-1038.
[31] M. Miyazaki, Y.C. Kim, M.P. Gray-Keller, A.D. Attie, J.M. Ntambi, The biosynthesis of hepatic cholesterol esters and triglycerides is impaired in mice with a disruption of the gene for stearoyl-CoA desaturase 1, J. Biol. Chem. 275 (2000) 30132-30138.
[32] C.W. Miller, J.M. Ntambi, Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression, Proc. Natl. Acad. Sci. U S A 93 (1996) 9443-9448.
[33] A. Brolinson, S. Fourcade, A. Jakobsson, A. Pujol, A. Jacobsson, Steroid hormones control circadian Elovl3 expression in mouse liver, Endocrinology 149 (2008) 3158-3166.
[34] T. Lemberger, R. Saladin, M. Vazquez, F. Assimacopoulos, B. Staels, B. Desvergne, W. Wahli, J. Auwerx, Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm, J. Biol. Chem. 271 (1996) 1764-1769.
[35] T. Lemberger, B. Staels, R. Saladin, B. Desvergne, J. Auwerx, W. Wahli, Regulation of the peroxisome proliferator-activated receptor alpha gene by glucocorticoids, J. Biol. Chem. 269 (1994) 24527-24530.
[36] F. Valianpour, J.J. Selhorst, L.E. van Lint, A.H. van Gennip, R.J. Wanders, S. Kemp, Analysis of very long-chain fatty acids using electrospray ionization mass spectrometry, Mol. Genet. Metab. 79 (2003) 189-196.
[37] M. Engelen, R. Ofman, P.A. Mooijer, B.T. Poll-The, R.J. Wanders, S. Kemp, Cholesterol-deprivation increases mono-unsaturated very long-chain fatty acids in skin fibroblasts from patients with X-linked adrenoleukodystrophy, Biochim. Biophys. Acta 1781 (2008) 105-111.
[38] W.B. Rizzo, P.A. Watkins, M.W. Phillips, D. Cranin, B. Campbell, J. Avigan, Adrenoleukodystrophy: oleic acid lowers fibroblast saturated C22-26 fatty acids, Neurology 36 (1986) 357-361.

Figure legends

Figure 1. The PPARα signaling cascade is not constitutively activated upon *Thb* ablation in mice. 6h-fasted WT and *Thb*-/- mice previously fed or not with Wy for 8-days were used. Liver RNA was isolated and RT-qPCR was performed. Error bars represent SEM (n=5). Differences between WT and *Thb*-/- mice fed Wy were evaluated using one-way Anova; * $p<0.05$, ** $p<0.01$; Gene expression levels from the animals receiving vehicle only were set at 1. Abcd1: ATP-binding cassette, sub-family D, member 1; Abcd2: ATP-binding cassette, sub-family D, member 2. Cyp4a14: Cytochrome P450 4a14; Vlcad: Very long-chain acyl-CoA dehydrogenase; Aldh3a2/Faldh: Fatty aldehyde dehydrogenase; Cyp4a10: Cytochrome P450 4a10; Fatp-1: Fatty acid transport protein-1; L-fabp: Liver-fatty acid binding protein; Lpl: Lipoprotein lipase; Pparα: Peroxisome proliferator-activated receptor α.

Figure 2. Plasma and hepatic cholesterol levels are normal in *Thb*$^{-/-}$ mice.

(a) Plasma cholesterol concentration in liver of 6h-fasted WT and *Thb*$^{-/-}$ mice previously fed or not the PPARα agonist Wy (30 mg/kg of body weight) for 8-days and (b) lipoprotein profiling by FPLC. Fractions were assayed for cholesterol in 6h-fasted WT (n=9) and *Thb*$^{-/-}$ (n=9) mice (c) Hepatic cholesterol contents in liver of 6h-fasted WT and *Thb*$^{-/-}$ mice previously fed or not the PPARα agonist Wy (30 mg/kg of body weight) for 8-days Error bars represent ± SEM. Numbers of animals used are indicated in each bar for (a) and (c).

Figure 3. The hypotriglyceridemic action of Wy is hampered in *Thb*$^{-/-}$ mice.

(a) Plasma blood triglycerides in 6h-fasted WT and *Thb*$^{-/-}$mice previously fed or not the PPARα agonist Wy (30 mg/kg of body weight) for 8-days and (b) lipoprotein profiling by FPLC. Fractions were assayed for triglycerides in WT (n=9) and *Thb*$^{-/-}$ (n=9) mice. (c) Hepatic triglycerides contents assessed by gas chromatography in liver of 6h-fasted WT and *Thb*$^{-/-}$ mice previously fed or not the PPARα agonist Wy (30 mg/kg of body weight) for 8-day. Error bars represent ± SEM. * $p<0.05$, ** $p<0.01$. Numbers of animals used are indicated in each bar for (a) and (c).

Figure 4. Wy magnifies the effect of *Thb* deletion on lipid metabolism

Histological sections of liver stained for neutral lipids using Oil Red O. (a) Frozen liver sections of a representative WT and *Thb-/-* mouse fed or not Wy were examined for neutral lipid accumulation as described in materials and Methods. Bars indicate 10 μm. Original magnification: 40X. (b) Haematoxylin and Eosin staining of a representative WT and *Thb-/-* mouse fed or not Wy. Bars indicate 10 μm. Original magnification: 40X.

Figure 5. Expression of hepatic genes involved in fatty acid and TG synthesis is comparatively increased in *Thb*$^{-/-}$ mice fed Wy, yet nuclear form of SREBP1 remains unaltered.

(a) 6h-fasted WT and *Thb-/-* mice previously fed or not with Wy for 8-days were used. Liver RNA was isolated and RT-qPCR was performed. Error bars represent SEM (n=5). Differences between WT and *Thb*$^{-/-}$ mice fed Wy were evaluated using one-way Anova; * $p<0.05$, ** $p<0.01$; Gene expression levels from the animals receiving vehicle only were set at 1. Cycle times for the highest expressing group for each gene are shown in the corresponding bar. Wy = Wy14,643. *Acly*: ATP citrate lyase; *Acc*α: Acetyl-CoA carboxylase α; *Fas*: Fatty acid synthase; *Scd1*: Stearoyl CoA desaturase-1; *Scd2*: Stearoyl CoA desaturase-2; *Srebp1c*: Sterol regulatory element-binding protein 1c; *Dgat1*: Acyl-CoA:diacylglycerol acyltransferase 1; *Dgat2*: Acyl-CoA:diacylglycerol acyltransferase 2; *Gpam*: mitochondrial glycerol-3-phosphate acyltransferase; *Mgat1*: Monoacylgycerol acyltransferase 1; *Gyk*: Glycerol kinase; *Insig1*: Insulin-induced gene 1 ; *Srebp1a*: Sterol regulatory element-binding protein 1a; *Lxr*α: Liver x receptor α; *Chrebp*: Carbohydrate response element-binding protein.

(b) Equal amounts of nuclear protein (50 µg/lane) from the liver of WT and *Thb*$^{-/-}$ mice fed or not Wy were Western blotted with anti-SREBP1 antibody. mSREBP1: mature (nuclear) form of SREBP1. Pooled hepatic lysates (n=5) were used for each condition. Histone H4 expression was evaluated as a control of loading amount.

(c) Equal amounts of microsomal protein (50 µg/lane) isolated from the liver of WT and *Thb*$^{-/-}$ mice fed or not Wy were Western blotted with anti-SCD1 antibody. Pooled hepatic lysates (n=5) were used for each condition. Actin expression was evaluated as a control of loading amount.

Figure 6. Effect of *Thb* ablation on hepatic lipid desaturation index, on microsomal SCD1 protein content and on mRNA expression of selected genes involved in elongation of fatty acids.

(a) Shown are the ratios of the levels of MUFAs verses saturated fatty acids in liver. **p<0.01, significantly different from WT mice. Errors bars reflect S.E.M. (WT n=7, KO n=8, WTWy n=6, KOWy n= 8). *(b)* Gene expression was determined by RTqPCR. Errors bars reflect S.E.M. (WT n=5, KO n=8, WTWy n=6, KOWy n= 7). Differences between the different groups of mice were evaluated by one-way Anova test, * p <0.05, significantly different from WT mice. Cycle times for the highest expressing group for each gene are shown in the corresponding bar. Wy = Wy14,643.

Table 1.

Fatty acid composition (mass %) of total MUFAs in liver of 5-month-old mice. Values are expressed as mean ± SEM. Significant differences between both genotypes were evaluated with Anova one way test. *p<0.05; **p<0.01; ***p<0.001. In bold, saturated and monounsaturated fatty acids related, as precursors or substrates, to Scd1.

Table 2.

Fatty acid composition (mass %) of total PUFAs in liver of 5-month-old mice. Values are expressed as mean ± SEM. Significant differences between both genotypes were evaluated with Anova one way test. * p<0.05; ** p<0.01; *** p<0.001.

Table 3.

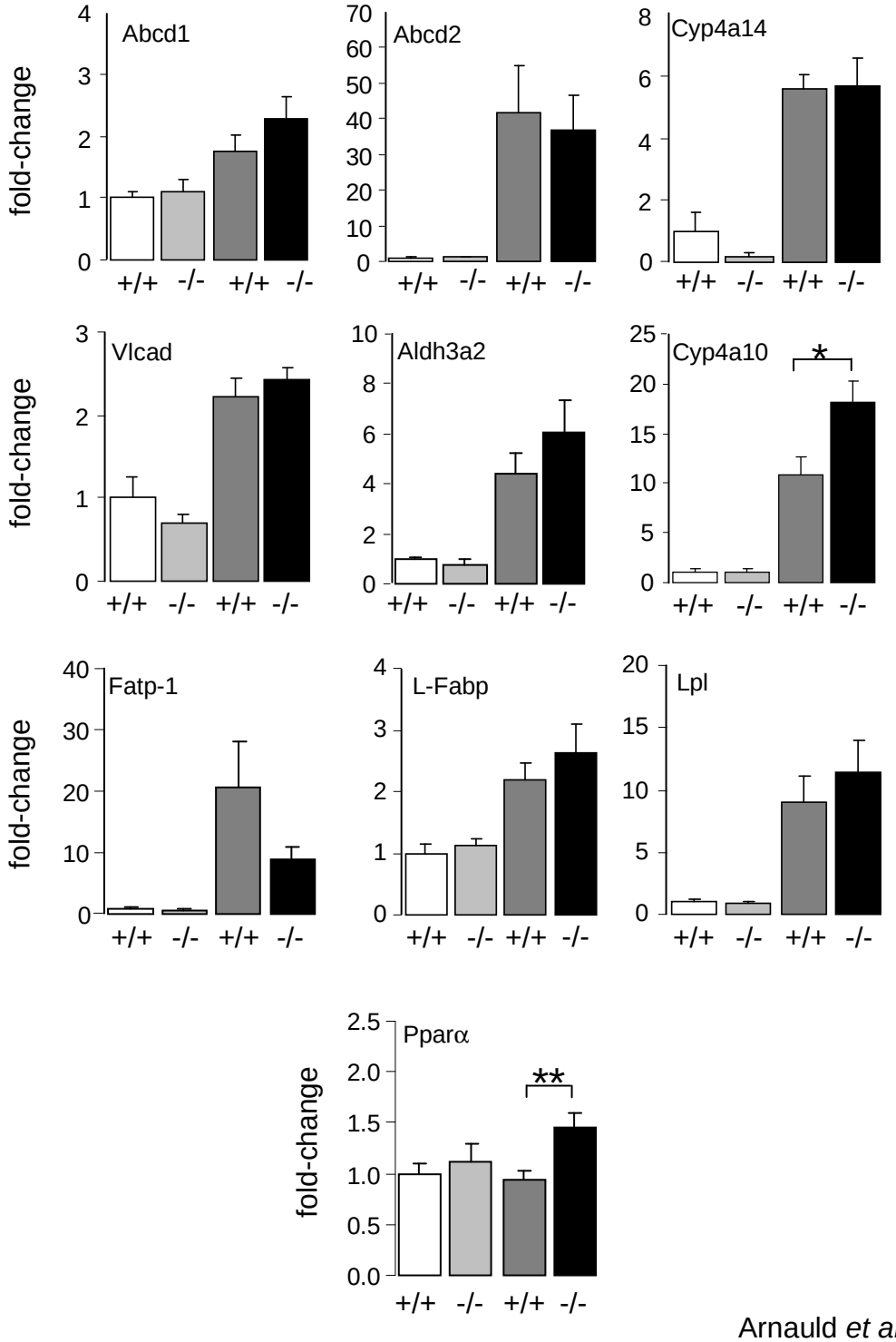
Fatty acid composition (mass %) of total Saturated FAs in liver of 5-month-old mice. Values are expressed as mean ± SEM. Significant differences between both genotypes were evaluated with Anova one way test. * p<0.05; ** p<0.01; *** p<0.001.

Supplementary data, Table 4.

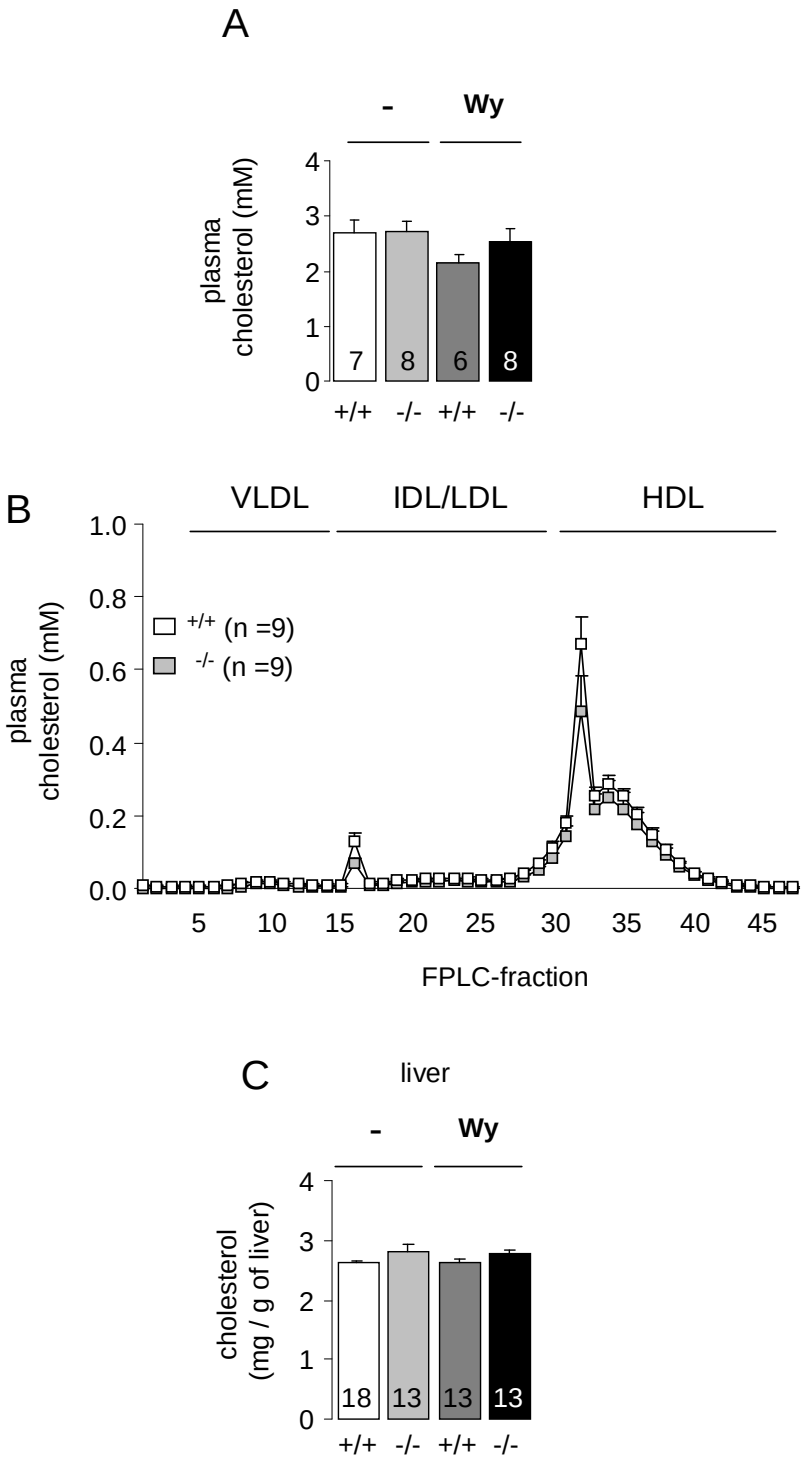
Fatty acid composition (mass %) of total Fatty Acids in plasma of 5-month-old mice. Values are expressed as mean ± SEM. Significant differences between both genotypes were evaluated using the one-way Anova test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. In bold, saturated and monounsaturated fatty acids related, as precursors or substrates, to Scd1.

Supplementary data, Table 5.

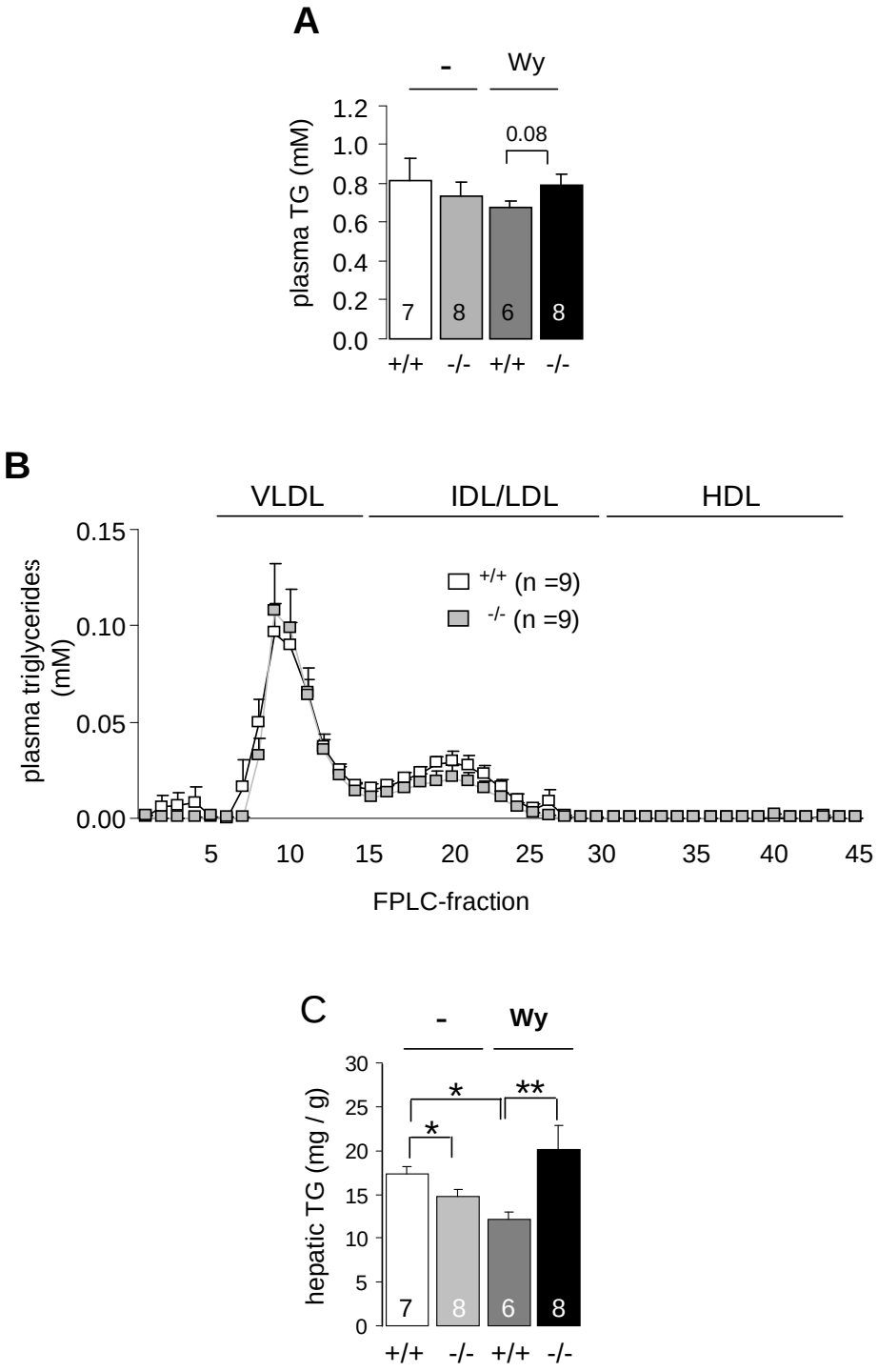
Plasma levels pristanic and phytanic acids, as determined by GC-MS. Values are expressed as mean ± SEM. Differences between both genotypes were evaluated using the one-way Anova test.



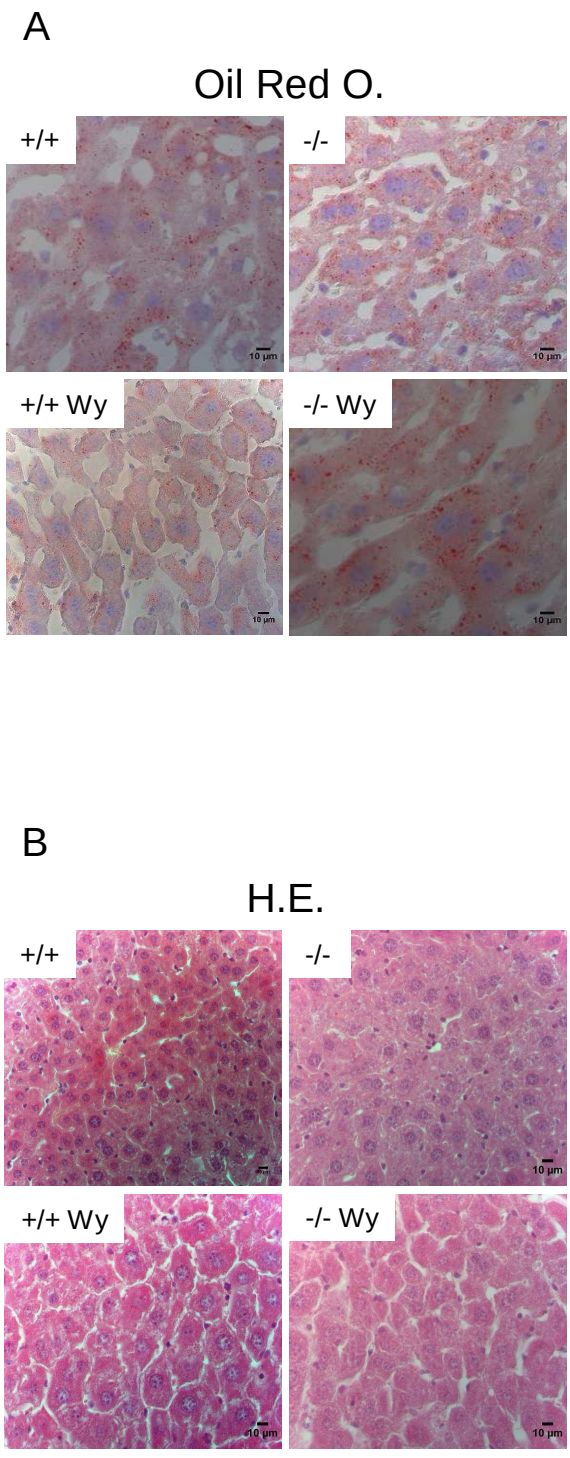
Arnauld *et al.,* Figure 1



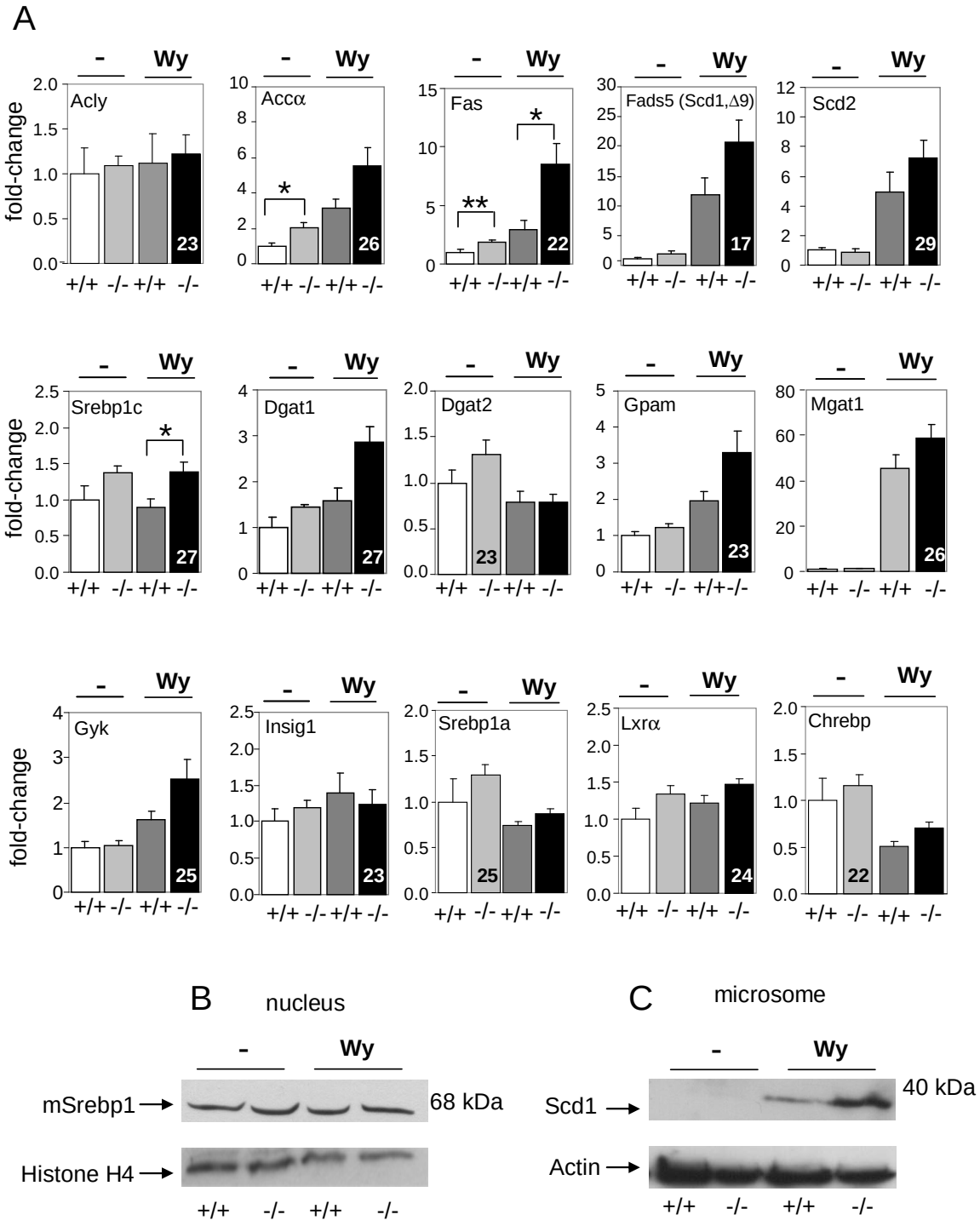
Arnauld *et al.,* Figure 2



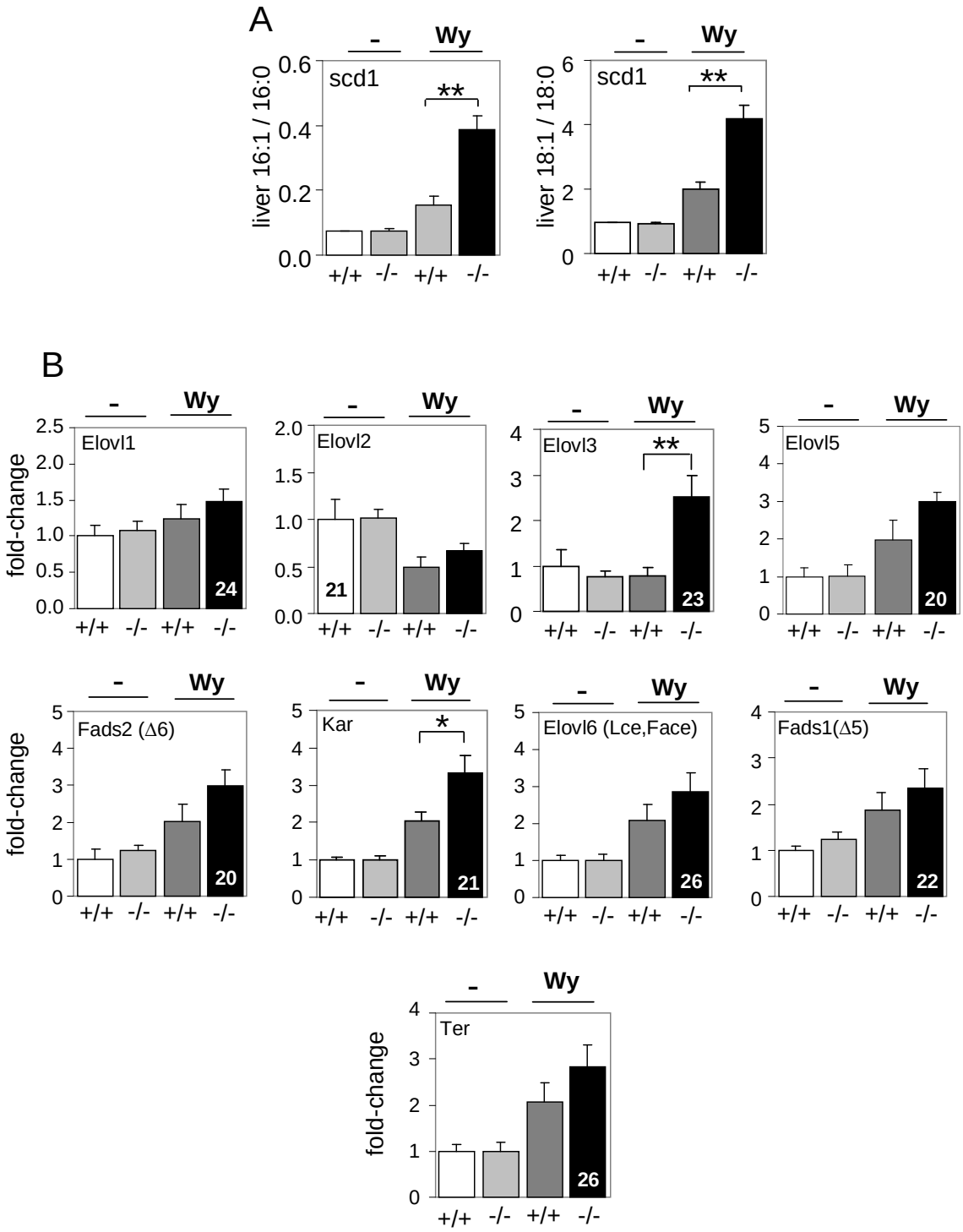
Arnauld *et al.,* Figure 3



Arnauld *et al.,* Figure 4



Arnauld *et al.*, Figure 5



Arnauld *et al.,* Figure 6

MUFA (%)	WT (n=7)	KO (n=8)	*p value*	WTWy (n=6)	KOWy (n=8)	*p value*
C16:1n-7	**0.69±0.075**	**0.69±0.05**	***1.000***	**1.041±0.123**	**1.78±0.121****	**0.001**
C18:1n-7	**1.03±0.067**	**1.20±0.085**	***0.109***	**2.28±0.256**	**2.73±0.150**	**0.122**
C20:1n-7	0.066±0.006	0.065±0,0068	*0.865*	0.091±0,016	0.139±0,013*	0.047
C22:1n-7	0.0094±0.003	0.0123±0,0047	*0.195*	0.0083±0,001	0.0218±0,0025**	0.001
C24:1n-7	0.0016±0.000	0.0023±0,00085	*0.061*	0.0013±0,00016	0.0025±0,0003**	0.006
∑ MUFA n-7	**1.81±0.05**	**1.98±0.14**	***0.321***	**3.42±0.37**	**4.68±0.25***	**0.013**
C16:1n-9	**0.17±0.03**	**0.17±0.05**	***0.865***	**0.37±0.02**	**0.46±0.02****	**0.029**
C18:1n-9	**10.46±0.34**	**9.83±0.49**	***0.335***	**16.16±0.83**	**21.73±1.16****	**0.003**
C20:1n-9	0.24±0.06	0.23±0.01	*0.756*	0.27±0.01	0.35±0.02*	0.012
C22:1n-9	0.02±0.02	0.02±0.00	*0.921*	0.017±0.00	0.03±0.00**	0.003
C24:1n-9	0.0071±0.0003	0.0088±0.0004*	0.011	0.0058±0.0004	0.0059±0.0004	*0.889*
∑ MUFA n-9	**10.90±0.36**	**10.27±0.49**	***0.335***	**16.82±0.85**	**22.58±1.19****	**0.003**
∑ MUFA	**12.72±0.38**	**12.25±0.56**	***0.518***	**20.25±0.98**	**27.26±1.30****	**0.001**

Arnauld *et al.,* Table 1

PUFA (%)	WT (n=7)	KO (n=8)	*p value*	WTWy (n=6)	KOWy (n=8)	*p value*
C18:3n-3	0.367±0.018	0.286±0.014**	0.003	0.123±0.010	0.183±0.020***	<0.0001
C20:3n-3	0.602±0.025	0.822±0.063**	0.009	2.233±0.244	1.992±0.136	*0.374*
C20:4n-3	0.043±0.0027	0.055±0.0036*	0.018	0.062±0.006	0.065±0.005	*0.745*
C20:5n-3	0.419±0.0238	0.57±0.0309**	0.002	0.350±0.043	0.324±0.036	*0.640*
C22:5n-3	0.395±0.017	0.507±0.021**	0.001	0.492±0.035	0.461±0.041	*0.587*
C22:6n-3	7.639±0.23	7.987±0.26	*0.340*	5.948±0.28	4.258±0.24***	<0.0001
C24:5n-3	0.0208±0.0022	0.0199±0.0013	*0.826*	0.005±0.001	0.004±0.000	*0.795*
C24:6n-3	0.0131±0.0016	0.0136±0.0008	*0.670*	0.006±0.001	0.008±0.001	*0.062*
∑ PUFA n-3	9.501±0.24	10.263±0.34	*0,101*	9.22±0.32	7.296±0.33**	0.001
C22:6n-3/C24:6n-3	389.86±40.5	415.50±32.4	*0.625*	1013.12±161.5	514.84±39.3**	0.005
C18:2n-6	22,81±1,359	20,75±0,910**	0.003	15,39±0,555	15,71±1,021	*0.795*
C18:3n-6	0.172±0,046	0,119±0,027*	0.015	0,102±0,005	0,098±0,003	*0.554*
C20:3n-6	0,036±0,006	0,049±0,013*	0.046	0,045±0,005	0,037±0,003	*0.204*
C20:4n-6	7,44±0,705	8,57±0,493**	0.002	8,47±0,462	6,12±0,384**	0.001
C22:4n-6	0.103±0.009	0.122±0.016*	0.014	0.129±0.006	0.133±0.007	0.662
C24:4n-6	0.003±0.001	0.004±0.001	*0.347*	0.0021±0.000	0.0018±0.000	*0.501*
C24:5n-6	0.00018±0.0000	0.00024±0.0001	*0.177*	0.00014±0.000	0.00018±0.000	*0.066*
∑ PUFA n-6	30.57±0.55	29.61±0.24	*0.122*	24.14±0.54	22.11±0.91	*0.104*
∑ PUFA	39.67±0.58	39.37±0.39	*0.67*	32.87±0.65	28.94±0.86**	0.005

Arnauld *et al.,* Table 2

SFA (%)	WT (n=7)	KO (n=8)	*p value*	WTWy (n=6)	KOWy (n=8)	*p value*
C12:0	0.016±0.02	0.014±0.05	*0.921*	0.0064±0.0005	0.00507±0.00043	*0.058*
C14:0	0.158±0.028	0.125±0.011	*0.055*	0.101±0.0053	0.117±0.0064	*0.095*
C16:0	**34.71±0.55**	**35.61±0.61**	***0.301***	**36.63±0.50**	**37.00±1.13**	**0.795**
C18:0	**12.14±0.245**	**11.90±0.352**	***0.605***	**9.54±0.696**	**6.09±0.379*** **	**<0.0001**
C20:0	0.155±0.08	0.175±0.009	*0.142*	0.076±0.0057	0.088±0.008	*0.289*
C22:0	0.014±0.001	0.017±0.001	*0.086*	0.0065±0.0005	0.0075±0.0008	*0.369*
C24:0	0.007±0.000	0.0092±0.001	*0.059*	0.0046±0.00037	0.00386±0.00038	*0.203*
C26:0	0.00096±0.000	0.00110±0.000	*0.734*	0.00078±0.00006	0.00037±0.00005***	<0.0001
∑ SFA	**47.21±0.58**	**47.86±0.54**	***0.078***	**46.38±0.52**	**43.32±1.21**	***0.061***
C26:0/C22:0	0.072±0.016	0.021±0.015	*0.612*	0.122±0.013	0.052±0.006***	0.0002
C26:0/C24:0	0.148±0.032	0.109±0.016	*0.278*	0.172±0.014	0.100±0.012**	0.002

Arnauld *et al.*, Table 3

SFA (%)	**WT (n=7)**	**KO (n=8)**	***p value***	**WTWy (n=6)**	**KOWy (n=8)**	***p value***
C12:0	0.0188±0.000	0.0220±0.003	*0.390*	0.0199±0.002	0.0159±0.002	*0.245*
C14:0	0.165±0.015	0.180±0.006	*0.345*	0.183±0.030	0.147±0.005	*0.204*
C16:0	**25.62±0.67**	**26.03±0.49**	***0.625***	**29.73±0.66**	**28.89±0.59**	***0.369***
C18:0	**10.06±1.29**	**7.22±0.26***	**0.039**	**6.26±0.30**	**4.95±0.29***	**0.010**
C20:0	0.1421±0.006	0.1354±0.008	*0.543*	0.0907±0.013	0.0904±0.006	*1.00*
C22:0	0.0153±0.001	0.0171±0.001	*0.257*	0.0159±0.005	0.0152±0.001	*0.889*
C24:0	0.0087±0.000	0.0103±0.000	*0.076*	0.0117±0.003	0.0085±0.000	*0.348*
C26:0	0.0008±0.000	0.00102±0.000	*0.424*	0.00138±0.000	0.00114±0.000	*0.476*
∑ SFA	**36.039±1.09**	**33.631±0.56**	***0.062***	**36.327±0.82**	**34.128±0.37***	**0.020**
C26:0/C22:0	0.056±0.005	0.059±0.010	*0.756*	0.100±0.018	0.073±0.005	*0.125*
C26:0/C24:0	0.098±0.010	0.095±0.009	*0.844*	0.13±0.011	0.13±0.011	*1.000*
MUFA (%)	**WT (n=7)**	**KO (n=8)**	***p value***	**WTWy (n=6)**	**KOWy (n=8)**	***p value***
C16:1n-7	**0.89±0.052**	**0.97±0.074**	***0.393***	**1.64±0.274**	**1.75±0.076**	***0.654***
C18:1n-7	**0.86±0.016**	**0.92±0.055**	***0.317***	**1.70±0.168**	**1.75±0.098**	***0.795***
C20:1n-7	0.086±0.005	0.085±0.009	*0.921*	0.111±0.015	0.155±0.016	*0.085*
C22:1n-7	0.009±0.001	0.012±0.002	*0.262*	0.009±0.001	0.022±0.002**	**0.001**
C24:1n-7	0.0017±0.001	0.0020±0.000	*0.249*	0.0017±0.000	0.0028±0.000	*0.051*
C26:1n-7	0.00064±0.000	0.00060±0.000	*0.889*	0.00036±0.000	0.00148±0.000*	**0.035**
∑ MUFA n-7	**1.84±0.064**	**1.99±0.138**	***0.367***	**3.46±0.413**	**3.69±0.142**	***0.576***
C16:1n-9	**0.17±0.012**	**0.18±0.017**	***0.618***	**0.59±0.139**	**0.38±0.022**	***0.120***
C18:1n-9	**11.38±0.677**	**11.49±0.500**	***0.889***	**17.69±0.979**	**18.36±0.515**	***0.524***
C20:1n-9	0.28±0.017	0.24±0.014	*0.229*	0.33±0.02	0.34±0.02	*0.782*
C22:1n-9	0.022±0.003	0.023±0.002	*0.756*	0.024±0.003	0.034±0.004	*0.106*
C24:1n-9	0.0083±0.000	0.0095±0.000	*0.118*	0.0076±0.000	0.0097±0.000	*0.094*
C26:1n-9	0.0030±0.000	0.0028±0.000	*0.756*	0.0028±0.000	0.0039±0.000	*0.292*
∑ MUFA n-9	**11.86±0.703**	**11.95±0.526**	***0.921***	**18.64±0.858**	**19.13±0.505**	***0.606***
∑ MUFA	**13.71±0.75**	**13.95±0.62**	***0.809***	**22.11±0.72**	**22.83±0.58**	***0.446***
PUFA (%)	**WT (n=7)**	**KO (n=8)**	***p value***	**WTWy (n=6)**	**KOWy (n=8)**	***p value***
C18:3n-3	0.38±0.031	0.41±0.017	*0.393*	0.21±0.021	0.25±0.021	*0.171*
C20:3n-3	0.61±0.031	0.77±0.044*	0.010	2.18±0.237	2.06±0.168	*0.678*
C20:4n-3	0.045±0.002	0.051±0.003	*0.152*	0.041±0.003	0.050±0.002*	0.028
C20:5n-3	0.76±0.046	0.91±0.026*	0.014	0.50±0.034	0.48±0.033	*0.633*
C22:5n-3	0.27±0.018	0.37±0.016**	0.002	0.35±0.011	0.35±0.02	*1.000*
C22:6n-3	4.85±0.266	4.94±0.198	*0.795*	3.77±0.311	2.83±0.166*	0.014
C24:5n-3	0.008±0.001	0.009±0.001	*0.548*	0.003±0.000	0.002±0.000	*0.412*
C24:6n-3	0.023±0.002	0.022±0.001	*0.605*	0.008±0.001	0.008±0.001	*0.724*
∑ PUFA n-3	6.97±0.285	7.49±0.178	*0.130*	7.08±0.226	6.05±0.192**	0.004
C22:6n-3/C24:6n-3	235.55±44.11	228.93±18.10	*0.889*	486.37±61.89	384.77±52.27	*0.231*
C18:2n-6	34.99±0.71	37.83±0.78*	0.020	27.53±0.93	31.23±0.45**	0.002
C18:3n-6	0.100±0.001	0.107±0.002*	0.016	0.080±0.003	0.09±0.001*	0.013
C20:4n-6	7.81±0.72	6.62±0.97	*0.352*	6.56±0.20	5.41±0.48	*0.073*
C22:4n-6	0.054±0.002	0.067±0.003*	0.017	0.090±0.005	0.096±0.006	*0.538*
C22:5n6	0.31±0.015	0.29±0.007	*0.108*	0.21±0.022	0.16±0.009	*0.062*
C24:4n-6	0.002±0.000	0.002±0.000	*0.471*	0.001±0.000	0.001±0.000	*0.138*
C24:5n-6	0.002±0.000	0.001±0.000	*0.105*	0.001±0.000	0.001±0.000	*0.745*
∑ PUFA n-6	43.28±1.39	44.92±0.68	*0.289*	34.48±0.87	36.99±0.70*	0.042
∑ PUFA	50.25±1.56	52.42±0.73	*0.213*	41.56±0.80	43.04±0.76	*0.210*

Arnauld *et al.,* suppementary data Table 4

	WT (n=3)	KO (n=4)	*p*	WTWy (n=3)	KOWy (n=4)	*p*
Pristanic acid (µmol/L)	0.072±0.012	0.118±0.017	*0.09*	0.087±0.008	0.097±0.007	*0.64*
Phytanic acid (µmol/L)	1.580±0.215	1.694±0.121	*0.37*	1.398±0.177	1.396 ±0.110	*0.81*

Arnauld *et al.,* supplementary data Table 5