TNF-α INTERFERES WITH LIPID HOMEOSTASIS AND ACTIVATES ACUTE AND PRO-ATHEROGENIC PROCESSES

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Running head: **TNF-α affects the lipid transcriptome and metabolome**

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Abbreviations: LDL-low density lipoprotein; Ldlr-LDL receptor; HDL-high density lipoprotein; Srebp-sterol regulatory element-binding protein; Scap-Srebp cleavage activating protein; Lxr-liver X receptor; FFA-free fatty acids ; Fxr-farnesoid X activated receptor ; LPS-lipopolysacharide; Q-RT-PCR-quantitative real time PCR; FF-MAS-follicular fluid meiosis

activating sterol; T-MAS-testis meiosis activating sterol; TNF-α-tumor necrosis factor α. Gene symbol abbreviations follow the *Unigene* code.

Abstract

The interaction between disrupted lipid homeostasis and immune response is implicated in the pathogenesis of several diseases, but the molecular bridges between the major players are still a matter of controversy. Our systemic study of the inflammatory cytokine tumor necrosis factor alpha (TNF-α) in the livers of mice exposed to 20 h-cytokine/fasting for the first time shows that TNF-α interferes with adaptation to fasting and activates harmful pro-atherogenic pathways, partially through the interaction with the insulin-Insig-Srebp (sterol regulatory element binding protein) signaling pathway. In addition to the increased expression of acute phase inflammatory genes, the most prominent alterations represent modified lipid homeostasis observed on the gene expression and metabolite levels. These include reduction of HDL-cholesterol, increase of LDL cholesterol, elevation expression of cholesterolgenic genes, accompanied by increase of potentially harmful pre-cholesterol metabolites and suppression of cholesterol elimination through bile acids. On the transcriptional level, a shift from fatty oxidation towards fatty acid synthesis is observed. The concept of TNF-α influence on the Srebp regulatory network, followed by downstream effects on sterol metabolism, is novel. Observed acute alterations in lipid metabolism are in agreement with chronic disturbances found in patients.

Keywords: transcriptome, inflammation, lipid homeostasis, acute response, cholesterol

Introduction

The interplay between inflammation and lipid metabolism is recently in the focus of research aiming to understand the development of metabolic syndrome and mechanisms of atherogenesis (33, 74). One of the difficulties to study the interaction between inflammation and lipid metabolism *in vivo* is anorexia triggered by pro-inflammatory cytokines, including TNF-α, interleukins (IL) and the endotoxin lipopolysaccharide (LPS) (37). Studies that discuss the effects of inflammatory agents have so far mostly neglected the metabolic adaptation to fasting what contributed to the controversial conclusions (26, 29, 38, 39, 48).

Cholesterol synthesis and uptake are regulated by feedback inhibition (72) that depends on the transcriptional activators sterol regulatory element-binding proteins (Srebp-1a, 1c and 2) (12). Under conditions of sterol depletion, the activation of membrane-bound Srebps and their transport from endoplasmatic reticulum (ER) to Golgi is mediated by the Srebp cleavage activating protein (Scap) (24). In contrast, when cholesterol content rises, cholesterol binds to the sterol sensing domain of Scap (65). This enables Scap to bind the anchored Insig proteins, which prevents the intracellular transport and disables activation of Srebps (11, 78, 80). Insig-1 and Insig-2 are closely related polytopic membrane proteins of the endoplasmic reticulum. They regulate cholesterol homeostasis by binding in a sterol-dependent manner to two ER proteins, the rate limiting enzyme of cholesterol biosynthesis-HMG-CoA reductase and Scap. And by doing so, they prevent activation of Srebp on one side and direct degradation of HMG-CoA reductase on the other (34) and so inactivate the cholesterol synthesis pathway.

Cholesterol is eliminated from the body through conversion to bile acids in the liver. Liver X receptors (Lxr) and farnesoid X receptor (Fxr) are nuclear receptors that function as intracellular sensors for sterols and bile acids, respectively. In response to their ligands, these receptors induce transcriptional responses that maintain a balanced, finely tuned regulation of cholesterol and bile acid metabolism (16, 45). Lxrs are sterol sensors and bind oxysterols to regulate genes critical to cholesterol efflux (Abca1, Abcg1, and ApoE), bile acid synthesis (Cyp7A1), and cholesterol secretion into bile for excretion (Abcg5/g8) (67). Fxr is activated by bile acids that and negatively regulates their synthesis to prevent bile acid toxicity and maintain bile acid homeostasis (45).

There are indications that inflammation might be one of the processes that permit the bypass of the cholesterol negative feedback loop. The increased rate of *de novo* hepatic cholesterol synthesis has been observed in different animal models of infection (26, 28, 30, 60) which would suggest a coordinate up-regulation of cholesterogenic genes/enzymes and repression of the ones responsible for cholesterol excretion. However, other studies reported that LPS, TNF-α and interleukin-1 provoke a discordant regulation of cholesterogenic genes (26, 28, 61). In contrast to the above, a recent transcriptome study of the acute-phase response to LPS *in vivo* reported a coordinate decrease of the cholesterogenic gene expression (81).

We used a systemic approach to evaluate the effect of TNF-α treatment in the mouse *in vivo* by separating the acute phase inflammatory effects from the effects of metabolic adaptation in liver to fasting. Our findings provide novel evidence that TNF-α disturbs adaptation processes to caloric restriction and mediates acute lipid-related and other pro-atherogenic changes including modulation of the Srebp regulatory pathway.

Materials and Methods

Materials. Recombinant human TNF-α with a specific activity of 3.3 x 10^7 U/mg was generously provided by V. Menart (Lek, d.d.). TNF-α was freshly diluted in sterile 0.9% saline to 0.15 mg/ml. Rabbit polyclonal antibodies anti-human Cyp51 (59), anti-human Hmgcr (Upstate) and anti-actin (Sigma-Aldrich) have been applied together with goat-anti-rabbit-HRP (Amersham), and mouse monoclonal anti-Srebp-2 antibody (BD Pharmingen) with goat-anti-mouse-HRP (Amersham). Cholesterol, desmosterol, 7-dehydrocholesterol, lanosterol, lathosterol were from Steraloids. FF-MAS and T-MAS are laboratory standards from A.G. Byskov (Laboratory of Reproductive Biology, University Hospital of Copenhagen).

Animals and treatment. All *in vivo* procedures were in accordance with the Amsterdam Protocol on Animal Protection and Welfare and were approved by Veterinary Administration of the Republic of Slovenia. Experiments were performed on C57BL/6 male mice (Harlan), between 10 and 12 weeks of age. Mice were *i.v.* injected by a human recombinant TNF-α (30 μg/animal) or with a corresponding volume (200μl) of saline and 20 hours later mice were sacrificed. In fasted and fasted/TNF-α group food was withdrawn after TNF-α application (37). Details are provided in Supplementary data.

Plasma parameters analyses. Plasma total cholesterol, HDL-cholesterol, triglycerides, free fatty acids and glucose were measured by enzymatic assays using commercially available kits (CHOD/PAP, Roche; HDL-cholesterol direct, Randox; The Infinity Triglycerides-Thermo Scientific, Free fatty acids Colorimetric Assay Kit -Roche; Autokit Glucose 2-Waco). Insulin

concentrations were measured with Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem. Inc.). VLDL, IDL/LDL and HDL were isolated by fast performance liquid chromatography (FPLC). 120 μL of pooled plasma from 5 mice was subjected to Superose 6 HR 10/30 (Pharmacia) column. Cholesterol and triglyceride content in fractions were measured enzymatically.

RNA isolation and quantitative real-time PCR (Q-RT-PCR). Total RNA was isolated using TRI-reagent (Sigma), subjected to RNeasy Cleanup column (Qiagen) processed for real-time PCR on an ABI Prism 7900 HT system (Applied Biosystems) (57). Details are provided in the Supplementary Material.

cDNA microarray hybridization and data analysis. 10 μg of total RNA was labeled using Agilent Direct Labelling Kit with Cy5 (fasted and TNF-α treated) and Cy3 (control) fluorescent dyes (Amersham) and hybridized on Agilent cDNA arrays (G4104A) according to manufacture's instructions. Individual samples from fasted and TNF-α-treated animals were hybridized versus the pooled control sample. Statistical significance of differential gene expression change was evaluated according to the Local Pooled Error test (42) and the z-test (52). Data are deposited in the GEO database with accession number GSE6317. Details are provided in the Supplementary Materials.

Immunoblot analyses of liver microsomal proteins and nuclear extracts. Protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary polyclonal antibodies against Cyp51-1:300; Hmgcr-1:1000; actin-1:1000 and anti-Srebp-2 antibody (1 μg/mL), followed by one hour incubation with a peroxidase-

conjugated anti-rabbit IgG. Peroxidase activity was measured using the goat anti-rabbit IgG (1:1000) or anti-mouse IgG (1:1000) (Amerhsam) using CL-HRP substrate system (Pierce Biotechnology). Quantification was performed using UVI Soft- UVI Band software (UVI Tec). Details are provided in the Supplementary Materials.

Liver sterol extraction and LC-MS analysis. Total liver sterols were extracted as previously described (3, 31) and subjected to LC-MS analysis. Details are provided in the Supplementary Materials.

Statistics. One-way ANOVA followed by a Tukey's multiple comparison test was used to compare all three groups. Statistical analysis was performed using Graph Pad Prism 4.02 (Graph Pad Software, San Diego, USA) and a value of $p < 0.05$ was considered to be significant for all the parameters measured.

Results

TNF-α decreases HDL-cholesterol level in plasma. Serum parameters were analyzed as a part of systematic analysis to explore the effect of TNF-α on lipid metabolism. After a single dose of TNF-α administration, food was withdrawn in TNF-α/fasted and fasted groups, whereas control group received food *ad libitum*. 20 hours after TNF-α administration animals were sacrificed. As expected, glucose and insulin levels were lower in fasted group compared to the normal fed group (Table 1). TNF-α did not result in significant changes of serum glucose and insulin levels. Serum triglycerides and free fatty acids are higher in TNF-α treated group, but the changes are not statistically significant.

In the total plasma cholesterol level, no statistically significant increase has been observed after the TNF-α treatment compared to the fasting group (Table 1). However, the effect of TNF-α on lipoprotein distribution is significant. The FPLC lipoprotein analysis shows that TNF-α induced a shift from HDL to LDL+ ILD (Figure 1) resulting in significantly decreased HDL-cholesterol level in TNF-α treated animals compared to the fasting counterparts.

TNF-α interferes with the metabolic adaptation to fasting on the transcriptional level. To understand the molecular responses to TNF-α in fasting condition, microarray analysis has been applied using mouse Agilent cDNA chips and mRNA from liver of fasted, TNF-α treated and control animals. The effect of fasting on the liver transcriptome was evaluated by direct comparison (fasted animals/normal fed controls), while the effect of TNF-α in fasted condition was evaluated by indirect comparison using normal fed control group as a common reference. Approximately 10% of the 9595 genes present on the microarray was expressed above level of detection. Among those detected as present fasting modulated 12% of genes: 46 up-regulated and

64 down-regulated, whereas TNF-α treatment resulted in modulation of 22% genes, 50 being up-regulated and 107 repressed compared to the fasted counterparts (Figure 1 in Supplementary Materials). The expression of selected genes was confirmed by Q-RT-PCR (Figures 2, 3).

TNF-α activates the expression of cholesterol biosynthetic genes in fasting. As expected, during fasting the expression of cholesterogenic genes is coordinately down-regulated due to disconnection of anabolic pathways (cholesterol metabolism in Table 2, cholesterol biosynthesis in Figure 2A). We also observed repressed expression of LDL receptor mRNA (Figure 2A) and altered mRNA level of regulatory proteins Srebp (Figure 2B) and Insig (Figure 2B, Table 2). The mRNA expression of all three Srebps, as well as Insig-1 and Insig-2b is repressed, whereas Insig-2a mRNA is expectedly up-regulated (78) (Table 2, Figure 2B).

In contrast, despite fasting the TNF-α-treatment results in the up-regulation of genes involved in cholesterol synthesis. This cytokine coordinately mobilizes the expression of genes involved in the early (*Hmgcs, Hmgcr, Fdps, Fdft1*) and late parts of cholesterol synthesis (*Cyp51, Sc4mol, Dhcr7, Sc5d, Dhcr7, Dhcr24*) (Table 2, Figure 2A). The expression of LDL-receptor mRNA was not changed (Figure 2A). In addition to cholesterogenic genes, TNF-α induces alterations in the expression of genes encoding regulatory proteins, Srebps and Insigs, respectively. Compared to the fasting response, Srebp-1a, Srebp-2 and Insig-1 mRNAs are increased while Insig-2a mRNA level is reduced by TNF-α (Figure 2B). The only exemption to the rule is Srebp-1c, which transcription is not activated by TNF-α (Figure 2B).

TNF-α represses genes involved in reverse cholesterol transport (RTC) and bile acid synthesis. Bile acid synthesis occurs *via* either the neutral (classical) or acidic (alternate) pathway

(reviewed in 68). TNF-α strongly represses three enzymes critical to the bile acid synthesis, Cyp7a and Cyp8b from neutral and Cyp27 from the acidic pathway (Figure 2C). We observed slightly decreased Cyp7a1 mRNA level in fasting conditions (Figure 2C), but after TNF-α treatment its expression was almost completely blocked (Figure 2C). Another gene from the neutral branch of bile acid synthesis, Cyp8b1, increases by exposing mice to starvation (21) (Figure 2C). Similarly to Cyp7a1, TNF-α also strongly repressed Cyp8b1 mRNA level as well as Cyp27 mRNA, the rate-limiting enzyme of the acidic branch of bile acid synthesis. However, the expression of the other enzyme from this branch, Cyp7b1, was increased by TNF-α what is consistent with previous reports (20).

The bile acid synthesizing enzymes Cyp7a1, Cyp8b1, and Cyp27 are subject to negative feedback regulation by bile acids that serve as ligands for nuclear receptors Fxr and Pxr. Fxr mediates negative feedback regulation of bile acid synthesis involving activation of another nuclear receptor Shp (2, 35). The expression level of Fxr is down-regulated by inflammation (49) what we observed also in our study (Figure 3A). Fxr target gene Shp and Pxr are not changed by fasting and TNF-α (Figure 3A). Hepatic transporters are responsible for uptake and efflux of bile acids. Two other Fxr-target genes, bile salt export pump (Bsep) that is induced by Fxr and Na(+)-taurocholate co-transporting polypeptide (Ntcp, Slc10a1) that is repressed by Fxr are both strongly down-regulated by TNF-α (Figure 3A). Thus, our data support the hypothesis that TNF-α strongly inhibits bile acid synthesis and transport by the mechanism that is most probably independent of Fxr.

Storage of cholesterol and conversion of excess cholesterol to bile acids is regulated by nuclear receptors Lxr (67). Although no decrease in Lxr and its heterodimer partner Rxr was observed, down-regulation of Lxr target genes involved in reverse cholesterol transport (Abcg5, Apo-E – Figure 3B) has been detected. This is consistent with previously observed reduced transcriptional activity of Lxr-Rxr dimers during acute phase response (7).

Despite fasting TNF-α activates fatty acid synthesis and decrease fatty acid oxidation. Since Lxrs are also key regulators of hepatic fatty acid synthesis through transcriptional activation of Srebp-1c (66), we investigated some of the Lxr-target genes involved in this pathway. In fasting, we observed down-regulation of two key enzymes of fatty acid synthesis, fatty acid synthase (Fas) and acetyl-CoA carboxylase (Acc-a) (Figure 3B). The inhibition is most probably through inhibition of Srebp-1c mRNA level (Figure 2B). In contrast, TNF-α activates the expression of two lipogenic genes Fas and Acc-a (Figure 3B) despite fasting and independently of Lxr and SREBP-1c (Figure 2B). These data suggest that TNF-α activates fatty acid synthesis through activation of another isoform, likely Srebp-1a (Figure 2B).

Nuclear receptor Ppar-a, that is activated by free fatty acids, plays a crucial role in coordinating metabolic changes caused by fasting and starvation (46). When activated, it turns on fatty acid oxidation and ketone body synthesis. We observed that several genes encoding enzymes involved in fatty acid β-(*Cpt1-a, Cpt2, Peci, Ehhadh, Aco, Hadh2*) and ω-oxidation (*Cyp4a10, Cyp4a14*) as well as fatty acid transport and uptake by liver (*Fabp 1 and 2, Acsl1, Slc27a2)* are highly up-regulated in fasting (Table 2, Figure 3C). The ω-hydroxylases of fatty acids from the cytochrome P450 superfamily, *Cyp4a10* and *Cyp4a14*, are among the most up-regulated genes in fasting (Table 2, Figure 3C) (6). Despite nutrient deprivation, the same genes are repressed by

TNF-α (Table 2, Figure 3C), most probably due to the repression of Ppar-a expression itself (Figure 3C) (50).

Lipoprotein metabolism, acute phase response and inflammation. An increased expression of genes encoding HDL-apolipoproteins A-V and C-II (Table 2) has been observed during fasting, while the expression of *ApoF*, an inhibitor of cholesteryl ester transfer protein (Cetp) (77), is diminished. Fasting provokes declination in the acute phase and other inflammatory proteins. The expression of Saa3 mRNA, a well known acute phase response protein marker, is clearly reduced (Table 2, Figure 3D). In addition, TNF-α interferes with lipoprotein metabolism. We have observed down-regulation of ApoA1, ApoE and hepatic lipase (Lipc) mRNA expression and elevated levels of Acat mRNA (Table 2, Figure 3D). HDL associated apolipoproteins (*Apo A-I, A-II, C-IV*) as well as paraoxonase 1 (*Pon1*) represent one of the strongest down-regulated groups of genes after TNF-α application (Table 2, Figure 3D). In addition, five members of Serpin (serin protease inhibitors) protein superfamily, important in hemostasis, clotting, complement system and inflammation (reviewed in 32), were also significantly down-regulated by fasting. TNF-α markedly induces the mRNA expression of many acute phase proteins, such as Saa3, serum amyloid P-component, orosomucoid 2, orosomucoid 1, hemopexin, and complement component 3. Saa3 is the most up-regulated gene in our study.

The amount of Cyp51 and mature SREBP-2 protein is increased by TNF-α. The quantity of the post-lanosterol enzyme Cyp51 is significantly diminished in fasted animals and increased after TNF-α-treatment (Figure 4A and C) what is in accordance to the Cyp51 mRNA expression data (Figure 2A), suggesting that the TNF-α-mediated up-regulation of Cyp51 mRNA leads to the

elevated amount of the Cyp51 protein. This protein seems to be enzymatically active since an increase of sterol FF-MAS, which is the product of the Cyp51 catalyzed reaction, is also observed (Figure 5B). This indicates that Cyp51 is regulated primarily on the transcriptional level.

Surprisingly (28), the quantity of HMG-CoA reductase protein (Hmgcr) was not significantly altered neither in fasting nor 20 hours after the TNF-α stimulus (Figure 4A and C). Since Hmgcr is the rate limiting enzyme of cholesterol synthesis, the kinetics of Hmgcr might precede the changes of other enzymes. Additionally, it is established that Hmgcr is regulated primarily by post-transcriptional and post-translational sterol-dependent events (64). When sterols accumulate in the cell, Hmgr protein undergoes sterol-accelerated degradation, a process mediated by Insig proteins. And remarkably, cholesterol precursor lanosterol, that accumulates after TNF-α treatment (Figure 5B), is a more potent inducer of Hmgcr degradation than cholesterol itself (reviewed in 34).

Repeated western blots with 100 000 g membrane factions of mouse liver with the anti-Srebp-2 antibody did not show a detectable signal at 125 kDa where the membrane bound Srebp-2 should reside (12). However, the amount of 68 kDa nuclear Srebp-2 was reduced by fasting (40) and elevated by TNF-α (Figure 4B and D). This is in accordance with the observed changes in expression of cholesterogenic genes that are regulated by Srebp-2 where diminished expression in starvation and up-regulation after the TNF-α treatment has been observed. Our data suggest that TNF-α stimulates cholesterol biosynthesis at least in part by stimulating transcription and activation of the regulatory proteins from cholesterol negative feed-back loop.

TNF-α alters the liver sterol metabolic profile. To our knowledge, there is no comprehensive study of the effects of inflammation or inflammatory cytokine TNF-α on the liver sterol profile. Figure 5A shows a schematic outline of the late part of cholesterol synthesis. Two enzymes (Cyp51 and Dhcr24) compete for the first cyclic intermediate lanosterol. Consequently, the late part of the cholesterol pathway has two branches with Δ24 desaturated or saturated intermediates. In addition to cholesterol, we measured several cholesterol synthesis intermediates: follicular fluid meiosis activating sterol (FF-MAS), testis meiosis activating sterol (T-MAS), lathosterol, zymosterol, desmosterol and 7-dehydrocholesterol. As expected (75), in livers of normally fed animals the level of sterol intermediates is very low, cholesterol representing 99.5% of the total sterol pool (data not shown).

Interestingly, fasting did not provoke a significant change in the liver sterol profile (Figure 5B, gray bars). The quantity of cholesterol and lanosterol was not changed significantly, whereas FF-MAS, T-MAS, zymosterol and lathosterol were even below level of detection (0.05 ng/mg for FF-MAS and T-MAS; 0.01 ng/mg for zymosterol and lathosterol). TNF-α treatment resulted in an increase of all measured liver sterols except the 7-dehydrocholesterol and lathosterol (Figure 5B, black bars), indicating activation of cholesterol biosynthesis primarily through the branch with unsaturated Δ24 intermediates. Increased sterol intermediate level was statistically significant for FF-MAS, T-MAS, zymosterol and desmosterol. It is important to note that changes in the metabolic sterol profile agree with changes of respective post-lanosterol genes, as observed by real time PCR and microarray analysis (Figure 2A, Table 2).

Discussion

TNF-α is a multifunctional cytokine with different roles ranging from proliferation to inflammatory effects and mediation of the immune responses (reviewed in 58) and in general induces anorexia. Our systemic approach enabled separation of the acute TNF-α response from the metabolic adjustment of liver to fasting. This is an important issue since experiments *in vivo* include food removal after induction of inflammation.

Mammals have evolved a complex mechanism to survive food deprivation. Generally, the hallmark of fasting is up-regulation of processes that are linked to energy production for gluconeogenesis, together with disconnection of all anabolic pathways. In accordance with this, genes involved in the β- and ω-oxidation of fatty acids as well as their transport are highly up-regulated while expression of cholesterol and bile acid synthesis genes is reduced (Table 2, Figures 2 and 6). Fasting increases the expression of the protective HDL-apolipoproteins (A-V, C-II) and inhibits the expression of the inflammatory apolipoprotein gene Saa3 that displaces ApoA-I from HDL and converts HDL from protective to pro-atherogenic (14). The expression of the acute phase proteins is also reduced, which supports the view that caloric restriction induces protective processes (5). Adaptation to fasting includes down-regulation of Srebp proteins that coordinately regulate the expression of genes involved in cholesterol and fatty acid biosynthesis. Down-regulation of Srebp-2-dependent cholesterogenic genes is expected since fasting diminished also the amount of the nuclear Srebp-2 protein (Figure 4B and D).

In contrast, TNF-α exerts multiple lipid-related pro-atherogenic and pro-oxidative alterations which can be mediated through receptors TNFRI and TNFRII (4). Human recombinant TNF-α

was injected in our study, effects are likely due to the activation of TNFRI, since mouse TNFRII shows strong specificity for the murine TNF-α (53). First of all, a coordinate up-regulation of the early and late genes of cholesterol synthesis was observed (Figure 2A), similarly as in rats (71) and after chronic LPS treatment in the mouse (81). At least three genes of this pathway (*Fdps, Fdft, Cyp51*) are up-regulated also by lead nitrate that induces hypercholesterolemia through TNF-α and interleukin-1 (51). Importantly, the observed transcriptional changes of the post-lanosterol genes are in accordance with the observed sterol metabolic profile (Figure 5B). This indicates that changes on the transcriptional level (increased expression of cholesterogenic genes mediated by elevated levels of the nuclear Srebp-2) are followed by modulation of the respective enzyme activities, suggesting further that the post-lanosterol cholesterol synthesis is regulated primarily on the level of transcription. TNF-α results in increase of Δ24 unsaturated post-lanosterol cholesterol intermediates which are not detected in the livers of regularly fed (75) and fasted animals (Figure 5B). It is worth to mention that these sterols are potentially harmful since they are not incorporated into the membranes due to redundant methyl groups (8). The exceptions are 7-dehydrocholesterol, found in membranes of patients with the Smith-Lemli-Opitz syndrome (69) and desmosterol, residing in spermatozoa and astrocyte membranes (56, 63). Other sterols, such as FF-MAS and T-MAS, have not yet fully understood signalling properties. They re-initiate the oocyte meiosis in vitro (13), contribute to maturation of the oocyte in humans in vivo (9) and are synthesized in sperm *in situ* (17). They are also non-specific ligands of the liver X- receptor (44) and are suggested to work through membrane G-protein coupled receptors (36). In addition to higher rate of cholesterol production, our and other data show that cholesterol elimination through bile acid synthesis and export is strongly inhibited (Figure 2C) (47). Cholesterol and its precursors can be so (10) subjected to harmful oxidation

which can intensify atherogenesis (55). Our data indicate that the oxidation potential is increased after TNF-α stimulus, since many genes involved in defense mechanisms against oxidative stress are down-regulated (Table 2 in Supplementary data).

Furthermore, response to TNF-α includes the shift from fatty acid oxidation (Table 2, Figure 3C and 6) to fatty acid synthesis (Table 2, Figure 3B and 6). Notably, the decrease of genes from fatty acid oxidation is a result of repressed Ppar-a expression (Figure 3C) (50) that plays a central role in fatty acid metabolism in fasting by directly stimulating the transcription of genes involved in fatty acid oxidation (46). Fatty acid synthesis is normally activated in high fed state through insulin mediated activation of Lxr-Srebp-1c transcription factors (66). However, the TNF-α-induced activation of fatty acid synthesis in low insulin/glucagon ration seems to be Lxr and Srebp-1c independent and might rely on activation of Srebp-1a (Figure 2A).

On the systemic level, the transcriptome data seem to be in accordance with the plasma lipoprotein profile. TNF-α administration results in increased expression of genes involved in cholesterol synthesis and reduced expression of genes, responsible for cholesterol elimination through bile acids, which together contribute to increase in LDL-cholesterol and reduction of HDL-cholesterol. Importantly, TNF-α inhibits the expression of paraoxonase (Pon1 - Table 2, Figure 3D), the major apolipoprotein that protects LDL from the oxidative stress (27) and is a valuable marker of atherogenic changes also in humans (54). A possible mechanism to explain the observed lipid disbalance is through interaction of TNF-α with the insulin-Insig-Srebp pathway that represents a bridge between cholesterol and energy homeostasis (23). The expression profile of Srebps and Insigs in the liver of fasted and TNF-α-treated animals

resembles the one during fasting-refeeding protocol (79). During fasting the expression of all three Srebps is repressed, but insulin stimulus during carbohydrate diet refeeding (22, 34, 79), or TNF-α despite low insulin/glucagon ratio (our study), activate their expression (Figure 2B) with exception of Srebp-1c. The expression of Srebp-1c is induced by insulin (19, 73) but not by TNF-α (Figure 2B). In addition to Srebps, the expression of Insig mRNA after TNF-α stimulus also follows the pattern of Insig mRNA expression after refeeding. Insig-2a expression is inhibited by insulin and is repressed by refeeding as well as by TNF-α (Figure 2B). Insig-1 can replace Insig-2a (22, 34, 79) (Figure 2B). Expression of the two Insigs is regulated in a different manner. Insig-1 is an obligatory Srebp target gene (43). Elevated levels of Insig-1 indicate a higher Srebp transcriptional activity after TNF-α application, what coincides with a higher Srebp-2 protein level (Figure 4B and D).

Even if we have not measured all proteins and enzyme activities, the correlation between mRNA and sterol metabolite levels suggests the regulation of the post-lanosterol cholesterol synthesis primarily at the transcriptional level. However, this seems not to be true for the early part of the pathway. While HMG-Co A reductase transcription is regulated by Srebp-2 (down-regulation of Srebp-2 and HMG-Co A reductase mRNAs in starvation) the protein is further subjected to sterol-dependent regulation. In sterol-depleted conditions (fasting), the stability of HMG-Co A reductase protein is likely increased (Figure 4A) and TNF-α in fasting does not seem to have an influence on the HMG-Co A reductase protein.

The observed TNF-α-mediated perturbations are in agreement with metabolic changes in obese subjects (62) and patients with metabolic syndrome (15). It is important to underline that the TNF-α action on liver can be either direct, or indirect through stimulated lipolysis in adipose

tissue (70), since this cytokine is implicated in the insulin resistance of adipose tissue in obese animals and humans (41, 76). Additionally, the pathophysiology of other diseases (cancer, sepsis, HIV, etc.) include TNF-α-induced anorexia and cachexia (1, 25).

In closing, our systemic study indicates that TNF-α severely disrupts the lipid homeostasis in fasting conditions which modulates metabolism of cholesterol, fatty acids, bile acids and lipoproteins. Despite caloric restriction, TNF-α coordinately induces expression of genes involved in synthesis of cholesterol and at the same time abolishes its elimination. The TNF-α activation of cholesterogenic genes results at least in part by interacting with the insulin-Insig-Srebp signaling pathway. In contrast to fasting that is accompanied with protective alterations, the acute TNF-α inflammatory response activates harmful metabolic pathways that are involved also in chronic pro-atherogenic and related pathological processes.

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Figure legends

Figure 1: **TNF-α decreases serum HDL level and increases serum levels of LDL and IDL.** 120 μL of pooled plasma from 5 mice from control (x), fasted (■) and TNF-α (▲) treated group was subjected to Superose 6 HR 10/30 (Pharmacia) column for lipoprotein separation. Cholesterol and triglyceride content in fractions were measured enzymatically. Arrows indicate TNF-α induced change lipoprotein composition from decrease in HDL to increase in LDL + IDL.

Figure 2: **TNF-α activates the expression of genes involved in cholesterol synthesis and inhibits expression of bile acid synthesis.** Equal amounts of liver RNA from 3 animals per group were pooled and converted to cDNA. The expression of genes involved in cholesterol synthesis (A), cholesterol negative feedback loop (B) and bile acid synthesis (C) was measured by Q-RT-PR in livers of normal-fed (white bars), fasted (gray bars) and TNF-α-treated/fasted (black bars) animals. mRNA values were normalized to 18S rRNA and plotted relative to normal-fed controls as the mean of triplicate measurements ± standard deviation error bars. UniGene symbols were used as abbreviations.

Figure 3: **TNF-α modulates the expression of genes involved in bile acid and fatty acid homeostasis, lipoprotein metabolism and acute phase response.** The expression of genes involved in bile acid homeostasis (A), fatty acid synthesis (B) and oxidation (C), lipoprotein metabolism and acute phase response (D) was measured by Q-RT-PR in livers of normal-fed (white bars), fasted (gray bars) and TNF-α-treated/fasted (black bars) animals. mRNA values were normalized to 18S rRNA and plotted relative to normal-fed controls as the mean of

triplicate measurements ± standard deviation error bars. UniGene symbols were used as abbreviations.

Figure 4: **TNF-α augments Cyp51 protein level and nuclear content of SREBP-2.** Representative immunoblot of liver microsomal (A) and nuclear (B) proteins from control (1-white bars), fasted (2-gray bars) and fasted/TNF-α-treated (3-black bars) mice. (A) 40 μg of microsomal proteins were subjected to SDS-PAGE and immunoblot analysis against HMG-CoA-reductase (Hmgcr) and lanosterol 14α-demethylase (Cyp51). Data represent pools from 3 animals. (B) 60 μg of pooled nuclear extracts from two animals were subjected to SDS-PAGE and immunoblot analysis against Srebp-2 was performed. (C and D). Intensity of bands from at least two independent replicas were quantified, normalized to actin and fold induction calculated relative to control that was set to 1.

Figure 5: **The outline of the late part of cholesterol biosynthesis (A) and the liver sterol metabolome in fasting (grey bars) and after TNF-α application (black bars) (B).** (A) Enzymatic steps are labeled by numbers. Sterols measured in our study are presented by formulas. Two enzymes Cyp51 (lanosterol 14α-demethylase, 1) and Dhcr24 (24-dehydrocholesterol reductase, 7) compete for lanosterol, the first cyclic intermediate of late part of cholesterol synthesis. This leads to two possible branches with sterol intermediates containing either saturated (right branch) or unsaturated (left branch) Δ24-bond. 2- Dhcr14 (14-dehydrocholesterol reductase); 3- C-4 demethylation by Sc4mol (C-4 demethylation by sterol C4-methyl oxidase), Nsdhl (3-β-hydroxy-Δ5-steroid dehydrogenase) and Hsd3b3 (3β-keto-steroid reductase), 4- Ebp- Δ8, Δ7- sterol isomerase; 5-Sc5d- sterol-C5 desaturase; 6- Dhcr7 (7-

dehydrocholesterol reductase). Abbreviations of enzyme names correspond to UniGene Symbols. FF-MAS- follicular fluid meiosis activating sterol, 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol; T-MAS- testis meiosis activating sterol, 4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol. (B) Liver sterol metabolome represented as log2 ratio of fasted/normally fed animals (grey bars) and TNF-α/fasted *versus* fasted animals (black bars). Data represent mean log2 ratio ± SD of six animals of both genders. * $P<0.05$, One-way ANOVA followed by a Tukey's multiple comparison test. # Sterols were below level of detection in fasted animals. Fold change was calculated according to the detection level threshold for each sterol. Lathosterol was below level of detection in our samples.

Figure 6: **Schematic overview of cholesterol homeostasis and the effects of TNF-α in the liver of fasted mice.** (A) Cholesterol biosynthesis is regulated by the negative cholesterol feedback loop and transcription factors Srebp. Activated Srebps trigger transcription of cholesterol biosynthetic genes leading to higher cholesterol production. In contrast, cholesterol loading prevents Srebp activation mediated by Insigs. Excess cholesterol is eliminated from peripheral organs through reverse cholesterol transport (RTC) by HDL particles. Cholesterol is converted to bile acids for excretion.

Fasting (grey arrows) inhibits cholesterol and fatty acid synthesis by inhibiting expression of regulatory proteins involved in the cholesterol negative feed-back loop (Srebps, Insig2a). This consequently leads to reduced expression of cholesterogenic genes and Cyp51 protein. To provide energy, fatty acid oxidation is activated in nutrient depleted condition.

In contrast, TNF-α (black arrows) induces the expression of genes encoding regulatory proteins Srebp-1a and -2 that leads to up-regulation of cholesterogenic genes as well as genes involved in

fatty acid synthesis. The expression of two major liver Insig proteins is reciprocal. In fasting, Insig-2a is up-regulated while Insig-1 is reduced while TNF-α reverses the expression of both. TNF-α even further inhibits expression of genes in bile acid synthesis and transport. At the same time, TNF-α blocks elimination of cholesterol through bile acid synthesis and inhibits fatty acid oxidation.

(B) In contrast to fasting, that provokes protective, anti-atherogenic, protective changes, TNF-α induces pro-atherogenic alterations in the liver.

Table legends

Table 1: Serum parameters in control, fasted and TNF-α treated mice. Each value represents mean ± SD of 5 values. [a] Level of statistical significance compared to control group, [b] Level of statistical significance compared to fasting group (One-way ANOVA followed by a Tukey's multiple comparison test, $p<0.05$).

Table 2: Differentially expressed genes in the mouse liver in fasted condition and after TNF-α treatment as detected by microarray analysis. Data represent log2 ratios of fasted *versus* normal fed control and TNF-α/fasted *versus* fasted. Genes are arranged into functional groups and sorted ascending according to log2 of the TNF-α/fasted group. P-z-test; n.d.- not detected. Genes confirmed by Q-RT-PCR are in bold.

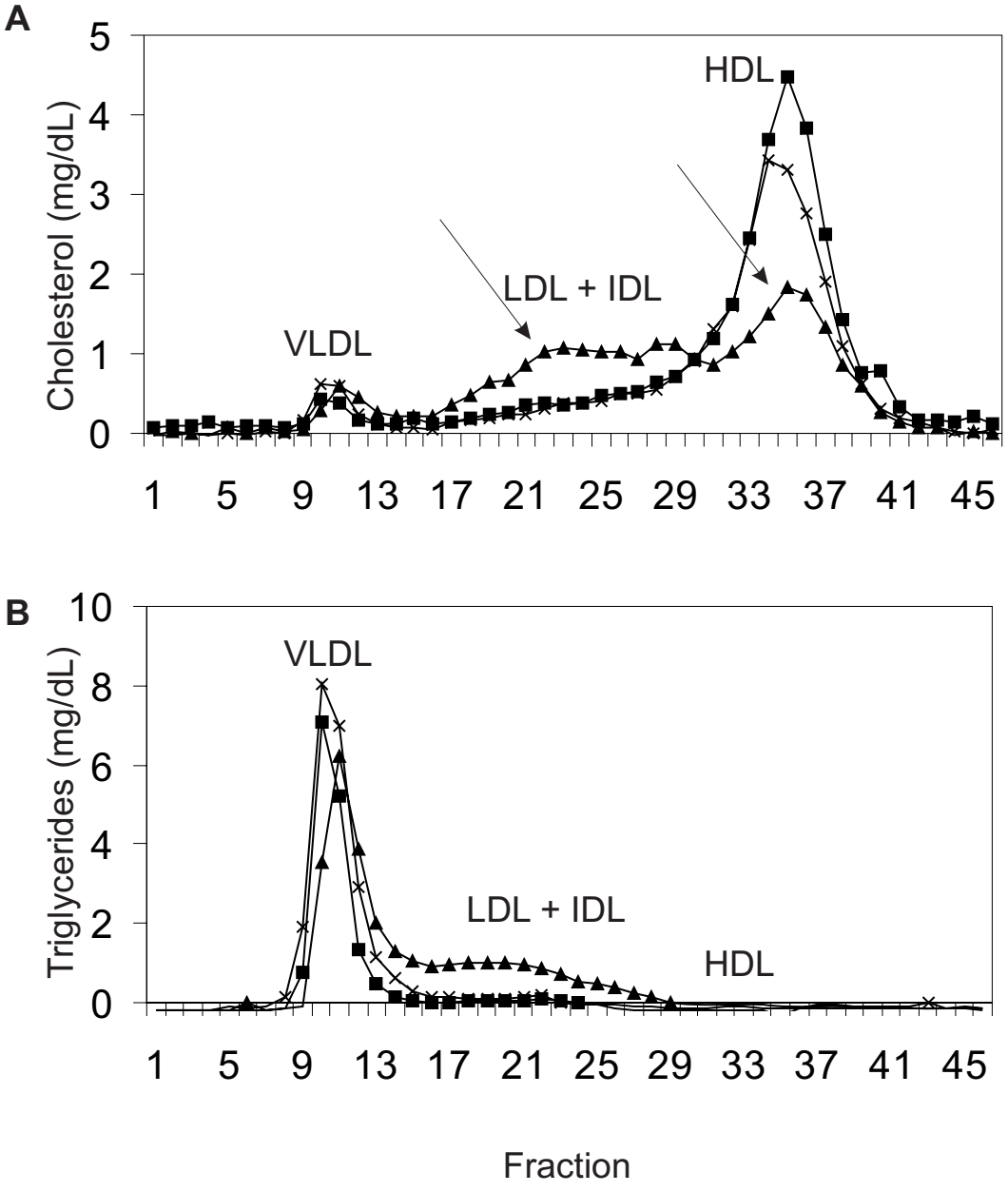

A
Cholesterol (mg/dL)
0
1
2
3
4
5
HDL
LDL + IDL
VLDL
1 5 9 13 17 21 25 29 33 37 41 45
B
Triglycerides (mg/dL)
0
2
4
6
8
10
VLDL
LDL + IDL
HDL
1 5 9 13 17 21 25 29 33 37 41 45
Fraction

Figure 1

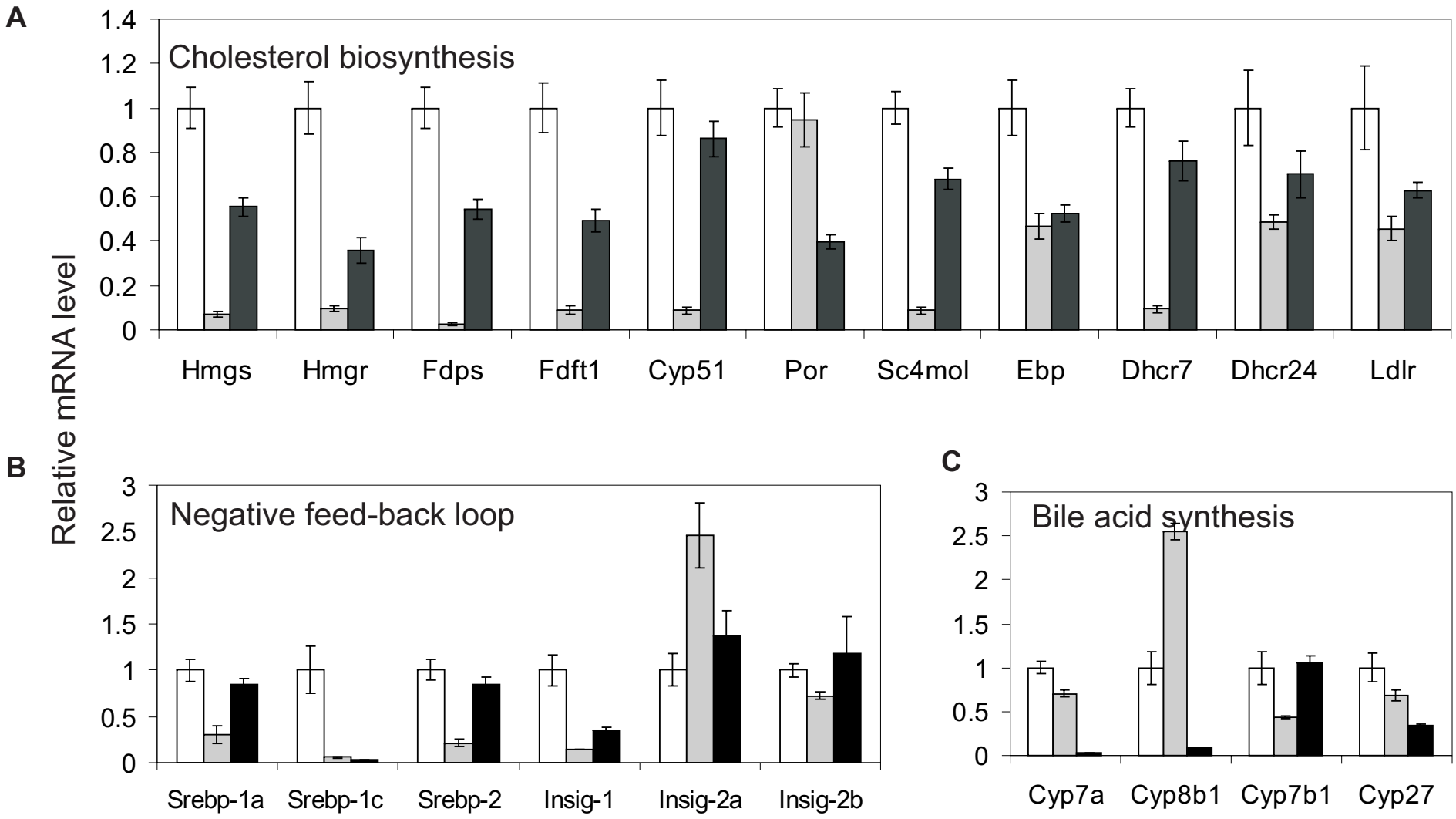

A
Cholesterol biosynthesis
Relative mRNA level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Hmgs
Hmgr
Fdps
Fdft1
Cyp51
Por
Sc4mol
Ebp
Dhcr7
Dhcr24
Ldlr
B
Negative feed-back loop
3
2.5
2
1.5
1
0.5
0
Srebp-1a
Srebp-1c
Srebp-2
Insig-1
Insig-2a
Insig-2b
C
Bile acid synthesis
3
2.5
2
1.5
1
0.5
0
Cyp7a
Cyp8b1
Cyp7b1
Cyp27

Figure 2

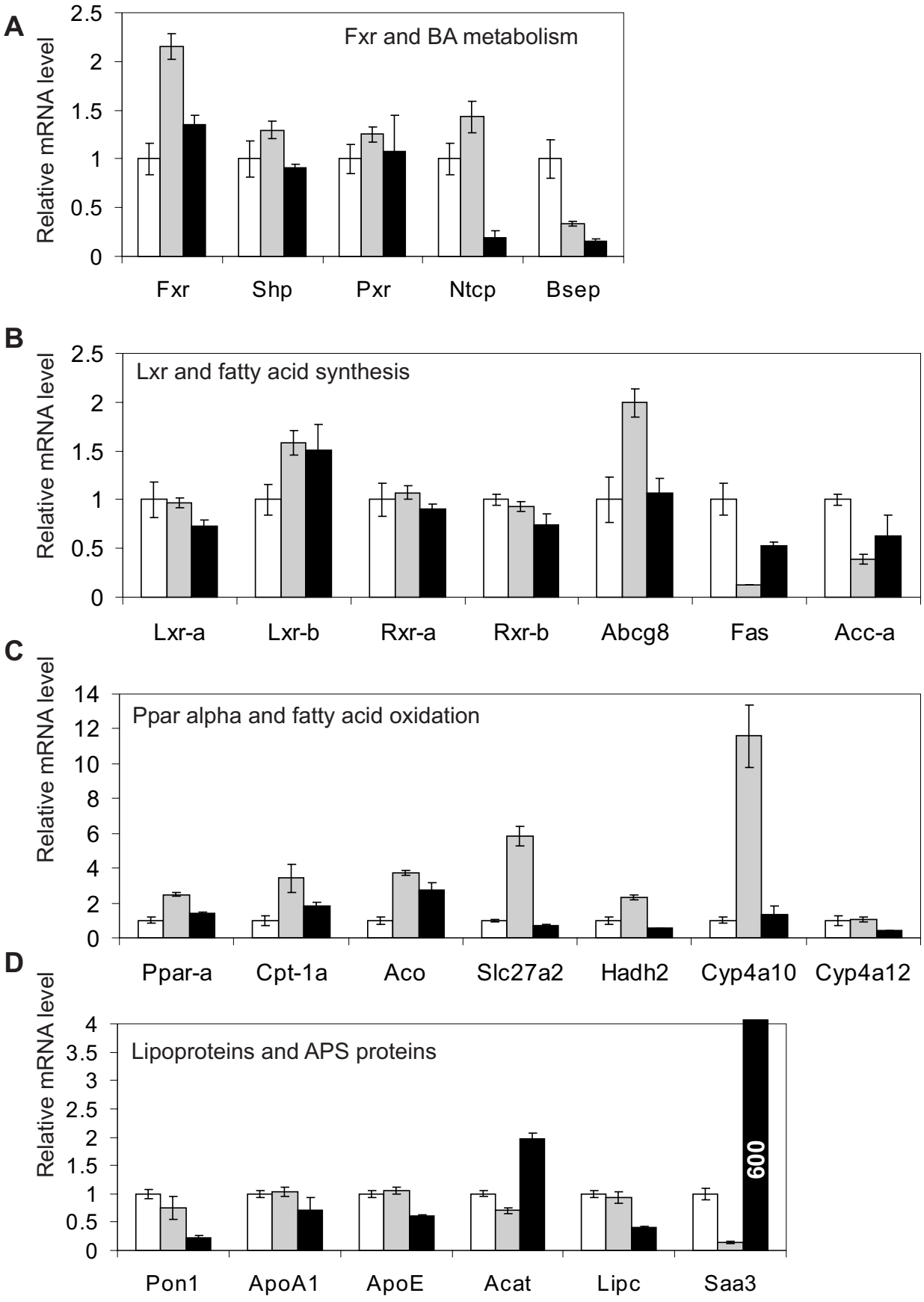

A
Fxr and BA metabolism
Relative mRNA level
2.5
2
1.5
1
0.5
0
Fxr
Shp
Pxr
Ntcp
Bsep
B
Lxr and fatty acid synthesis
Relative mRNA level
2.5
2
1.5
1
0.5
0
Lxr-a
Lxr-b
Rxr-a
Rxr-b
Abcg8
Fas
Acc-a
C
Ppar alpha and fatty acid oxidation
Relative mRNA level
14
12
10
8
6
4
2
0
Ppar-a
Cpt-1a
Aco
Slc27a2
Hadh2
Cyp4a10
Cyp4a12
D
Lipoproteins and APS proteins
Relative mRNA level
4
3.5
3
2.5
2
1.5
1
0.5
0
600
Pon1
ApoA1
ApoE
Acat
Lipc
Saa3

Figure 3

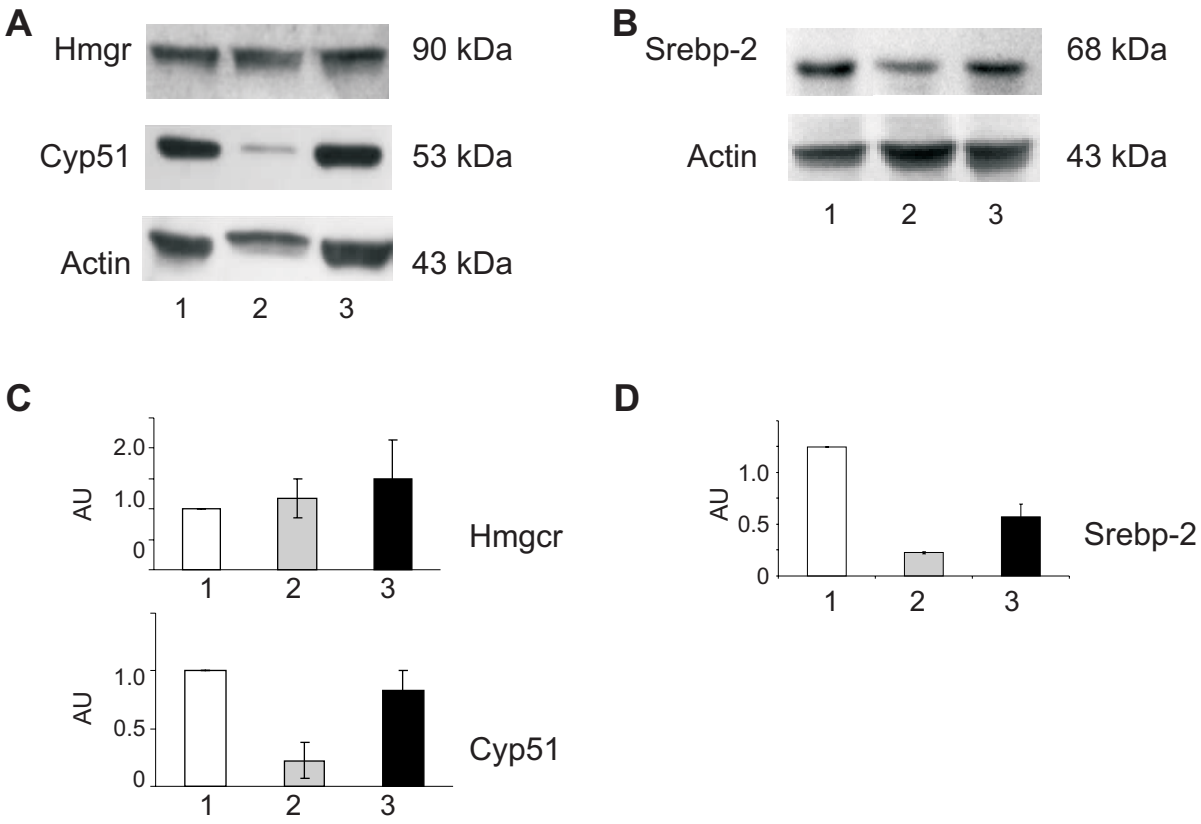

A
Hmgr
90 kDa
Cyp51
53 kDa
Actin
43 kDa
1 2 3
B
Srebp-2
68 kDa
Actin
43 kDa
1 2 3
C
AU
2.0
1.0
0
1 2 3
Hmgcr
AU
1.0
0.5
0
1 2 3
Cyp51
D
AU
1.0
0.5
0
1 2 3
Srebp-2

Figure 4

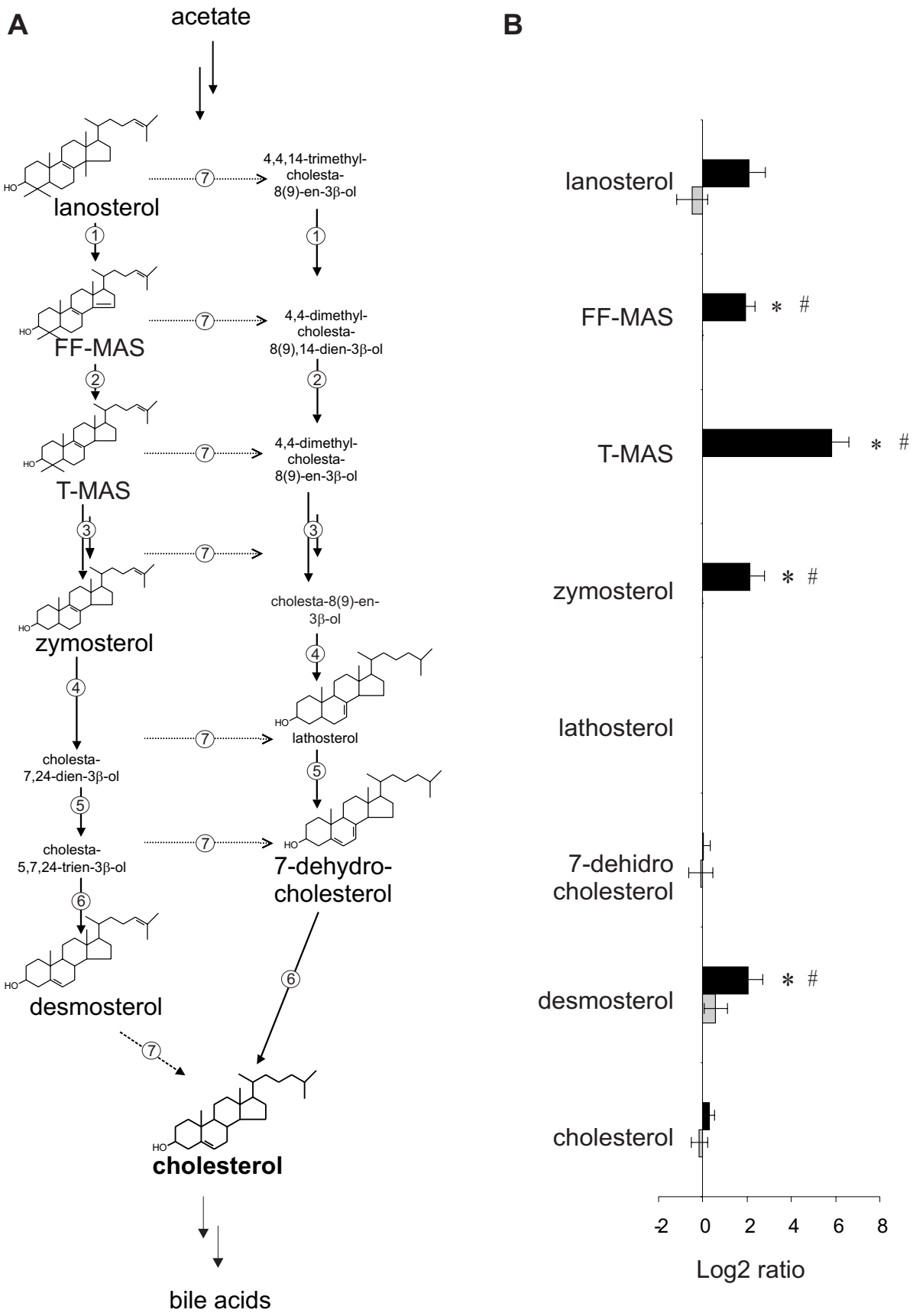
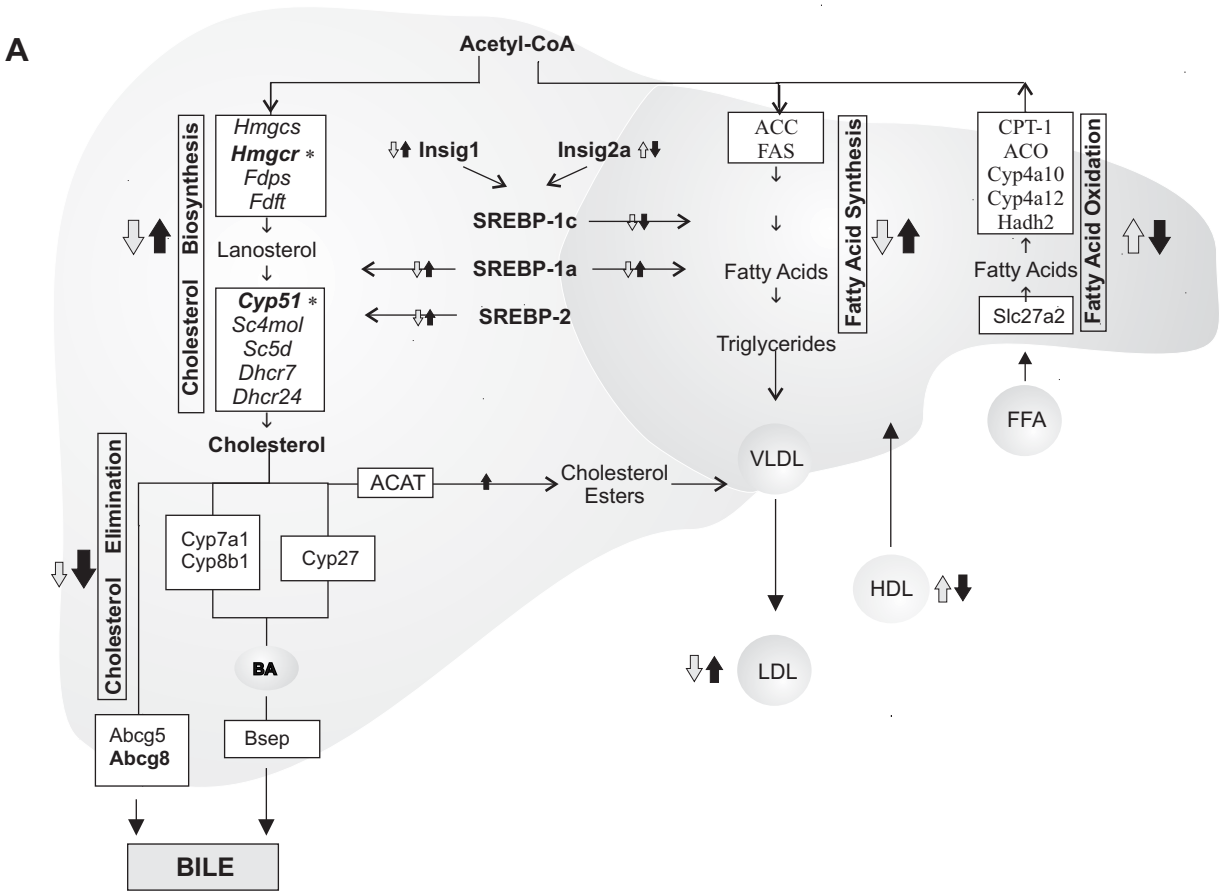

A
acetate
lanosterol
4,4,14-trimethyl-cholesta-8(9)-en-3β-ol
FF-MAS
4,4-dimethyl-cholesta-8(9),14-dien-3β-ol
T-MAS
4,4-dimethyl-cholesta-8(9)-en-3β-ol
zymosterol
cholesta-8(9)-en-3β-ol
lathosterol
cholesta-7,24-dien-3β-ol
cholesta-5,7,24-trien-3β-ol
7-dehydro-cholesterol
desmosterol
cholesterol
bile acids
B
lanosterol
FF-MAS
T-MAS
zymosterol
lathosterol
7-dehidro cholesterol
desmosterol
cholesterol
-2
0
2
4
6
8
Log2 ratio

Figure 5


A
Acetyl-CoA
Hmgcs
Hmgcr *
Fdps
Fdft
Lanosterol
Cyp51 *
Sc4mol
Sc5d
Dhcr7
Dhcr24
Cholesterol
Cholesterol Biosynthesis
Insig1
Insig2a
SREBP-1c
SREBP-1a
SREBP-2
ACC
FAS
Fatty Acids
Triglycerides
Fatty Acid Synthesis
CPT-1
ACO
Cyp4a10
Cyp4a12
Hadh2
Fatty Acids
Slc27a2
Fatty Acid Oxidation
FFA
VLDL
HDL
LDL
ACAT
Cholesterol Esters
Cholesterol Elimination
Cyp7a1
Cyp8b1
Cyp27
BA
Abcg5
Abcg8
Bsep
BILE

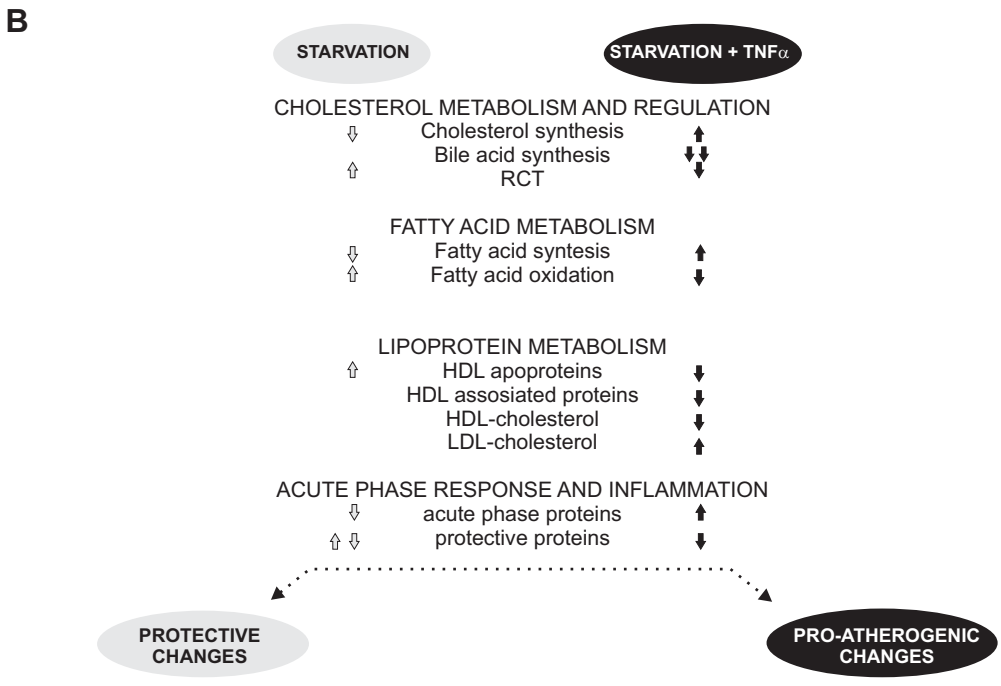

B
STARVATION
STARVATION + TNFα
CHOLESTEROL METABOLISM AND REGULATION
Cholesterol synthesis
Bile acid synthesis
RCT
FATTY ACID METABOLISM
Fatty acid syntesis
Fatty acid oxidation
LIPOPROTEIN METABOLISM
HDL apoproteins
HDL assosiated proteins
HDL-cholesterol
LDL-cholesterol
ACUTE PHASE RESPONSE AND INFLAMMATION
acute phase proteins
protective proteins
PROTECTIVE CHANGES
PRO-ATHEROGENIC CHANGES

Figure 6

Fig.1: **Fold change (log2) of gene expression and local pool error (LPE) *p*-values *(*−log2*)* for starving mice (A) and for TNF-α treated mice (B).** The two horizontal lines mark the 2-fold change threshold and the vertical line marks the threshold of cutoff *p*-value = 0.01. Genes shown in black color are identified as significantly up regulated, while genes in gray are identified as significantly down regulated. Numbers shown in each sector represent the number of genes in that sector. Total number of genes on array is 9595.