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Danielle Naville[*,†,§], Claudie Pinteur[*,†,§,1],Nathalie Vega[*,†,§,1], Yoan Menade[*,†,§], Michèle Vigier[*,†,§], Alexandre Le Bourdais[*,†,§], Emmanuel Labaronne[*,†,§], Cyrille Debard[*,†,§], Céline Luquain-Costaz[*,†,§,‡], Martine Bégeot[*,†,§], Hubert Vidal[*,†,§,‡], Brigitte Le Magueresse-Battistoni[*,†,§].

[*]Inserm, U1060, CarMeN laboratory, Oullins, F-69921, France; [†]INRA 1362, Oullins, F-69921, France; [§]Université Lyon 1, Villeurbanne, F-69622, France; [‡]INSA-Lyon, IMBL, Villeurbanne, F-69621, France.

[1]these authors contributed equally to the manuscript

Corresponding author: Brigitte Le Magueresse-Battistoni, INSERM U1060 (CarMeN), Faculté Médecine Lyon-Sud, 165 chemin du Grand Revoyet, F-69921 Oullins,

Phone 33 (0)426235919, Fax 33 (0)426235916, E-mail : brigitte.lemagueresse@inserm.fr

Short title: Pollutant mixture and metabolic disorders

Abbreviations: 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD; area-under-curve, AUC; bisphenol A, BPA; diethylhexyl phthalate, DEHP; dimethylsulfoxide, DMSO; No-Observed-Adverse-Effect Level, NOAEL; Persistent organic pollutants, POP; polychlorinated biphenyl, PCB ; Tolerable Daily Intake, TDI.

ABSTRACT

Environmental contaminants are suspected to be involved in the epidemic incidence of metabolic disorders, food ingestion being a primarily route of exposure. We hypothesized that life-long consumption of a high-fat diet that contains low-doses of pollutants will aggravate metabolic disorders induced by obesity itself. Mice were challenged from preconception throughout life with a high-fat diet containing pollutants commonly present in food (2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated-biphenyl-153, diethylhexyl-phthalate and bisphenol-A), added at low doses, in the Tolerable Daily Intake range. We measured several blood parameters, glucose and insulin tolerance, hepatic lipid accumulation and gene expression in adult mice. Pollutant-exposed mice exhibited significant sex-dependent metabolic disorders in the absence of toxicity and weight gain. In males, pollutants increased the expression of hepatic genes (from 36 to 88%) encoding proteins related to cholesterol biosynthesis and decreased (40%) hepatic total cholesterol levels. In females, there was a marked deterioration of glucose tolerance which, may be related to the 2-fold induction of estrogen-sulfotransferase and reduced expression of estrogen receptor α (25%) and estrogen target genes (>34%). Because of the very low doses of pollutants used in the mixture, these findings may have strong implications in terms of understanding the potential role of environmental contaminants in food in the development of metabolic diseases.

Keywords: BPA, DEHP, Persistent Organic Pollutant, Estrogen sulfotransferase, cholesterol biosynthesis

INTRODUCTION

Obesity is a significant health problem because of its association with increased risks for metabolic disorders including type 2 diabetes, and the current prevalence of these chronic diseases has reached epidemic proportions worldwide (1). Apart from genetic alterations and behavior linked to excessive food intake and low physical activity, which do not explain the magnitude of the disease or its kinetics, environmental pollutants have emerged as new "actors" for their suspected endocrine and metabolic disruption activity (2). This assumption was based on epidemiological studies, which associated the prevalence of type 2 diabetes with elevated body burdens of chemicals (3-6), and experimental studies in rodents, which established a causal relationship between exposure to chemicals and obesity-related metabolic dysfunction such as insulin resistance (7, 8). Based on their resistance to biodegradation, pollutants are classified as persistent organic pollutants (POPs) or short-lived pollutants. POPs are chemicals created by industrial activities either intentionally [polychlorinated biphenyls, (PCBs)] or as by-products (dioxins). They are lipophilic and accumulate higher up the food chain through processes of bioaccumulation, being present in virtually all categories of foods especially in fatty foods (9). Bisphenol A (BPA) and phthalates are short-lived chemicals, but because of their massive production in the manufacture of plastic goods, as well as epoxy resins for BPA, they are omnipresent and can leach from food and beverage containers and packaging to cause contamination (10, 11). Consequently, exposure in the general population is characterized by life-time exposure to a complex mixture of various chemical agents, the resulting effects of which could not be predicted from the effects of individual pollutant (12). In addition, pollutants can transfer from mother to fetus through the placenta during pregnancy and through breast feeding (13).

However, human health risk assessments have focused primarily on single chemicals by setting up Tolerable Daily Intake (TDI) reference doses defined by international agencies, such as the US Environmental Protection Agency (EPA) or the European Food Safety Agency (EFSA), which are based on no-observed-adverse-effect levels (NOAELs) or lowest-observed-adverse-effect levels in animal studies (14). Furthermore, the mean exposures for the general population were found to be below the current levels determined to be safe for chemicals, as demonstrated for BPA and phthalates (10), but adverse effects of BPA were shown for doses lower than the TDI reference dose (15, 16). Finally, interactions between obesity and POPs on the prevalence of type 2 diabetes in the US general population were seen (17), and experimental studies showed that intake of a high-fat diet could be a trigger initiating adverse metabolic effects, as shown with BPA in rats (18) and PCB153 in mice (19).

These data prompted us to explore the hypothesis that during their life-course, obese populations exposed to a mixture of low doses of food contaminants are particularly at risk of developing metabolic disorders, especially from exposure to contaminants defined to be of great concern for human health (2, 6). The choice of the mixture for this study was based on the persistence of pollutants, their recognized endocrine disrupting properties, the large extent of their occurrence in food, the broad range of activated signaling pathways (2, 13, 16), and their link with metabolic diseases in epidemiological and experimental studies (3, 6). Accordingly, in the present study, we determined the effect on metabolism of a mixture composed of representative persistent [2,3,7,8-tetrachlorodibenzo-p-dioxin, (TCDD), PCB 153] and short-lived pollutants (Di[2-ethylhexyl] phthalate and BPA) added at low doses (in the TDI range) to a high-fat diet provided lifelong to mice of both sexes.

MATERIALS AND METHODS

Diets and Animals

All procedures were performed with the approval of the Regional Committee of Ethics for Animal Experiments.

C57Bl/6J mice were fed a high-fat, high sucrose diet (HFSD; Harlan, Le Marcoulet, France; Supplemental Table 1) containing 36.1% fat (20). This diet was supplemented with a mixture of TCDD (LGC-Promochem, Molsheim, France), PCB153, BPA and DEHP (Sigma-Aldrich, Lyon, France) dissolved in dimethyl sulfoxide (DMSO). Each pollutant was used at a dose grossly corresponding to the TDI reference dose of either the pollutant itself [BPA, (16) and DEHP (21)] or representative congeners of dioxins and dioxin-like PCBs with TCDD (22) and non-dioxin like PCBs with PCB153 (23). The mixture was referred to as TDIΔ (Table 1). In preliminary studies, we also exposed animals to higher doses, as reported in Table 1. An equal volume of DMSO, either containing or not containing the mixture of pollutants was added to the HFSD in corn oil (30ml/100g of diet). Therefore, all animals fed the HFSD received the same amount of DMSO and corn oil. To ensure that animals ingested the correct amount of polluted food, we distributed 1g contaminated food/17g body weight/d, and extra pollutant-free HFSD was provided to animals *ad libitum.*

The protocol was as follows. Female C57Bl/6J mice, 4wk old, were purchased from Harlan and housed in separated polypropylene cages (to avoid BPA leaching from polycarbonate cages) at 21°C with a normal light-dark cycle and free access to water (polypropylene bottles) and standard chow. After a 1-week acclimatization, mice were randomized and divided into 5 groups, a group fed the HFSD without pollutant [F0-obese (F0-Ob)] and groups fed the HFSD containing the mixture of pollutants at TDIΔ (F0-ObTDIΔ) and higher doses than TDIΔ as specified in Table 1. The fifth group of mice was fed standard chow. These diets were maintained 5 wk before mating with 8-wk-old standard chow-fed males and then during gestation and lactation. After weaning, F1 descendants were fed the same diet as their dams until 12 wk of age (Supplemental Fig. 1). Body weight and

food intake were recorded weekly, and total energy expenditure was measured in 10-wk-old F1 mice by indirect calorimetry (24).

Metabolic tests

Glucose tolerance tests (GTT) and insulin sensitivity tests (IST) were performed in F1 mice at 11 wk of age exactly as previously described (25). GTTs were also performed in F0 dams after 5 wk of the HFSD with or without pollutants (i.e., before mating) and 12 wk of the HFSD with or without pollutants (i.e., 1 wk postweaning).

Blood and tissue collection

After 6h of food withdrawal, 12-wk-old mice were euthanized. Blood was collected, and liver was removed and snap-frozen in liquid nitrogen. A small piece of liver was fixed in 4% formaldehyde and frozen in liquid nitrogen. Sections were stained with Oil Red O for lipid visualization, and nuclei were colored by using hematoxylin staining. We measured blood glucose concentrations (OneTouchUltra glucometer, Lifescan, Issy-Les-Moulineaux, France) and plasma levels of insulin (Alpco; Eurobio, Courtaboeuf, France), leptin (RayBio, CliniSciences, Nanterre, France), triglycerides (TG; BioMérieux, Marcy-l'Etoile, France), total cholesterol and cholesteryl esters (CEs; Abcam, Paris, France), testosterone and 17β-estradiol (Interchim, Montluçon, France).

TG, free cholesterol (FC) and CE levels were measured in liver samples after lipid extraction by the method of Bligh and Dyer (26). For FC and CEs, lipids were separated by TLC (silica gel) using the solvent system hexane-diethylether-acetic acid (80:20:1, v/v). FC was extracted with hexane-diethylether (1:1, v/v). The samples were dried and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide. The resulting sterol derivatives were resuspended in hexane and were analyzed by gas chromatography/tandem mass spectrometry

using an Econo-Cap EC-5 capillary column with helium as the carrier gas and quantified using stigmasterol as the internal standard. CEs were transmethylated by heating at 100°C for 90 min in methanol containing 5% H_2SO_4. The resulting fatty acid (FA) methyl esters were analyzed by gas chromatography, and the percentage and mass of each FA were calculated using the internal standard (pentadecanoic acid methyl ester) as described previously (27).

Quantitative RT-PCR

Total RNA was extracted from the frozen liver tissue samples. RNA was analysed by real-time PCR exactly as described previously (28) in the presence of specific primer pairs (Supplemental Table 2) with data normalized relative to β-glucuronidase mRNA expression levels.

Western-Blotting analysis

Proteins (10µg) prepared from mouse liver were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using rabbit polyclonal antibodies directed against estrogen receptor (ER) α (sc-542; Santa-Cruz Biotechnology, CliniSciences, Nanterre, France), estrogen sulfotransferase (EST; sc-292049; Santa-Cruz Biotechnology) or mouse monoclonal antibodies directed against α-tubulin (sc-5286 from Santa-Cruz Biotechnology). After incubation with either anti-rabbit or anti-mouse IgG Horseradish peroxidase (HRP) conjugate (BioRad, Marnes-la-Coquette, France), blots were revealed using Luminata *Classico* Western HRP substrate (Millipore, Molsheim, France), detected using the *ChemiDoc*^{TM}XRS+ Imaging system (BioRad), and analyzed with Image Lab software (BioRad). EST and ERα data were normalized relatively to α-tubulin.

Statistics

All statistical analyses were performed using 1-way ANOVA, followed by *post hoc* testing with Fisher's protected least square difference test. Results are expressed as means ± SE, and differences were considered significant at values of $P < 0.05$ using the Ob group as reference.

RESULTS

Determination of the pollutant dosage resulting in no or little toxicity in offspring

F0 females fed the HFSD were heavier than standard chow-fed females, and their pups were also heavier than pups from standard chow-fed dams (Table 2). Consistent with pollutant contamination, the activating drug-metabolizing enzymes cytochrome P450 (CYP) 1A1 and CYP4A14 showed enhanced hepatic gene expression in dams exposed to the 2 highest doses of the mixtures tested (referred to as M1 and M10 in Table 2). The TDIΔ mixture dosage did not affect the body weight of F0 females at mating (Table 2). It also did not affect glucose tolerance of F0 females according to GTTs performed either before mating (5 wk of diet) or at 1 wk post weaning (12 wk of diet; data not shown). Glycemia measured in F0 females exposed to pollutants (either dosage) was in the normal range, and the sex ratio was normal among litters. However, depending on the mixture dosage, litter size, pup survival, and weight at 10 d were found to be altered (Table 2). We therefore studied the metabolic phenotype of the F1 progeny exposed to the TDIΔ mixture dosage because of no apparent toxicity that could result in nonspecific metabolic effects.

Pollutant-induced metabolic alterations in adult offspring are sex-dependent

With the HFSD, the onset of obesity was gradual (Fig. 1) and 12-wk-old mice were obese with high levels of blood glucose and enhanced levels of plasma insulin, leptin, and cholesterol but no change in plasma TGs (Table 3). Metabolic profiles were completed with

GTTs and ISTs consistently showing that obese mice were less glucose tolerant than lean mice and that obese males (but not females) were less sensitive to insulin than lean males (Fig. 1). Pollutants did not affect the plasma parameters studied (Table 3), body weight (Fig. 1), and daily food intake or energy expenditure (data not shown). Examination of liver slices indicated no gross modifications in Oil Red O staining between samples recovered from mice fed the HFSD and the HFSD plus pollutants within each sex (Supplemental Fig. 2). In pollutant-exposed females but not males, there was a marked deterioration of glucose tolerance, with a significant increase (25%, P= 0.002) of the area under curve (AUC) during GTTs without an indication of extra-hepatic insulin resistance assessed by ISTs (Fig. 1). These data indicated that lifelong consumption of a TDIΔ dose of pollutants added to the HFSD further aggravated glucose metabolic disorders of obese 12-wk-old female but not male mice. Because liver is the major site of detoxification, we focused hereafter on this organ. Furthermore, drug disposition is known to be altered in fatty liver (29) and dependent on sex (30).

Hepatic expression of transcription factors/nuclear receptors in response to pollutants in an obesity context is sex-dependent

Because transcription factors/nuclear receptors are known to control hepatic lipid metabolism and drug disposition (31), we measured the expression of xenosensors [aryl hydrocarbon receptor (AHR), constitutive androstane receptor (CAR; *Nr1i3*), and pregnane X receptor (PXR; *Nr1i2*), lipid sensors *Nr1h3* and *Nr1h2* encoding liver X receptors (LXRs) α and β, respectively; and *Nr1c1* encoding peroxisome proliferator-activated receptor α (PPARα). We showed sex-dimorphic expression of these genes with globally higher levels in chow-fed females than in males. LXRs, CAR, PPARα, and PXR (in males only) showed significant (P<0.05) enhanced gene expression levels in the HFSD-fed mice (Fig. 2A). In males but not

in females, AHR, PPARα, and LXRα mRNA expression levels were significantly up-regulated in the presence of pollutants by 160% (*P*=0.007), 60% (*P*=0.005), and 36% (*P*=0.036), respectively (Fig. 2A), whereas the LXRβ mRNA level was down-regulated in females by 32% (*P*=0.02). These data suggested that pollutants interacted with the expression of hepatic genes controlling lipogenesis, cholesterol homeostasis and AHR signaling in males but not in females.

Cholesterol metabolism-related genes are altered in males but not in females exposed to pollutants

Because pollutants affected PPARα and LXRα mRNA levels in male liver, we measured expression of target genes, including sterol regulatory element binding protein 1c (*SrebF1*), a master regulator of *de novo* lipogenesis, and several genes involved in lipid metabolism including *de novo* fatty acid (FA) synthesis such as FA Synthase (*Fasn*), acetyl-CoA carboxylase 1 (*Acaca*) and diacylglycerol O-acyltransferases (*Dgat1*and *Dgat2*); in fat uptake and transport (*Cd36*); and in cholesterol metabolism [sterol response element binding Protein 2 (*SrebF2*), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*Hmgcr*), cholesterol 7 α-hydroxylase (*Cyp7a1*), and ATP-binding cassettes (ABC) transporters (*Abca1*, *Abcg5*, *Abcg8*); (31, 32)]. As for nuclear receptors, most genes displayed sex-dimorphic expression with globally higher expression in females than in males in chow-fed conditions, and most genes had their expression levels altered with diet depending on sex (Fig. 2B-C). In pollutant-exposed males, no modification was observed in the expression level of *SrebF1*, *Fasn*, *Dgat1*, and *Dgat2*. However, *Cd36*, a target of LXRα, PPARα, and AHR (33) had its expression levels enhanced by 86% (*P*=0.047); *Acaca* expression levels were also enhanced (+46%, *P*=0.01; Fig. 2B). Notably, several genes related to cholesterol metabolism had their expression levels significantly increased on pollutant exposure (Fig. 2C). These included

SrebF2 (+64%, *P*=0.006), its downstream target gene *Hmgcr* (+65%, *P*=0.007) encoding the rate-limiting enzyme for cholesterol synthesis, and *Cyp7a1* (+88%, *P*=0.03), *Abca1* (+48%, *P*=0.03) and *Abcg8* (+49%, *P*=0.04), which are known LXRα target genes (34). These modifications, indicative of altered cholesterol biosynthesis and efflux, were not observed in pollutant-exposed females. In contrast, these exhibited reduced levels of *SrebF1* (28%, *P*=0.02) and *Dgat2* (44%, *P*=0.003), 2 genes related to FA metabolism, and a trend of 33% was observed for *Cd36* (although it did not reach significance with *P*=0.07; Fig 2B). Finally, expression of genes involved in glucose metabolism (glucose-6-phosphatase and hosphoenolpyruvate carboxykinase) or encoding inflammatory markers (tumor necrosis factor α and interleukin 6) remained unaltered by the mixture of pollutants in either sex (data not shown).

Sex-related differences in hepatic lipid content of pollutant exposed animals

To evaluate the consequences of modified expression of genes related to cholesterol metabolism, we quantified the hepatic concentrations of FC and CEs. We observed a decrease in the hepatic FC level and a trend for CEs, resulting in a significant decrease in total cholesterol levels by 40% (*P*=0.02) in F1-ObTDIΔ male mice (Fig. 3A). In females, total cholesterol levels were not affected despite a significant reduction in hepatic CEs levels (Fig. 3B). Hepatic TG accumulation (greater in the HFSD-fed than in standard chow-fed mice) was not affected by pollutants in either sex (Fig. 3C), and there were no marked changes in liver weight (not shown). These data are consistent with the absence of changes in Oil Red O staining between pollutant-exposed and non-exposed animals (Supplemental Fig.. S2).

Estrogen signaling and metabolism are altered in pollutant-exposed females but not in males

Because estrogens regulate positively hepatic insulin sensitivity (35) with ERα having a central role in energy homeostasis (36), we hypothesized that the marked alteration of glucose tolerance in pollutant-exposed females may be related to alterations in the estrogen signaling pathway. We therefore surveyed the expression of ERα at both mRNA (*Esr1*) and protein levels, and found a significant down-regulation of 25% (P=0.007) and 20% (*P*=0.01), respectively (Fig. 4A, C). Effects on *Esr1* gene expression were liver specific and were not observed in adipose tissues (not shown). In addition, the expression levels of 2 estrogen-regulated genes, selenoprotein P (*Sepp1*) (37) and insulin-like growth factor (*Igf1*) (38), were decreased by 51% (*P*=0.02) and 34% (*P*=0.03), respectively (Fig. 4A). We also measured the expression levels of drug-metabolizing enzymes involved in estrogen metabolism (39). These included NAD(P)H-dehydrogenase quinone 1 (*Nqo1*), UDP-glucuronyltransferase 1a1 (*Ugt1a1*), and the EST encoded by sulfotransferase family 1E, estrogen-preferring, member 1 (*Sult1e1*), which is the primary enzyme responsible for the inactivation of estrogens (40). In contrast with *Nqo1* and *Ugt1a1*, whose expression levels were down-regulated by 45% (*P*=0.01) and 33% (*P*=0.03), respectively, both *Sult1e1* mRNA expression and protein levels were significantly up-regulated 150%, *P*=0.02 and 120%, *P*=0.02), respectively (Fig. 4 B-C), indicating that EST may contribute to reducing hepatic insulin responsiveness in females.

Finally, these genes, whose expression levels were significantly higher in female than in male mice fed standard chow, were not altered in pollutant-exposed males except for *Esr1* encoding ERα, whose expression was up-regulated by 57% (*P*=0.01) (Fig. 4A-B).

DISCUSSION

In the present study, we developed a model allowing exploration of the effects of a mixture of low-doses of food pollutants on metabolic disorders in the progeny of obese adult

mice, under conditions of lifelong contamination encompassing maternal exposure (gestation and lactation). Within this model, exposed females but not males exhibited aggravated glucose intolerance. We also found hepatic gene alterations targeting cholesterol biosynthesis in males and estrogen metabolism in females. These events were observed in the absence of weight gain and apparent toxicity.

Because the human population is widely exposed to low levels of chemicals, it is necessary to examine the effects of pollutants not only as unique compounds but also in a mixture of persistent and short-lived chemicals activating a broad range of signaling pathways in an attempt to mimic real-world exposure. However, not all combinations could be assessed. Thus, we have chosen a mixture of persistent and nonpersistent food pollutants already described as endocrine disruptors and known to activate different signaling pathways with established links with metabolic diseases in epidemiological and experimental studies (2, 3, 6, 13, 16). We therefore dissected the metabolic phenotypes of animals exposed to a very low dose of pollutants (TDIΔ), which grossly corresponded to the TDI supposedly “safe dose” for humans. Accordingly, our data constitute a proof-of-concept model addressing the hypothesis of possible cumulative metabolic adverse effects of a pollutant mixture as suggested with reprotoxicity studies (41, 42). This is especially important considering the doses used in the present study which are $\geq$ 3 orders of magnitude lower than doses commonly used in toxicological studies with the exception of BPA (2, 19, 43-45) and relatively close to the doses to which human beings may be exposed to (16, 21, 23, 46).

This study focused on liver because it is the major site of detoxification, and it is known that drug disposition is altered in fatty liver (29). Because metabolic disruption could result from inappropriate activation of transcription factors and nuclear receptors (2, 5), we reasoned that they may be primary targets under conditions of exposure to the HFSD, pollutants orchestrating phenotypic changes through alteration in the expression of

315 downstream genes. Notably, we found a sex-dimorphic metabolic response to the pollutant
316 mixture in an obesity context. Furthermore, most genes shown to be altered by the mixture of
317 pollutants were expressed in a sex-dimorphic manner with higher levels in females than in
318 males under standard chow condition (this last point extends previous data) (47). This finding
319 highlights the necessity for conducting toxicological experiments on the 2 sexes.

320 In males, although pollutants did not alter glucose and insulin metabolic tests or
321 plasma cholesterol levels, several genes were stimulated in liver including LXRα and
322 PPARα and their target genes, HMGCoAR encoding the rate-limiting enzyme in cholesterol
323 synthesis and SREBP2 (31, 32), together with enhanced expression of genes encoding
324 CYP7A1 and ABC transporters (*Abca1* and *Abcg8*). There was also a decrease in hepatic total
325 cholesterol. It is noteworthy that all genes altered converged to cholesterol synthesis and
326 efflux directed toward the bile salt pathway. Therefore, further studies will be needed to
327 distinguish between adverse and adaptive effects in liver, particularly focusing on older
328 animals because deleterious effects may appear later in life. Finally, the meaning of the
329 induced expression of ERα will have to be explored in that scheme.

330 Contrasting with males, females became more glucose intolerant in the presence of
331 pollutants at the TDIΔ dose than the unexposed females with no changes in the expression of
332 genes involved in hepatic glucose production or inflammation. It is known that estrogens
333 protect females from obesity and diabetes, probably acting through ERα in the liver because
334 it is the dominant ER in this organ (48). We therefore postulated that the metabolic alteration
335 could result from the observed down-regulation of *Esr1* expression, which would provoke a
336 reduction in estrogen signaling. Indeed, disruption of the estrogen signaling pathway results in
337 metabolic dysregulation and hepatic insulin resistance (36, 49-51). One well-described
338 mechanism is conjugation of a sulfonate group to estrogens by EST, thereby inactivating them
339 and preventing their binding to the ER (40, 52). Notably, induction of hepatic EST is a

common feature of type 2 diabetes (35, 40), and loss of EST in female but not male mice has recently been shown to improve metabolic function in diabetic mice (40). Because plasma levels of estrogens were not altered, these data prompted us to investigate EST expression. The observation of its enhanced mRNA and protein levels in the liver of female mice was thus consistent with a decreased estrogenic signaling. We can therefore suggest that pollutant-related induction of EST may reduce estrogen bioavaibility specifically in the liver, hence down-regulating ERα and the expression of target genes including *Igf1* and *Sepp1*.

The sex-dependent action of the mixture of pollutants in liver is probably related to the endocrine disrupting activity of the pollutants, with dioxins having proestrogen and antiestrogen activity, depending on the hormonal context, phthalates generally described as antiandrogens, and BPA and PCBs bearing estrogen-mimetic activities (2-5, 53). This emphasized the advantage of using a combination of pollutants rather than pollutants individually, to unravel the resulting endocrine/metabolic-disrupting activity of the mixture in the biological model investigated. In our experimental model, no alteration in body weight of the adult progeny was observed, thus indicating that the mixture was not obesogenic under our experimental conditions. The term obesogen was coined by Grün and Blumberg (54) to design chemicals that generate obesity. Notably, each pollutant of the mixture has been described as potentially adipogenic (2, 55). For example, increased body weight is observed in rats with low concentrations of dioxins, whereas high doses resulted in decreased body weight (43). Moreover, Taxvig *et al.* (55) demonstrated differential effects of food contaminants on adipogenesis using the *in vitro* model of 3T3-L1 with BPA, PCB 153, and the DEHP major metabolite mono-(2-ethylhexyl) phthalate, increasing adipogenesis. Because it was shown that intake of a high-fat diet intake could be a trigger initiating the adverse metabolic effects of BPA (18) or PCB 153 (19), it will be relevant in future studies to determine whether similar alterations could be generated with standard-fed animals or if

obese populations are particularly sensitive to pollutants. It will also be interesting to explore the adipose tissue even though we did not observe any weight changes in fat pads, at least in males, because it is a storage tissue for pollutants.

The protocol we chose did not allow us to discriminate among the different windows of exposure to which animals were the most susceptible, because animals have experienced lifelong exposure. This is an extremely complex issue because it is highly endpoint and pollutant dependent as reported previously (2-4, 56). In addition, it is known from Barker's hypothesis (57) on the developmental origins of health and diseases that the developmental period is a period of high vulnerability, and exposure to pollutants during gestation and lactation may be responsible for an increase in the outcome of metabolic disorders later in life as discussed Barouki *et al*.(58).

Moreover, males and females probably show different effects because pollutants have been described as endocrine disruptors. To circumvent these differences, we exposed animals life-long to a high fat diet containing a mixture of food pollutants widely distributed in our environment at doses considered to be safe for humans because there were grossly equal to the TDI (and even lower for BPA) and relatively close to the environmental doses to which human beings may be exposed, as mentioned above. With this worst case scenario, we demonstrated for the first time sex-specific metabolic alterations in the absence of general toxicity and body weight gain.

However, pollutants in the mixture have not been tested individually, making it difficult to conclude whether the effects of the mixture are additive, synergic, or antagonist. To overcome this problem and avoid excessive animal use in experiments, *in vitro* experiments will be required. Nonetheless, we here demonstrated the lack of zero effect when using a mixture of very low doses of food pollutants. This study therefore fuels the concept of rethinking the way to address the question of risk assessment used to date, based on NOAELs

for single chemicals, and may have strong implications in terms of recommendations for food market security. It also adds new information for understanding the effect of environmental contaminants in food in the development of metabolic diseases.

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582 **LEGENDS**
583

584 **Fig. 1**: **(A/B).** Body weight curve from weaning to 12 weeks of age for F1 male (A) and
585 female (B) mice fed either HFSD (F1-Ob) or HFSD containing the mixture of pollutants at the
586 TDIΔ dose (F1-ObTDIΔ). Results are expressed as mean ± SEM with n ≥ 9 for each point.
587 **(C/D)**. Glucose tolerance tests (GTT) performed on F1 male (C) and female (D) mice. **(E)**
588 Areas under curves (AUCs) calculated from curves corresponding to GTT (C, D). Results are
589 expressed as mean ± SEM with n ≥ 6 in F1-Ob and F1-Ob TDIΔ groups and n = 3 for
590 standard mice. **(F/G)**. Insulin sensitivity tests (IST) performed on F1 male (F) and female (G)
591 mice. Results are expressed as mean ± SEM with n ≥ 5.

592 *: $p < 0.05$ compared to male F1-Ob and **: $p < 0.05$ compared to female F1-Ob.

593

594 **Fig. 2:** Impact of the mixture of pollutants at the TDIΔ dose on male and female hepatic
595 expression of (A) transcription factors and nuclear receptors including Aryl hydrocarbon
596 receptor (Ahr), Constitutive Androstane Receptor (Nr1i3), Pregnane X receptor (Nr1i2),
597 Peroxisome proliferator-activated receptor α (Nr1c1), Liver X receptors alpha (Nr1h3) and
598 beta (Nr1h2); (B) genes encoding proteins related to lipogenesis: Sterol regulatory element 1c
599 (SrebF1), Fatty Acid Synthase (Fasn), Acetyl-CoA carboxylase 1 (Acaca), Diacylglycerol O-
600 acyltransferase (Dgat1 and 2); fat uptake and transport, Cd36 and (C) cholesterol metabolism:
601 Sterol regulatory element 2 (SrebF2), 3-hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr),
602 Cytochrome P450 7A1 (Cyp7a1) and transport: ATP-binding cassettes (ABC) transporters,
603 Abca1, Abcg5, Abcg8.
604 Results are expressed as mean ± SEM with n = 5-8.

605 *: $p < 0.05$ compared to male F1-Ob and **: $p < 0.05$ compared to female F1-Ob.

606

Fig. 3: Hepatic cholesterol, cholesteryl ester and triglyceride levels. Results are expressed as mean ± SEM with n = 5-8.

*: $p<0.05$ compared to male F1-Ob and **: $p<0.05$ compared to female F1-Ob.

Fig. 4: Impact of the mixture of pollutants at the TDIΔ dose on **(A)** hepatic expression of Estrogen receptor alpha (Esr1) and genes encoding proteins under estrogen regulation including Selenoprotein P (Sepp1) and Insulin-like growth factor 1 (Igf1), and **(B)** phase II detoxification enzymes: NAD(P)H dehydrogenase, quinone 1 (Nqo1), UDP glucuronosyltransferase 1 family, polypeptide A1 (Ugt1a1) and sulfotransferase family 1E, estrogen-preferring, member 1 (Sult1e1)**.** Results are expressed as mean ± SEM with n = 5-8. **(C)** Western blotting analysis of the effect of pollutant exposure on ERα and EST protein expression in female liver. For histograms results are expressed as mean ± SEM with n=7 in each group for ERα and n=4 in each group for EST.

*: $p<0.05$ compared to male F1-Ob and **: $p<0.05$ compared to female F1-Ob

Table 1: Reference dose of the pollutants used in the mixture and doses added to the HFHS diet. In addition to the TDI**Δ** mixture dosage group, 2 higher dosages than TDI**Δ** were tested, in preliminary studies, referred to as Mixture 1 (M 1) and 10 times M1 (M10).

	TCDD	**PCB153**	**BPA**	**DEHP**
TDI	1-4 pg/kg	20 ng/kg	50 µg/kg	50 µg/kg
TDIΔ	2 pg/kg	80 ng/kg	5 µg/kg	50 µg/kg
M1	2 ng/kg	40µg/kg	5mg/kg	25mg/kg
M10	20 ng/kg	400µg/kg	50mg/kg	250mg/kg

Table 2: Reproductive parameters, characterization of F0 females and F1 offspring before weaning. Results are expressed as mean ± SEM. (ND not determined)

* Significant differences compared to Ob

		Standard	Ob	ObTDIΔ	ObM1	ObM10
n (F0 females)		13	**32**	32	41	9
Weight F0 females at mating (gr)		19.2 ± 0.3*	**21.3 ± 0.4**	21.9 ± 0.4	23.6 ± 0.5*	20.2 ± 1.0
liver gene expression (specific gene/GUSB mRNA ratio) at mating	CYP1A1	1.44 ± 0.29	**0.71 ± 0.06**	0.64 ± 0.19	22.9 ± 8.2*	1029 ± 83*
	CYP4A14	44.2 ± 10.2*	**129.3 ± 12.0**	129.0 ± 25.6	236.6 ± 34.4*	467.5 ± 64.7*
average number of pups per mouse		6.5 ± 0.5 [4-9]	**7.3 ± 0.2 [4-9]**	8.0 ± 0.2 [5-10]	5.9 ± 0.3* [4-9]	6.1 ± 0.3* [5-7]
sex-ratio		0.48 ± 0.06	**0.52 ± 0.04**	0.53 ± 0.04	0.53 ± 0.04	0.50 (n=2)
% of F1 survivors/mouse		95 ± 3	**86 ± 5**	75 ± 5*	65 ± 9*	22 ± 13*
glycaemia F0 females (mmol/l) 1 week post F1 weaning (6 hours fasting)		6.8 ± 0.2	**7.7 ± 0.4**	8.2 ± 0.5	8.3 ± 0.6	7.4 ± 0.5
Weight F1 offspring at Day10 after birth (gr) n ≥ 12	**Females**	4.5 ± 0.2*	**4.9 ± 0.1**	4.8 ± 0.1	5.4 ± 0.1*	ND
	Males	4.4 ± 0.2*	**4.9 ± 0.1**	4.9 ± 0.1	5.1 ± 0.1	ND

650 **Table 3**: Biochemical characterization of F1 mice. Results are expressed as mean ± SEM.
651 (ND= Not Determined) * Significant differences compared to F1-Ob

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653

Females	Standard	F1-Ob	F1-ObTDIΔ
glycaemia (mmol/L)	8.4 ± 0.4* (n=13)	**10.4 ± 0.5 (n=22)**	10.0 ± 0.5 (n=16)
insulinemia (ng/ml)	0.4 ± 0.1* (n=13)	**1.3 ± 0.5 (n=11)**	0.6 ± 0.1 (n=11)
HOMA-IR	3.6 ± 0.8*	**15.3 ± 6.0**	7.5 ± 1.2
leptin (ng/ml)	0.9 ± 0.3* (n=5)	**4.0 ± 0.7 (n=8)**	4.6 ± 0.8 (n=8)
triglycerides (mmol/L)	1.3 ± 0.1 (n=6)	**1.1 ± 0.1 (n=6)**	1.2 ± 0.1 (n=6)
total cholesterol (mg/ml)	1.0 ± 0.1* (n=11)	**1.4 ± 0.1 (n=11)**	1.5 ± 0.2 (n=10)
Cholesteryl ester (mg/ml)	0.7 ± 0.1* (n=11)	**1.0 ± 0.1 (n=11)**	1.1 ± 0.1 (n=10)
estradiol (pg/ml)	24.1 ± 10.1 (n=4)	**30.9 ± 7.2 (n=7)**	23.7 ± 3.5 (n=4)

Males	Standard	F1-Ob	F1-ObTDIΔ
glycaemia (mmol/L)	9.6 ± 0.6* (n=8)	**11.5 ± 0.4 (n=18)**	12.5 ± 0.4 (n=16)
insulinemia (ng/ml)	0.6 ± 0.1* (n=8)	**1.2 ± 0.2 (n=14)**	1.2 ± 0.2 (n=10)
HOMA-IR	5.8 ± 0.9*	**15.4 ± 2.1**	18.0 ± 3.0
leptin (ng/ml)	0.9 ± 0.2* (n=5)	**4.0 ± 0.9 (n=9)**	5.8 ± 0.8 (n=8)
triglycerides (mmol/L)	1.4 ± 0.1 (n=6)	**1.4 ± 0.1 (n=6)**	1.3 ± 0.1 (n=6)
total cholesterol (mg/ml)	1.1 ± 0.1* (n=6)	**2.0 ± 0.2 (n=11)**	1.9 ± 0.2 (n=11)
Cholesteryl ester (mg/ml)	0.8 ± 0.1* (n=6)	**1.5 ± 0.2 (n=11)**	1.4 ± 0.1 (n=11)
testosterone (ng/ml)	ND	**6.8 ± 4.2 (n=5)**	6.7 ± 2.6 (n=6)

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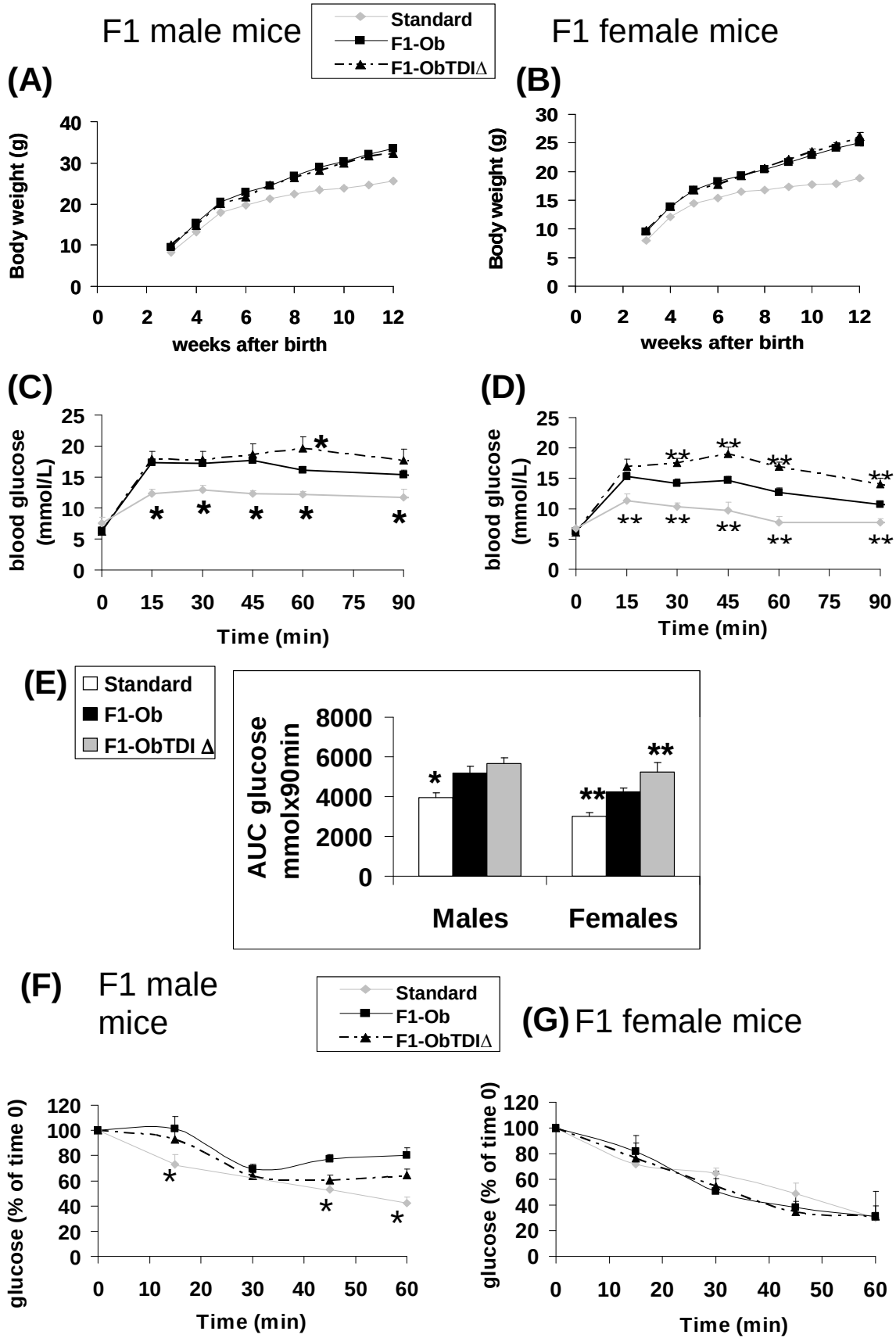
F1 male mice
Standard
F1-Ob
F1-ObTDIΔ
F1 female mice
(A)
Body weight (g)
weeks after birth
(B)
Body weight (g)
weeks after birth
(C)
blood glucose (mmol/L)
Time (min)
(D)
blood glucose (mmol/L)
Time (min)
(E)
Standard
F1-Ob
F1-ObTDI Δ
AUC glucose mmolx90min
Males
Females
(F) F1 male mice
Standard
F1-Ob
F1-ObTDIΔ
(G) F1 female mice
glucose (% of time 0)
Time (min)
glucose (% of time 0)
Time (min)

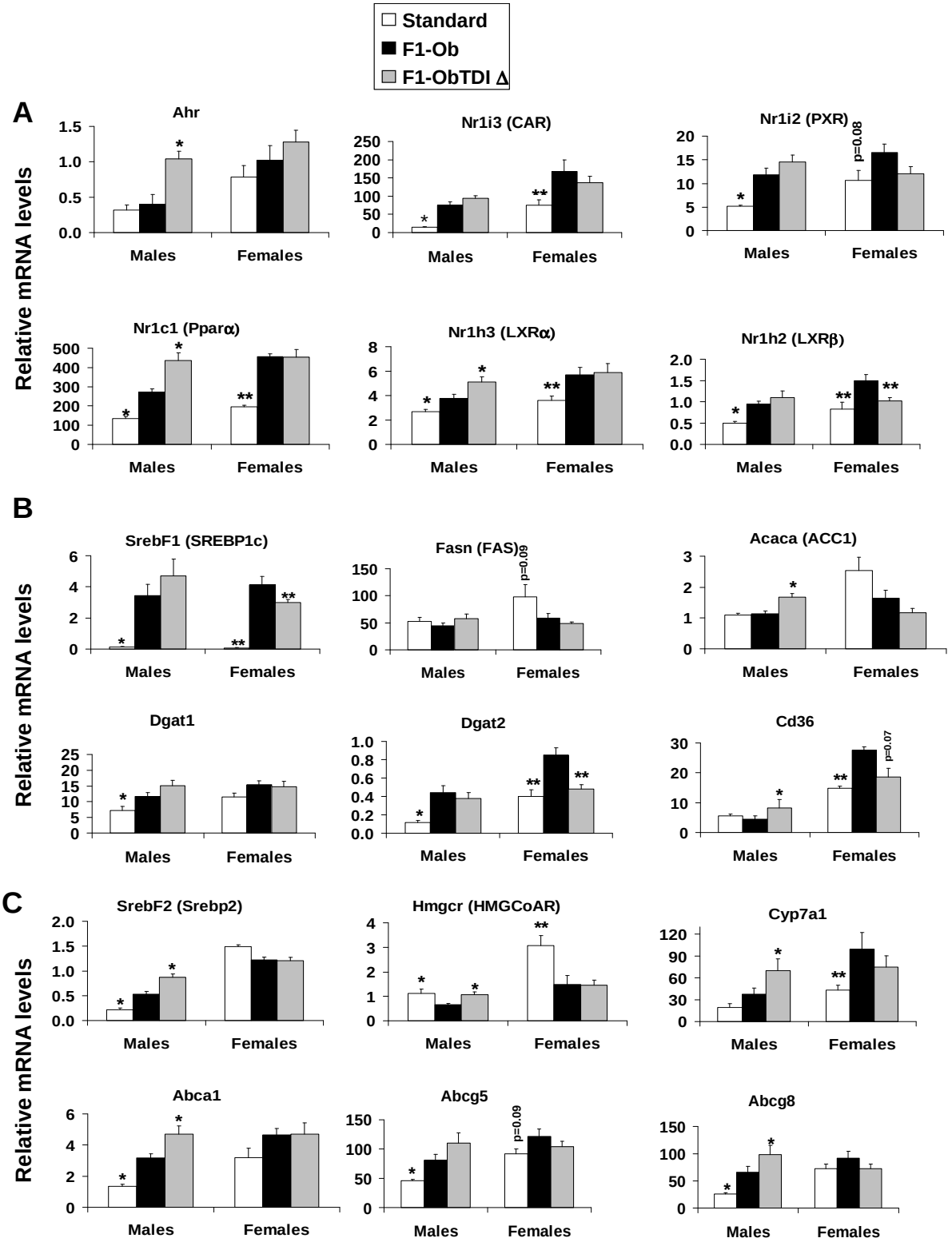
Standard
F1-Ob
F1-ObTDI Δ
A
B
C
Relative mRNA levels
Ahr
Nr1i3 (CAR)
Nr1i2 (PXR)
p=0.08
Nr1c1 (Pparα)
Nr1h3 (LXRα)
Nr1h2 (LXRβ)
SrebF1 (SREBP1c)
Fasn (FAS)
p=0.09
Acaca (ACC1)
Dgat1
Dgat2
Cd36
p=0.07
SrebF2 (Srebp2)
Hmgcr (HMGCoAR)
Cyp7a1
Abca1
Abcg5
p=0.09
Abcg8
Males
Females

Figure 2

662

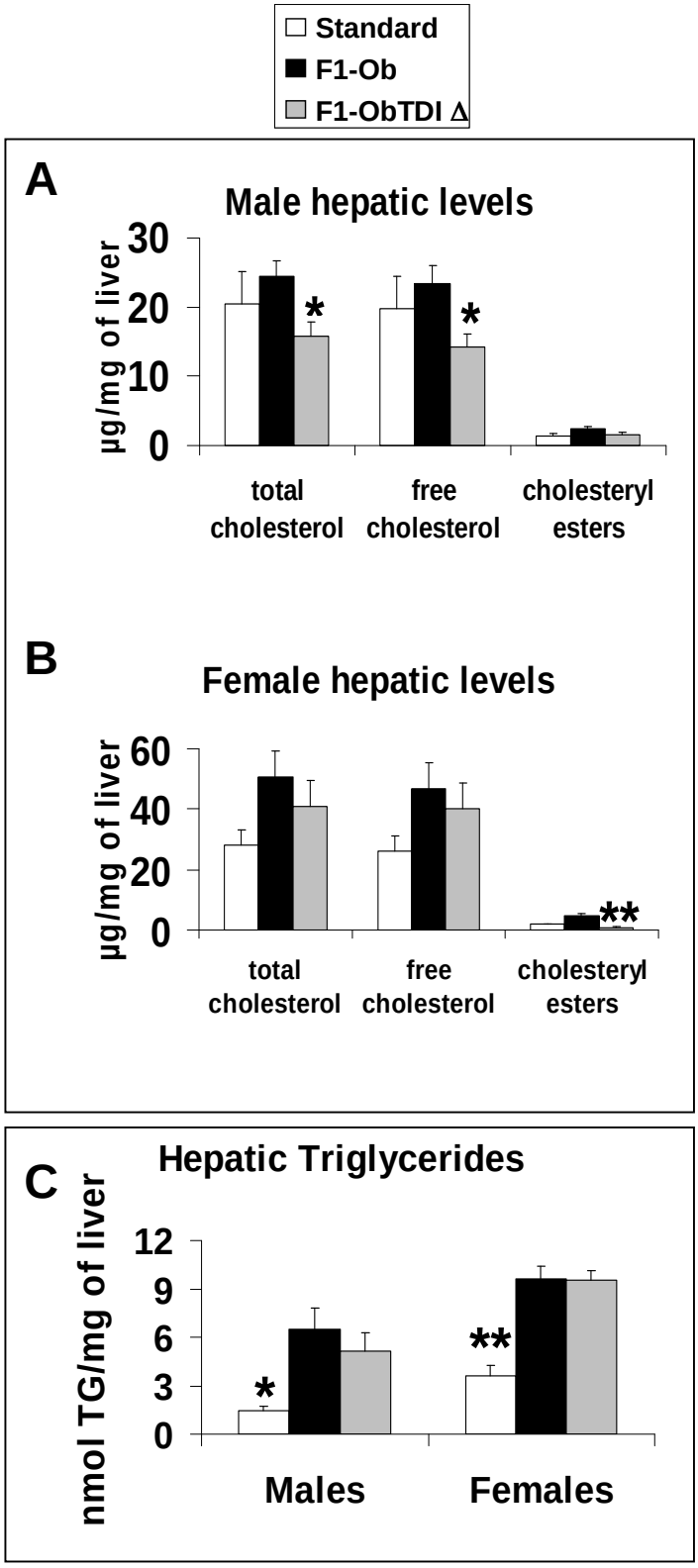
Standard
F1-Ob
F1-ObTDI Δ
A
Male hepatic levels
µg/mg of liver
30
20
10
0
*
*
total cholesterol
free cholesterol
cholesteryl esters
B
Female hepatic levels
µg/mg of liver
60
40
20
0
**
total cholesterol
free cholesterol
cholesteryl esters
C
Hepatic Triglycerides
nmol TG/mg of liver
12
9
6
3
0
*
**
Males
Females

Figure 3

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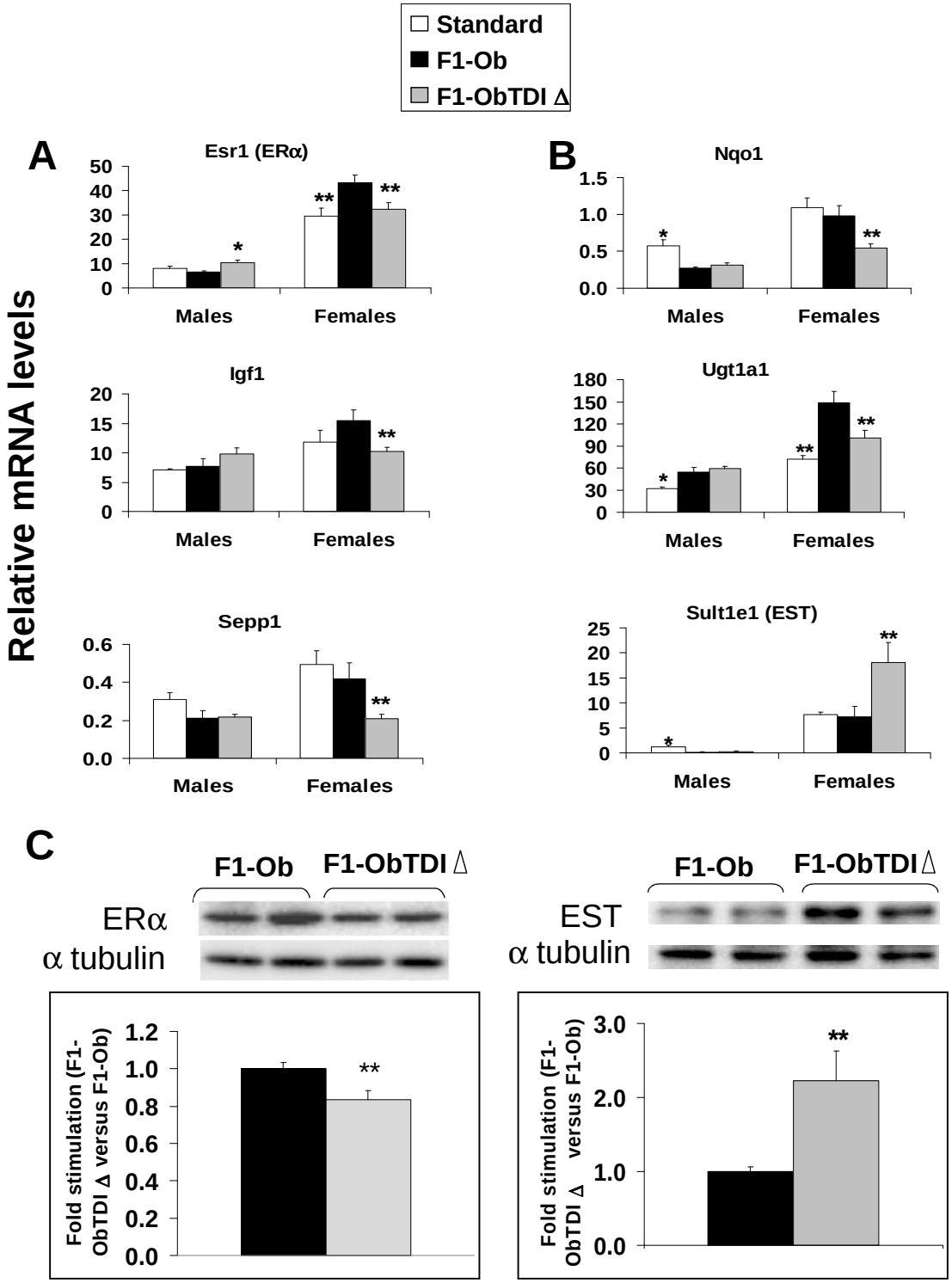

Standard
F1-Ob
F1-ObTDI Δ
A
Esr1 (ERα)
Nqo1
Igf1
Ugt1a1
Sepp1
Sult1e1 (EST)
Relative mRNA levels
Males
Females
B
C
F1-Ob
F1-ObTDI Δ
ERα
α tubulin
EST
Fold stimulation (F1-ObTDI Δ versus F1-Ob)

Figure 4

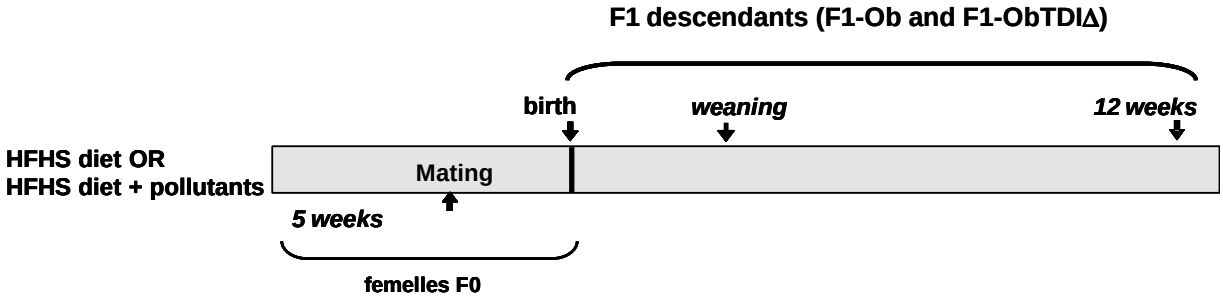
Supplemental Material

Legends of Supplemental figures

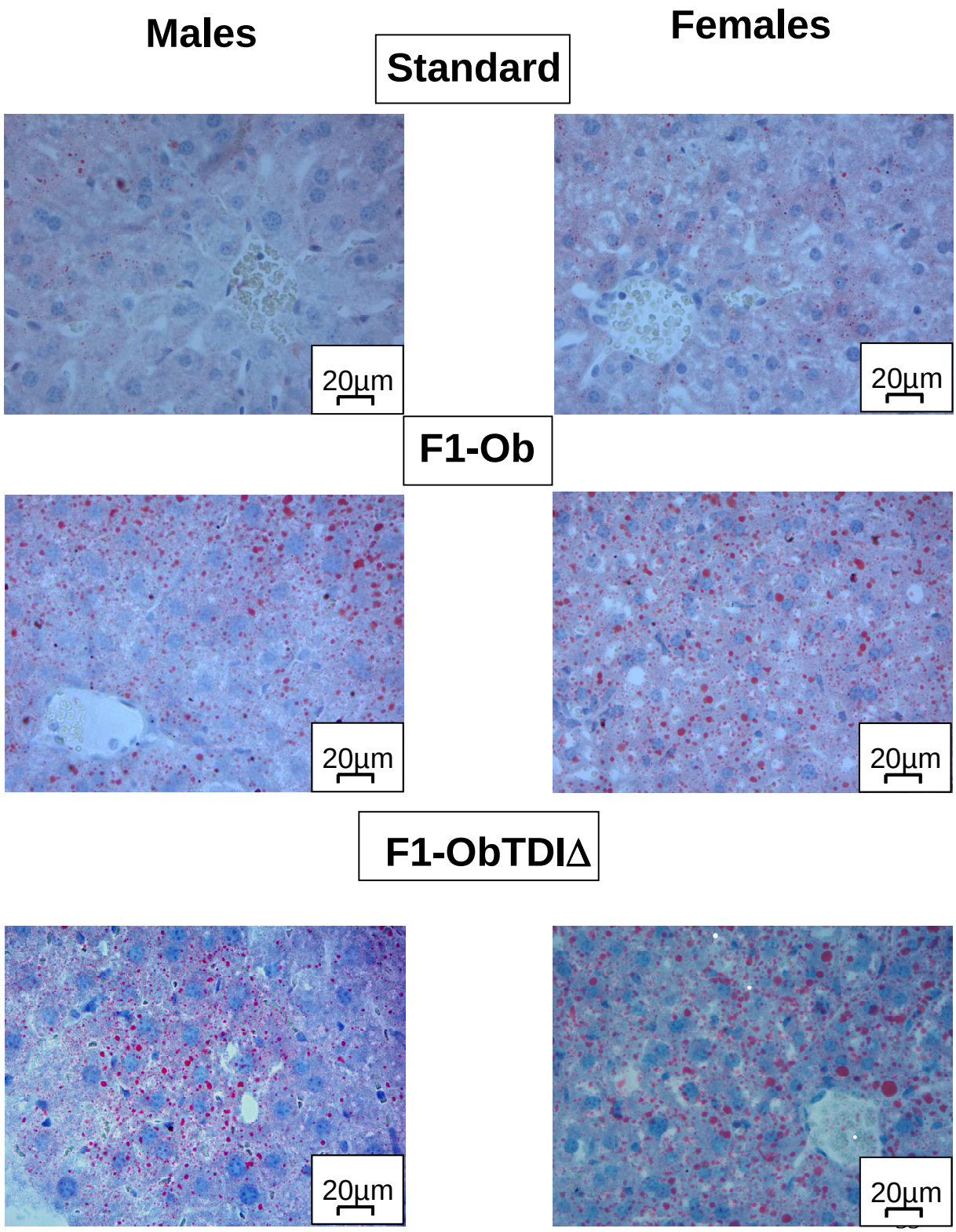
Supplemental Figure. 1: Experimental protocol to generate F1 offspring.

Supplemental Figure. 2: Oil Red O staining of liver sections obtained from F1 mice. Each picture is representative of three different sections of livers obtained from three different mice (original magnification x400)

Supplemental Figure 1



Supplemental Figure 2



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

	TD.99249 (Harlan)		Standard	
	% of mass	% kcal	% of mass	% kcal
Fat	36.1	58.7	5	12.2
Carbohydrate	35 (maltodextrin 50% + sucrose 50%)	25.5	60 (maltodextrin + starch)	65.3
Protein	19.8	15.7	18.9	22.5
kCal/g	5.4		3.3	

Supplemental Table 2: Primers used for RT-qPCR

		sequences: 5'->3'	
gene	**RefSeq Accession Number**	**sense**	**antisense**
Abca1	NM_013454	**CAG-GAG-GTG-ATG-TTT-CTG-ACC-A**	**TTG-GCT-GTT-CTC-CAT-GAA-GGT-C**
Abcg5	NM_031884	**AGC-CTC-GCT-CTG-AGC-TCT-TC**	**TTC-AGG-ACA-GGG-GTA-ACC-AC**
Abcg8	NM_026180	**AGC-CTC-GCT-CTG-ACA-TCT-TC**	**GTC-AAG-TCC-ACG-TAG-AAG-TC**
Acaca	NM_022193	**GAG-CAA-GGG-ATA-AGT-TTG-AG**	**AGG-TGC-ATC-TTG-TGA-TTA-GC**
Ahr	NM_013464.4	**TCA-TCT-GGT-TTC-CTG-GCA-ATG-AAT**	**ATA-AGC-TGC-CCT-TTG-GCA-TC**
Cd36	NM_001159558.1	**AAG-ATC-CAA-AAC-TGT-CTG-TA**	**GTC-CTG-GCT-GTG-TTT-GGA-GG**
Cyp7a1	NM_007824.2	**TAC-AGA-GTG-CTG-GCC-AAG-AG**	**AGT-GAA-GTC-CTC-CTT-AGC-TG**
Dgat1	NM_010046	**ACC-GCG-AGT-TCT-ACA-GAG-ATT-GGT**	**ACA-GCT-GCA-TTG-CCA-TAG-TTC-CCT**
Dgat2	NM_026384	**TGG-GTC-CAG-AAG-AAG-TTC-CAG-AAG-TA**	**ACC-TCA-GTC-TCT-GGA-AGG-CCA-AAT**
Esr1	NM_000125;NM_001122740;NM_001122741;NM_001122742	**TGT-TTG-CTC-CTA-ACT-TGC-TC**	**CCT-TCT-CTT-CCA-GAG-ACT-TC**
Fasn	NM_017332	**GTG-CAC-CCC-ATT-GAA-GGT-TCC**	**GGT-TTG-GAA-TGC-TGT-CCA-GGG**
Gusb	NM_010368	**CTT-CAT-GAC-GAA-CCA-GTC-AC**	**GCA-ATC-CTC-CAG-TAT-CTC-TC**
Hmgcr	NM_008255	**CCG-GCC-TGT-GTG-TCG-CTG-GT**	**CCA-GCG-ACT-ATG-AGC-GTG-AA**
Igf1	NM_001111276;NM_001111274;NM_184052	**ACC-AAA-ATG-ACC-GCA-CCT-GC**	**AAC-ACT-CAT-CCA-CAA-TGC-CTG-TC**
Nqo1	NM_008706	**GGC-CGA-TTC-AGA-GTG-GCA-TCC-TG**	**TCT-GCA-TGC-GGG-CAT-CTG-GTG**
Nr1h2	NM_007121	**AGG-ACC-AGA-TCG-CCC-TCC-TG**	**GGT-GGA-AGT-CGT-CCT-TGC-TGT-AGG**
Nr1h3	NM_005693;NM_001130101;NM_001130102	**CCG-GGA-AGA-CTT-TGC-CAA-AGC**	**GGA-GCT-GGT-CCT-GCA-CGT-TG**
Nr1i2	NM_010936.3	**AGG-AGG-AGT-ATG-TGC-TGA-TG**	**CTT-CAG-GAA-CAG-GAA-CCT-GTG**
Nr1i3	NM_001243063;NM_001243062;NM_009803	**GTC-CCA-TCT-GTC-CGT-TTG-C**	**AGG-GCT-TCT-GAC-AGT-ATC**
Nr1c1	NM_011144; NM_001113418.1	**AAG-GGC-TTC-TTT-CGG-CGA-AC**	**GTT-CAT-GTT-GAA-GTT-CTT-CAG**
Sepp1	NM_009155,3	**ATG-ACA-GAT-GTG-GCC-GTC-TTG-TGT**	**GCC-TCT-GAG-GGC-TCC-GCA-GT**
SrebF1	NM_011480	**ACG-GAG-CCA-TGG-ATT-GCA-CA**	**AAG-GGT-GCA-GGT-GTC-ACC-TT**
SrebF2	NM_033218	**CCT-GTG-ATG-ATG-GGG-CAA-CAG**	**CCT CAG AAC GCC AGA CTT G**
Sult1e1	NM-023135.2	**TCT-TGG-CAA-GGC-CAG-ATG-AC**	**TCC-CAA-AAT-GAT-GCT-GGA-AGG**
Ugt1a1	NM_201645.2	**GCA-TCT-ATC-TCG-CTG-ATG-AG**	**CAG-AGG-CGT-TGA-CAT-AGG**