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Abstract

Environmental pollution is increasingly considered an important factor involved in the obesity incidence. Endocrine disruptors (EDs) are important actors in the concept of DOHaD (Developmental Origins of Health and Disease), where epigenetic mechanisms play crucial roles. Bisphenol A (BPA), a monomer used in the manufacture of plastics and resins is one of the most studied obesogenic endocrine disruptor. Bisphenol S (BPS), a BPA substitute, has the same obesogenic properties, acting at low doses with a sex-specific effect following perinatal exposure. Since the liver is a major organ in regulating body lipid homeostasis, we investigated gene expression and DNA methylation under low-dose BPS exposure. The BPS obesogenic effect was associated with an increase of hepatic triglyceride content. These physiological disturbances were accompanied by genome-wide changes in gene expression (1,366 genes significantly modified more than 1.5-fold). Gene ontology analysis revealed alteration of gene cascades involved in protein translation and complement regulation. It was associated with hepatic DNA hypomethylation in autosomes and hypermethylation in sex chromosomes. Although no systematic correlation has been found between gene repression and hypermethylation, several genes related to liver metabolism were either hypermethylated (Acsl4, Gpr40, Cel, Pparδ, Abca6, Ces3a, Sgms2) or hypomethylated (Soga1, Gpihbp1, Nr1d2, Mlxipl, Rps6kb2, Esrrb, Thra, Cidec). In specific cases (Hapln4, ApoA4, Cidec, genes involved in lipid metabolism and liver fibrosis) mRNA upregulation was associated with hypomethylation. In conclusion, we show for the first time wide disruptive physiological effects of low-dose of BPS, which raises the question of its harmlessness as an industrial substitute for BPA.

Keywords: bisphenol S, obesogen, perinatal chronic exposure, liver, DNA methylation, transcriptome.

1. Introduction

Obesity is steadily increasing in many countries around the world. The positive correlation between obesity incidence and the increase in environmental pollution [1] initiated the concept of obesogens [2]. By promoting adipogenesis, these molecules potentiate a pre-existing imbalance in the energy homeostasis [3, 4]. Most obesogens are known to be endocrine disruptors (EDs). As EDs, they can act at low doses (corresponding to environmental doses) and following perinatal exposures [5]. EDs are a common factor in the DOHaD concept (Developmental Origins of Health and Disease) through potential effects of the gestational microenvironment during embryonic and/or fetal development on the occurrence of pathology during adulthood [6].

Bisphenol S (BPS), a structural analogue of Bisphenol A (BPA), is commonly used as a substitute for BPA in food contact material (including baby bottles), papers products, paints and varnishes, resins and glues, medical equipment and electronic and electrical components. BPS is ubiquitous in the environment as it was found in indoor air, surface water, sediment, sewage sludge and food). Dietary intake is the main source of BPS human exposure [7]. BPS exhibits a higher body burden, bioavailability and stability in environment than BPA [8]. BPA has obesogenic properties with disparities according to the exposure window, the doses, route of exposure and sex of the animals [3, 9]. Several studies suggested that obesogens induce an overweight only after perinatal exposure in rodent models [4]. BPS induces a similar obesogen effect to BPA following perinatal and chronic exposure at low doses (1.5 µg/kg bw/d) [10].

The BPS detection in U.S. adult urine samples increased from 25% to 74% between 2000 and 2014 [11]. Using data from BPS urine sample rates and pharmacokinetic models,

human BPS exposure could correspond to daily intakes (median values) of 0.023 µg/d for Korean, 0.316 µg/d for Americans and 1.67 µg/d for Japanese [12].

It is admitted than when a pregnant female is exposed to a chemical substance, this substance, if small enough, will be able to reach the fetuses via the blood flow and through the placenta. It is also supposed that when an endocrine disrupter mimics the structure of an hormone, it will hijack all its signaling cascade in a atopic and achronic fashion which may explain the pathogenic effect of the exposure.

Epigenetic mechanisms like DNA methylation have been associated in the development of obesity in the context of DOHaD [12]. DNA methylation of CpG islands is an important epigenetic mechanism associated with stable long-term changes in gene expression [13]. Several EDs are reported to induce epigenetic changes, notably after perinatal exposure. Thus, it has been described that BPA causes hypomethylation of liver DNA and alters the methylation level of certain CpG sites (notably around transcription start site) of genes involved in liver metabolism [13, 14, 15, 16, 17]. It has been shown for genes involved in glycolysis (hypermethylation of Glucokinase (Gck)), β-oxidation (hypo and hypermethylation of Carnitine Palmitoyltransferase (Cpt1a)) and lipid synthesis (hypomethylation of Nuclear factor E2-related factor (Nrf2) and sterol regulatory element binding protein (Srebf1 and 2)) [13, 15, 16, 17]. Recent articles suggest that genome-wide methylation analysis can identify epigenetic biomarkers (gene, transposon) that are indicative of a type of perinatal exposure and predict its consequences in adulthood [18, 19].

While the epigenetic effects of BPA are now well evaluated, those of BPS are missing. Here, we analyzed the liver transcriptome and DNA methylation induced by BPS exposure in obesogen conditions. Thus, we performed a perinatal and chronic exposure at BPS low-dose (1.5 µg/kg body weight/d) in male mice fed with a high fat diet.

2. Materials and methods

2.1. Animals and materials

Pregnant C57Bl/6J mice were purchased from Charles Rivers (L'Arbresle, France). Bisphenol S (BPS) was provided by Sigma-Aldrich (Saint Quentin Fallavier, France). The high fat diet (HFD) was based on the 4RF25 reproduction diet (Mucedola, Milano, Italia) with the addition of palm oil (La Vie Saine, Dijon, France) and cholesterol from Sigma Aldrich (Saint Quentin Fallavier, France).

2.2. Experimental design

BPS exposure was performed as previously described [10]. Briefly, eleven pregnant C57Bl/6J mice were divided into two groups and exposed to BPS or not in their drinking water from the first day of gestation (GD0, determined by vaginal plug detection). BPS was dissolved in absolute ethanol (0.1%). Control group drinking water contained only 0.1% ethanol. The treatment was continued during the lactation and in pups after weaning until they were 22-weeks old. At the weaning, the male offspring were divided into two groups. Seventeen and fifteen male mice were included in BPS exposed group and in the control group, respectively. At 8-weeks old, all pups were fed with a high fat diet (60% Kcal from lipids; composed by 4RF25 reproduction diet added with 30% palm oil and 0.42g/kg of cholesterol). A maximum of five mice were housed in each cage. The litters were mixed randomly after weaning in order to minimize a possible litter effect. In a cage, each mouse came from a different dam. Under our conventional animal facility conditions, BPS is stable for more than seven days in drinking water. So, we used a concentration of 12.76 ng/ml to obtain an average BPS intake of 0.99 (±0.057) µg/kg bw/d for an expected dose of 1.5 µg/kg bw/d. Cages and bottles were made of polypropylene (bisphenol-free). All mice (fasted 4h before) were sacrificed at 22-weeks old. Before sacrifice, the body weight was measured. At the sacrifice, the livers were

weighed, then immediately frozen in liquid nitrogen and stored at -80 °C. Experimental protocol was approved by the ministry and the University of Burgundy's ethic committee. Animal experiments have been carried out in accordance with EU Directive 2010/63/EU for animal experiments.

2.3. Liver triacylglycerol assay

Triacylglycerols (TG) were extracted from mouse liver (75 up to 190 mg) according to Schwartz D.M and Wolins N.E [20, 21] and as previously described [22]. Infinity TG® reagent (200 µl, Thermo Fischer Scientific, Asnière sur Seine, France) was used to reveal TG and absorbance at 500 nm was measured using a microplate reader (MultiskanGo®, Thermo-Scientific, France). A calibration curve was generated using a triglyceride standard (FS 200 mg/dl, DiaSys, Condom, France). Analysis was carried out in collaboration with the lipidomics platform of Université de Bourgogne Franche-Comté (Dr Jean-Paul Pais de Barros, Dijon, France).

2.4 RNA extraction and microarray analysis

Lysis of liver samples from each mouse was performed using Lysing Matrix D™ tubes (MP Biomedical, Illkirch-Graffenstaden, France) and the tissue homogenizer Precellys™24 (Bertin technologies, Montigny-le-Bretonneux, France). Total RNA was extracted using Tri-reagent™ (Sigma-Aldrich, Saint Quentin Fallavier, France). RNA quantification and quality were performed by capillary electrophoresis (Agilent bioanalyzer 2100, Les Ullis, France). Isolated cRNA of three points per condition was analyzed for global gene expression using Affymetrix Clariom S array hybridization, in the same batch, at the Genomics Platform of the Cochin Institute according to standard validated protocols. Each sample is a liver RNA pool prepared from three distinct mice originating from separate litters.

All genes significantly deregulated with a p-value ⩽0.05 were converted to ENSEMBL ID using Biomart and mm10 version of the mouse genome (http://www.ensembl.org/biomart). Liver RNA was extracted from the medial liver lobe whatever the mouse. The platform generated .CEL files that were analyzed using the TAC4.0 (Transcription Analysis Console) from Affymetrix (Applied Biosystems).

2.5 Bioinformatics analysis of the expression deregulations

Analysis of the transcriptome for microarray was carried out using two complementary approaches: DAVID analysis of deregulated genes and Functional Gene Set Enrichment using string (http://string-db.org/).

2.6. DNA extraction

Lysis of liver sample were performed using Lysing Matrix D™ tubes (MP Biomedical, Illkirch-Graffenstaden, France) and the tissue homogenizer Precellys™24 (Bertin technologies, Montigny-le-Bretonneux, France). All sample were treated with 20 µl of proteinase K (20 mg/ml) overnight and with 9 µl of RNase A (20 mg/ml) and 300 U of RNase T1, 30 min at 37 °C just before the DNA extraction using GenElute Mammalian Genomic DNA Miniprep kit™ (Sigma Aldrich, Saint Quentin Fallavier, France) in compliance with the manufacturer's guidelines. Liver DNA was extracted from the medial liver lobe whatever the mouse.

2.7. Reduced Representation Bisulfite Sequencing (RRBS)

Reduced Representation Bisulfite Sequencing was performed by Diagenode's DNA Methylation Profiling (RRBS Service) (Diagenode Cat# G02020000). For each group, three samples were analyzed. Each sample is a liver DNA pool prepared from three distinct mice

from separate litters. DNA concentration of the samples was measured using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific) and DNA quality was analyzed using the Fragment Analyzer™ and the DNF-487 Standard Sensitivity Genomic DNA Analysis Kit (Advanced Analytical). The library preparation, bisulfite conversion and amplification were performed using Diagenode's Premium RRBS Kit (Diagenode Cat# C02030033) by following manufacturer's recommendations. Finally, RRBS library pools were sequenced on a HiSeq3000 (Illumina) using 50 bp single-read sequencing (SR50).

The sequenced reads were controlled for quality of sequencing with FastQC [23]. Adapter removal was performed using Trim Galore! version 0.4.5_dev [24]. The cleaned reads were then aligned to the *Mus musculus* reference genome (Genome Reference Consortium m38 (mm10)) using bismark v0.16.1 [25]. Similarly, the sequenced reads were first transformed into the forward and reverse bisulfite converted versions and aligned to the four bisulfite genomes. The reads, producing a unique best hit to one of the bisulfite genomes, were then compared to the unconverted genome. The cytosine2coverage and bismark_methylation_extractor modules of bismark were used to infer the methylation state of all cytosines in a CpG context (for every single mappable read) and to compute the percentage methylation for each CpG site. The spike-in control sequences were used at this step to check the bisulfite conversion rates and to validate the efficiency of the bisulfite treatment (supplementary data A1).

Methylkit [26], a R/Bioconductor package, was used to perform the differential methylation analysis. The CpG data set was filtered for low coverage (CpGs with coverage less than 10X were discarded) and for extremely high coverage to discard reads with possible PCR bias (CpGs with coverage higher than the 99.9^{th} percentile were discarded). Pairwise comparisons were performed to identify differentially methylated CpGs (DMC) and differentially methylated regions (DMRs), the latter with a window and step size of 1000 bp. DMCs and

194 DMRs with a percent methylation difference higher than 25% and a q-value smaller than 0.01
195 were considered statistically significant. DMCs were annotated with the R/Bioconductor
196 package annotatr [27], with the mm10 refGene and CpG island annotations from UCSC [28].
197 The annotation comprised two categories: (i) distance to a CpG island and (ii) regional
198 annotation. The distance related annotation classified DMCs and DMRs whether they
199 overlapped a known CpG island, 2000 bp of the flanking regions of the CpG islands (shores),
200 2000bp of the flanking regions of the shores (shelves) or outside these regions (open sea). The
201 regional analysis classified DMCs in four groups, namely, exons, introns, promoters and
202 intergenic regions.

203

204 *2.8. Statistical analysis*

205 All data were expressed as mean ± standard error of mean (SEM). To determine the
206 statistically significant difference between two groups, a Student's unpaired *t*-test was used
207 after checking that the data assumed Gaussian distributions and equal variances with Graph
208 Pad Prism® software and RStudio® software. Results were considered statistically significant
209 at $p<0.05$.

210

211 **3. Results**

212

213 *3.1. BPS obesogenic effect is correlated with an increase of hepatic triglyceride*
214 *content.*

215 In a previous study, we reported a significant increase in body weight (from 38.93 g to
216 46.44 g, a fold increase of 1.2 ± 0.02; $p<0.001$) only in male mice fed a high fat diet and
217 exposed to BPS in comparison with unexposed male mice also fed with a high fat diet. This
218 obesogen effect of BPS was positively correlated with the body fat mass [10]. In male mice

fed with a fat diet, the BPS-induced overweight is associated with a hepatic triglyceride content significantly increased (268.23 mg to 731.79 mg, a 2.7 ± 0.5-fold increase; $p<0.05$) and a weak increase but not statistically significant on the liver weight (1.2-fold increase; supplementary data A2).

3.2 mRNA differential expression profiling of liver genes

Amongst 22,206 genes using a threshold of 1.5-fold and a p value <0.05, 1,366 genes were found deregulated (782 up-regulated and 584 down-regulated, Figure 1A). Among these genes, most belong to the categories Coding and Multiple Complex, logically since they are mainly interrogated by this microarray design. The most up-regulated mRNAs were Cytochrome P450 Family 2 Subfamily B Member 13 (Cyp2b13), Cytochrome P450 Family 2 Subfamily B Member 9 (Cyp2b9), Solute Carrier Family 22 Member 27 (Slc22a27), Glutathione S-Transferase Mu 3 (Gstm3) and Apolipoprotein A4 (ApoA4) (up-regulated by BPS in the HFD condition more than 4 fold), while the more strongly decreased mRNAs were Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 4 (Hsd3b4), Gm10800, Gm10801, C8 beta chain (C8b), Gm10715, Gm10720, Gm17535, Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 5 (Hsd3b5), Early growth response 1 (Egr1) and Gm11168 (that are decreased more than 4 fold), as shown in Figure 1B. To note, these Gm genes belong to two clusters located on chromosome 2 for Gm10800 and Gm10801 and on chromosome 9 for Gm10715, Gm10720, Gm17535 and Gm11168, suggesting that their deregulation may be associated with specific cis-elements. Sequence analysis of the proteins encoded by these genes suggest a membrane localization since they harbor four to five putative transmembrane domains. Using a semi-supervised analysis on the 1366 differential genes, it was possible to classify unambiguously the effects of BPS superimposed on the high fat diet (Figure 1C).

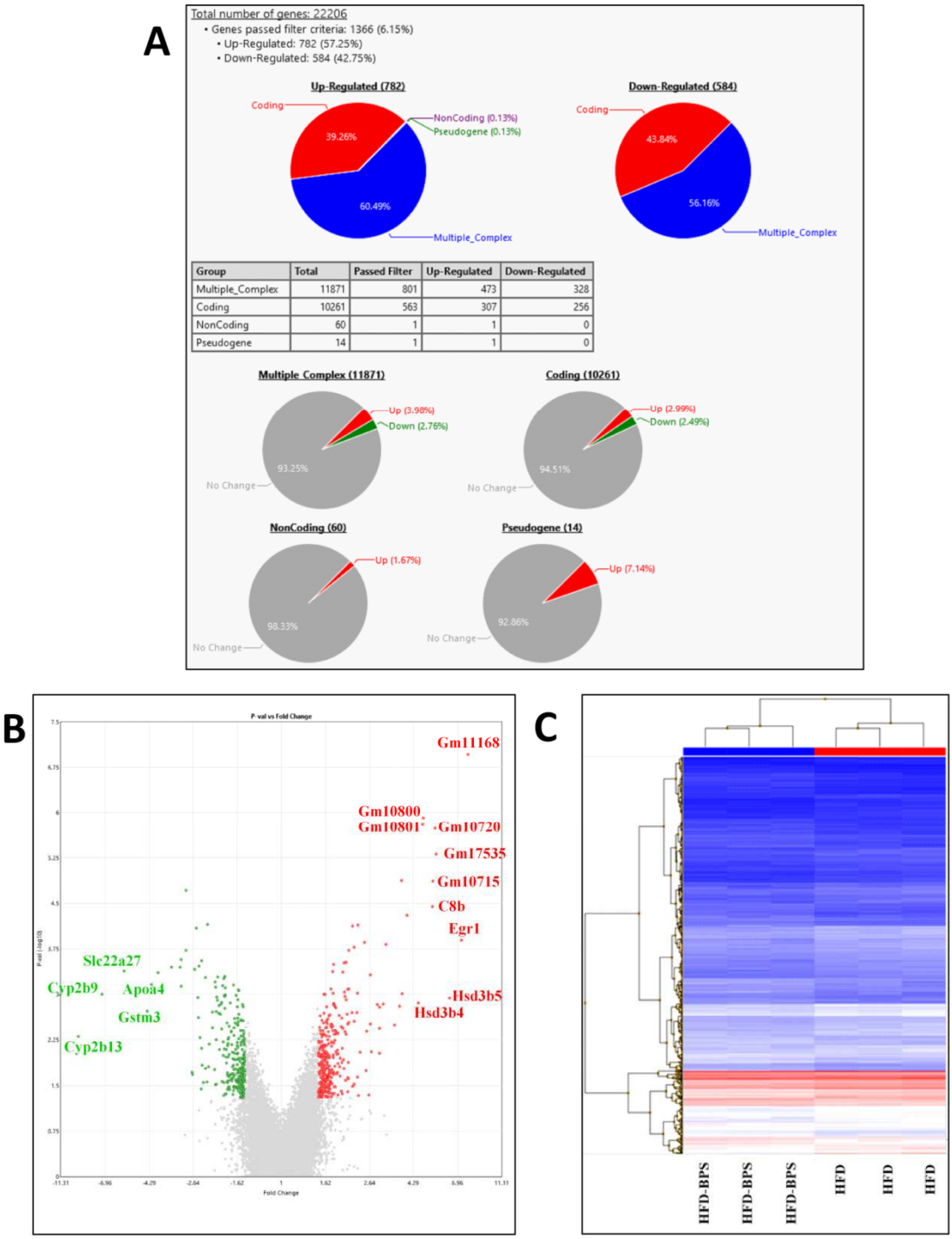


Figure 1. (A) Expression profiling of liver up-regulated or down-regulated genes according to their category and (B) their level of deregulation in volcano plot (up-regulated more than 4 fold in green (left panel) and down-regulated more than 4 fold in red (right panel)). (C) Heatmap of differently expressed genes (C) when liver mRNA of C57Bl/6J male mice fed with a high fat diet and after BPS exposure from GD0 to 22 weeks-old at 1.5 µg/kg bw/d (HFD BPS 1.5) was compared with liver mRNA of HFD control mice by microarray. n = 9/group.

To further understand the alterations of gene functions triggered by BPS treatment we performed a Gene Set Enrichment Analysis using the recently developed tool of the web-based String tool (https://string-db.org/cgi/input.pl?sessionId=Zdoso9cqhBbX). We performed two kinds of analyses using either the total gene set (Analysis A: 15,582 genes were recognized by the software), or the gene set limited to the 1,366 significant genes (Analysis B: 1,021 recognized genes). In the A analysis (summarized in Figure 2), a systematic keyword, 'ribosome', came out from various databases (Molecular Function, Cellular Component, Uniprot, KEGG), corresponding to genes that are almost systematically down-regulated involved in ribosomal function, and thus in protein translation and dynamics (Figure 2A). We could also note a systematic enrichment of complement function down-regulated genes, as well as Membrane Attack Complex [focused as well on the C9 complement component that corresponds to the generation of a membrane pore before the cell destruction (Figure 2B)]. Complement activation is a very general stress response pathway often activated following various exposures. By contrast, rarer pathways were found enriched in the Reactome database in genes that are overexpressed following BPS exposure especially associated to the transfer of sulfate residues and their implication in diseases (mental retardation, infantile epileptic encephalopathy, macular corneal dystrophy) as shown in Figure 2C.

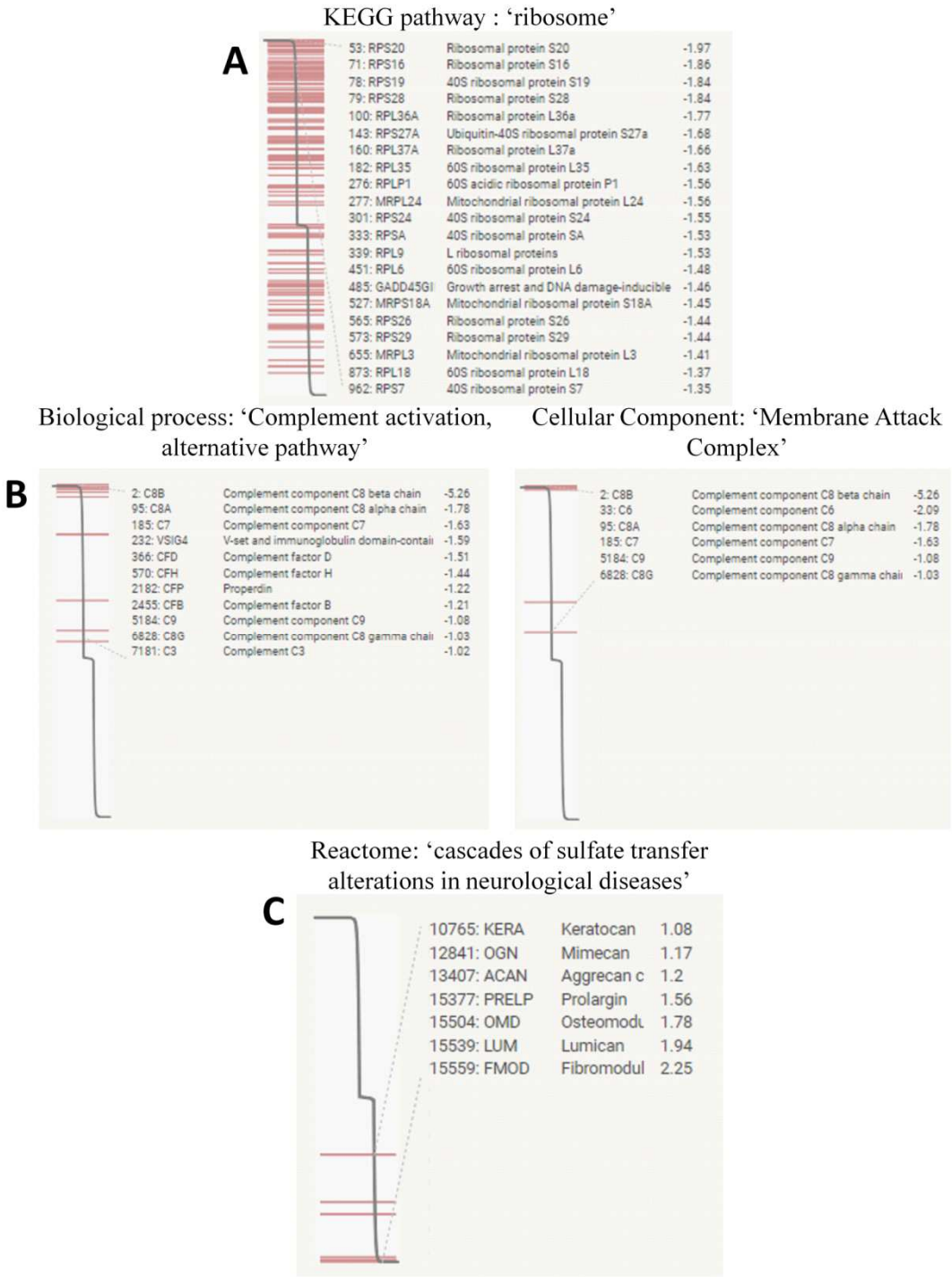
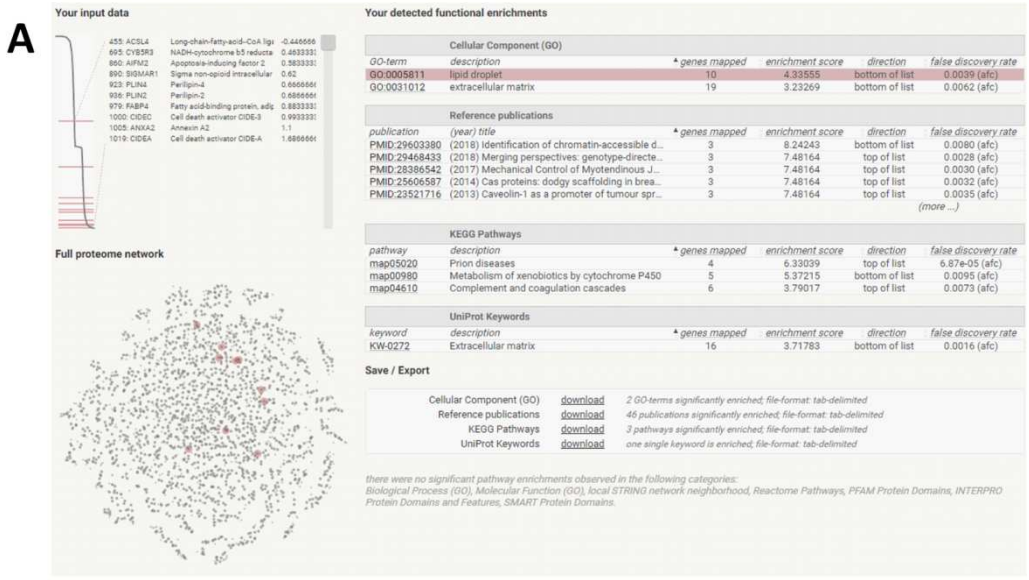


Figure 2. Functional Gene Set Enrichment Analysis for down-regulated genes (A, B), and overexpressed genes (C) using the total gene set when we compare liver mRNA of C57Bl/6J male mice fed with a high fat diet and after BPS exposure from GD0 to 22 weeks-old at 1.5 µg/kg bw/d (HFD BPS 1.5) with liver mRNA of HFD control mice by microarray. n = 9/group.

When the analysis was only focused on the 1,366 significant genes, lipid droplets component appeared as the most enriched cellular component in up-regulated genes (Figure

3A), which is consistent with the increase of hepatic triglycerides content, revealing specific alterations of lipid metabolism in the mice exposed to BPS under a high-fat diet. Amongst the KEGG pathways the alterations of the complement cascade were again found using the transcriptome data restricted to the significant genes (Figure 3B).



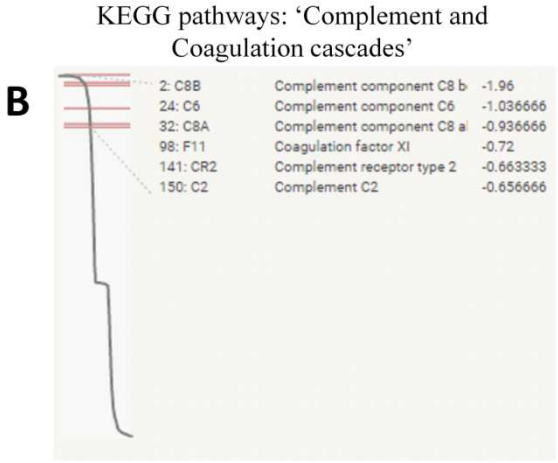


Figure 3. Functional Gene Set Enrichment Analysis for up-regulated genes (A), and down-regulated genes (B) focused on the significant genes when liver mRNA of C57Bl/6J male mice fed with a high fat diet and after BPS exposure from GD0 to 22 weeks-old at 1.5 µg/kg bw/d (HFD BPS 1.5) was compared with liver mRNA of HFD control mice by microarray. n = 9/group.

3.3. Untargeted assessment of genome-wide alterations in liver DNA methylation by Reduced Representation Bisulfite Sequencing (RRBS)

To investigate whether changes in DNA methylation in the liver were induced by BPS and HFD, we analyzed the liver DNA of these mice by RRBS allowing to analyze the genome part (≈1%) encompassing 70% of the CpGs islands in mice. Figures 5A and 5B depict the Differentially Methylated Regions (DMRs) that were identified in the data set, with a percent methylation difference cutoff of 25% and q-value of 0.01. The obesogenic effect of BPS in male mice fed with HFD was associated with a significant hypo or hyper methylation of 2,604 DMRs (width of 1,000 nucleotides per DMR), which corresponds to 10.42% of the DMRs assessed by the RRBS approach. Among these DMRs observed between the HFD BPS 1.5 and the HFD groups, those located in the autosome regions are mainly hypomethylated at 61.5% (against 38.5% for hypermethylated regions) (Figure 4A). By contrast, hypermethylation was predominant in the sex chromosomes (Figure 4A).

Non-supervised hierarchical classification revealed five clusters of Differentially Methylated Region (DMR) according to the direction of the BPS obesogenic effect (Figure 4B). The 1, 3 and 5 clusters contain regions down-methylated by BPS exposure. In contrast, the clusters 2 and 4 contain regions up-methylated by BPS exposure (Figure 4B).

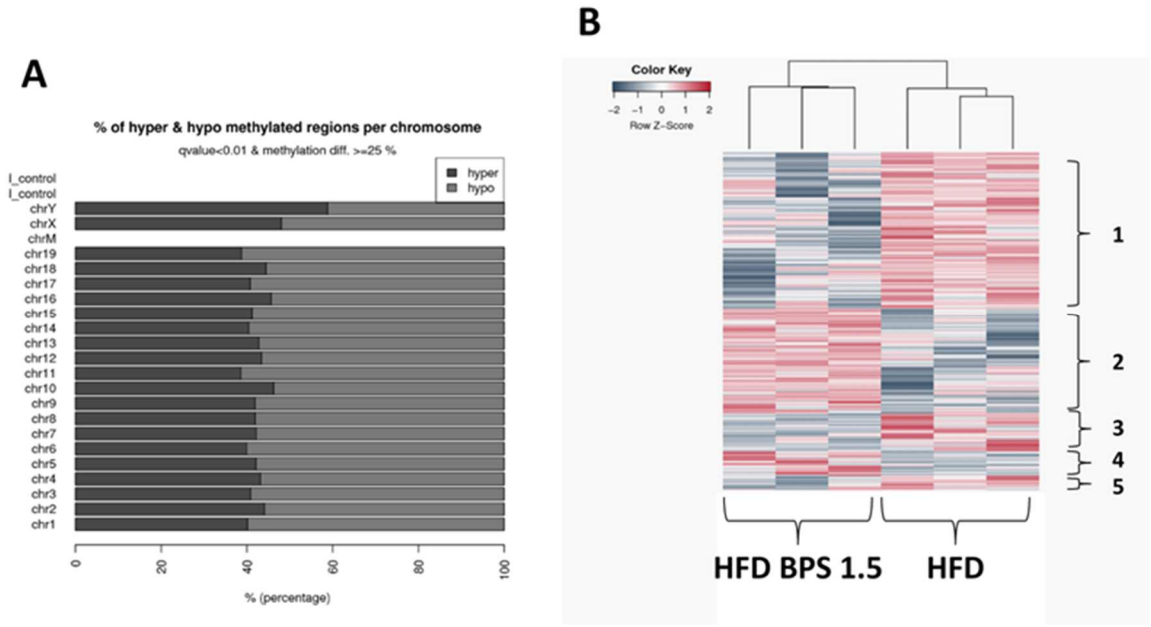


Figure 4. Percentage of hyper and hypomethylated regions per chromosome with a percent methylation difference cutoff of 25% and q-value of 0.01(A), and heatmap of Differentially Methylated Regions (DMRs) (width of 1000 nucleotides) (B) when liver DNA of C57Bl/6J male mice fed with a high fat diet and after BPS exposure from GD0 to 22 weeks-old at 1.5 µg/kg bw/d (HFD BPS 1.5) was compared with liver DNA of HFD control mice by RRBS. n = 9/group.

3.4. Genomic distribution of differentially methylated CpGs

In the current experiment, between 362 and 522 million of cytosine were analyzed per sample (data not shown). The methylated/non-methylated cytosine ratios are in the same range 43% for HFD mice and 42% for BPS exposed mice (data not shown). Among the cytosines analyzed, 20% is located inside CpGs islands, 22% is in a CpHpG context and 56% is in a CpHpH context (with H = A, C or T), and this is consistent across all samples and with standard RRBS results for a mouse sample. Although the cytosines analyzed in the CpG islands represent only 20% of the cytosines analyzed, more than 40% is methylated, while only 0.5% to 0.3% of the cytosines in a CpHpG or CpHpH context is methylated, respectively (data not shown).

For the HFD BPS 1.5 *vs* HFD comparison, 6.8% of differentially methylated CpGs were found located within CpG islands (defined by the following criteria: > 200 bp length, GC percentage > 50% and observed/expected CpG ratio > 60%), 4.3% within CpG shelves (2-4 kb from CpG island), 10.2% within CpG shores (regions up to 2 kb away from CpG islands) and 78.8% in open sea (isolated CpGs in the genome) [29] (Figure 5B). Concerning the gene localization of the alterations, we identified 17.4% of differentially methylated CpGs located in exons, 36.3% in intergenic regions, 41.5% in introns and 4.8% in promoter regions (Figure 5A).

Overall, 6,552 fragments were found significantly altered nearby 1,952 genes (some genes being associated with several differentially methylated fragments, Supplementary Table A.1). The genes for which the maximum number of modified fragment was counted, was the complex imprinted gene Gnas (55 fragments), Zinc Finger And SCAN Domain Containing 10 (Zscan10: 49 fragments), Apoptosis Associated Tyrosine Kinase (Aatk: 32 fragments), T-Box 3 (Tbx3: 32 fragments), Signal-Induced Proliferation-Associated 1 (Sipa1: 30 fragments), Nuclear Factor Of Activated T Cells 2 (Nfatc2: 28 fragments).

3.5. Identification and clustering of genes in differentially methylated regions located in promoter areas or exons

The differential methylation in the promoters, allowed us to identify three clusters. Clusters 1 and 3 (Figure 5C) correspond to genes located in hyper-methylated DMRs in the DNA of BPS exposed mice compared to that of unexposed animals. Cluster 2 represents genes located in hypo-methylated DMRs. Several genes involved in liver lipid metabolism are present in this subgroup. Acyl-coA Synthetase Long Chain Family Member 4 (Acsl4) involved in Non-Alcoholic Fatty Liver Disease (NAFLD), free fatty receptor 1 (Ffar1/GPR40) implicated in hepatic steatosis and carboxyl ester lipase (Cel) associated to

hepatic lipoprotein metabolism present a hyper-methylated promoter. Suppressor of glucose autophagy associated 1 (Soga1) which plays a role in adiponectin-mediated liver insulin sensitivity, glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (Gpihbp1) controlling hepatic triglyceride content and lipid biosynthetic gene expression, nuclear receptor subfamily 1 group D member 2 (Nr1d2/Rev-Erb-beta) involved in hepatic steatosis related to circadian clock disruption and Mlx interacting protein like (Mlxipl) regulating glycolysis, lipogenesis and gluconeogenesis had hypo-methylated promoters.

For exons, only two clusters could be observed (Figure 5D). Ribosomal protein S6 kinase beta-2 (Rps6kb2) playing a role in hepatic steatosis and insulin resistance, estrogen related receptor beta (Esrrb) and thyroid hormone receptor alpha (Thra), which are the receptors of two hormones strongly involved in the regulation of energy metabolism, are genes with one or more hypo-methylated exons. Peroxisome proliferator-activated receptor d (Ppard) coordinated regulation of glucose and fatty acid metabolism, ATP-binding cassette a6 (Abca6) having an important role in lipid trafficking, carboxylesterase 3a (Ces3a) involved in detoxification and energy homeostasis and sphingomyelin synthase 2 (Sgms2) acting on lipid droplet formation and type 2 diabetes, have one or more exons that are hyper-methylated.

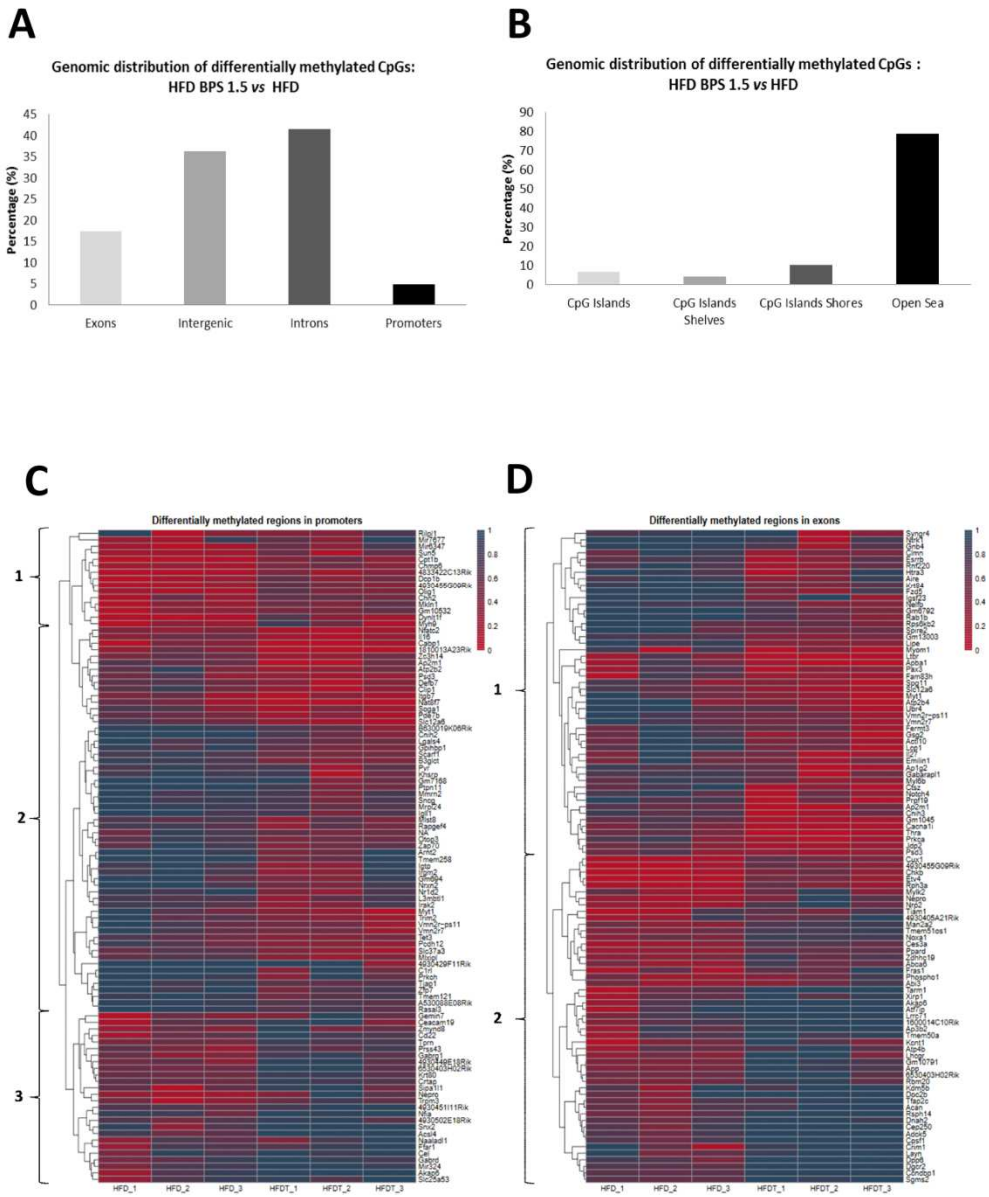
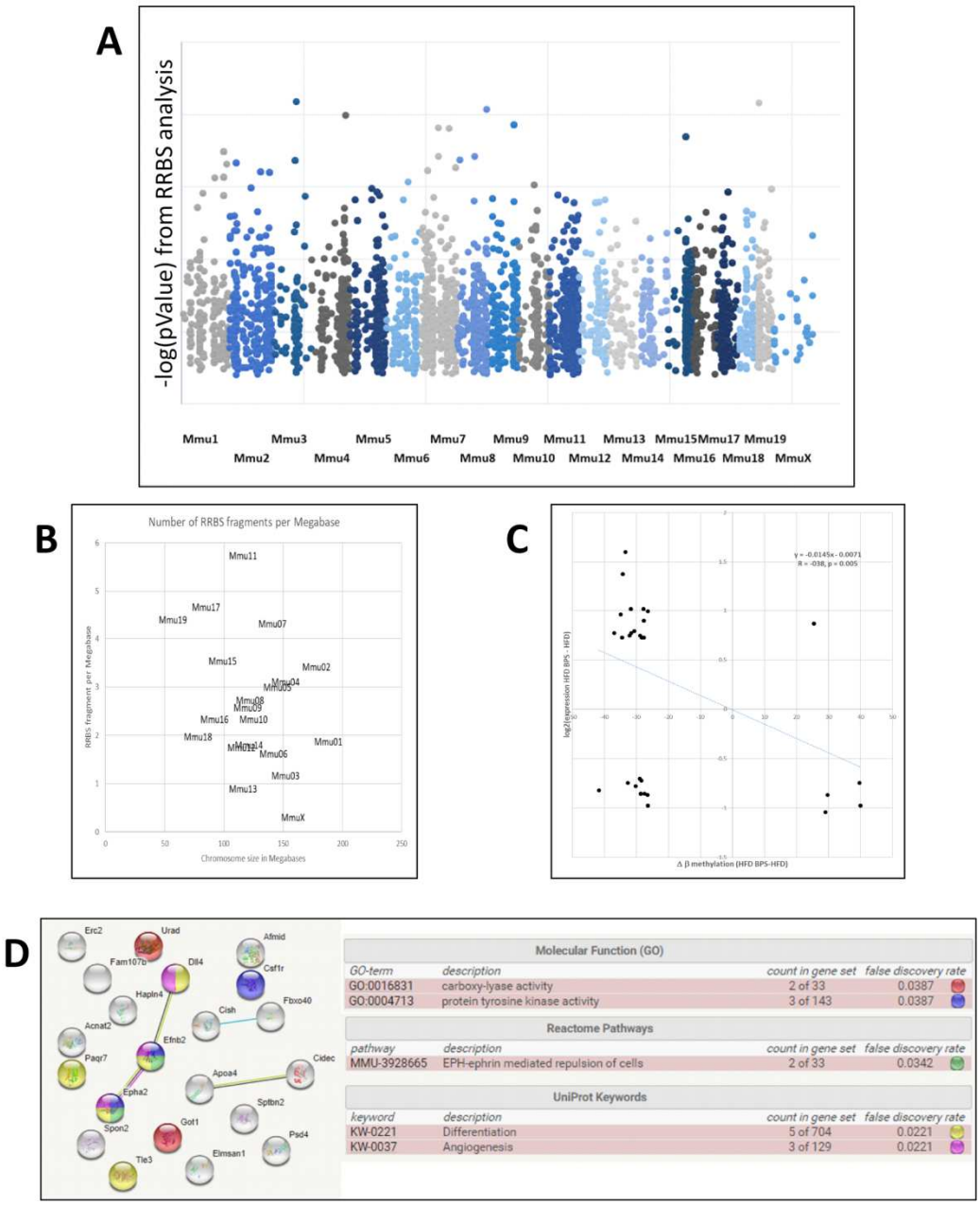


Figure 5. The differentially methylated CpGs annotated with different genomic regions (A), as well as CpG island and shore coordinates (B) and their distribution and heatmaps of top 100 Differentially Methylated Regions (DMRs) (width of 1000 nucleotides) located in promoters (C) or in exons (D) when liver DNA of C57Bl/6J male mice fed with a high fat diet and after BPS exposure from GD0 to 22 weeks-old at 1.5 µg/kg bw/d (HFD BPS 1.5) was compared with liver DNA of HFD control mice by RRBS. (HFD_1 : HFD control mice sample 1 ; HFDT_1 : HFD exposed to BPS mice sample 1). n = 9/group.

3.6. RRBS Hits and connections with gene expression deregulations.

Classically, gene expression is generally thought as down-regulated when CpGs are methylated. The complete set of differentially methylated RRBS fragments is presented in

Figure 6A by following the chromosome distribution (6,552 RRBS fragments). The number of RRBS fragment varied from 44 to 685 (chromosomes X and 11, respectively, Figure 6B). When adjusted to the chromosome size it appears clearly that the density of differential RRBS fragment is not homogeneous according to the chromosome, with the highest density of differential RRBS fragment located on chromosomes 11, 17, 19 and 7. In an attempt to correlate methylation with gene expression, we used a linear regression analysis (Pearson correlation coefficient was calculated and its significance evaluated). When the complete gene expression dataset was matched to the 6552 RRBS fragments, 1958 genes could be identified, but no significant correlation could be found between methylation and gene expression. Selecting genes that are deregulated at the expression level (log2 (Delta expression) > |0.7|, corresponding ~1.62 fold), 71 RRBS fragments were conserved and for them we observed a negative correlation between methylation level and expression for the genes nearby (21 genes, Figure 6C and 6D). These 21 genes were clusterized using String (https://string-db.org/), and shown to be involved in important cellular processes, carboxy-lyase and tyrosine kinase activity, cell repulsion mediated by ephrin, differentiation and angiogenesis (Figure 6D). These observations suggest that with HFD, BPS triggers important alterations of the methylation profile with an associated effect on gene expression, but limited to certain genes.



385 Figure 6. Manhattan plot showing the complete set of differentially methylated RRBS fragments following the
386 chromosome distribution (A), mapping of the density of differential RRBS fragment adjusted to the chromosome
387 size (B), correlation between DNA methylation and gene expression (C) and Functional Gene Set Enrichment
388 Analysis for genes with correlation between DNA methylation and expression level when liver mRNA and DNA
389 of C57Bl/6J male mice fed with a high fat diet and after BPS exposure from GD0 to 22 weeks-old at 1.5 µg/kg
390 bw/d (HFD BPS 1.5) was compared with liver mRNA and DNA of HFD control mice by microarray and RRBS.
391 n = 9/group.

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393

4. Discussion

In this study, we investigated liver DNA methylation and transcriptomic profiles associated with the obesogen effect of BPS. BPS potentiates the high fat diet-induced obesity in male mice, more specifically after perinatal exposure [30]. Previously, in the male mice fed with HFD and exposed to BPS (1.5 µg/kg bw/d), we showed that TG and glucose plasma levels were not modified, but insulin and cholesterol plasma levels and HOMA-IR index were significantly increased in comparison with unexposed male mice fed with HFD. So, these mice were in a state of metabolic disturbance characterized by systemic insulin resistance, dyslipidemia, and ectopic storage of fat mass in liver related to the obesogenic effect of BPS [10].

Here, we set to a hypothesis-free approaches to evaluate the consequences of the exposition in the liver, through mRNA expression and DNA methylation profiling. Obesity is associated with an increased risk of developing liver disease such as steatosis lesions. The liver plays a key role in lipid metabolism. In the liver, free fatty acids (FFAs) are derived, *via* the portal venous system, from diet, *de novo* lipogenesis from carbohydrates or amino acids and lipolysis in visceral adipose tissue (VAT; [31]. In the hepatocytes, FFAs can be metabolized (β-oxydation) or esterified (storage as triglycerides) or released into the blood flow as very low-density lipoprotein [31]. In addition, the adipokines secreted by VAT and the liver lipid accumulation are susceptible to create an inflammatory state that may promote the development of an insulin resistance context [31]. Furthermore, it's already known that visceral adipose tissue expansion is involved in non-alcoholic fatty liver disease (NAFLD) development [32]. Interestingly, in our study, we observed that the VAT weight was increased in HFD BPS 1.5 mice vs HFD control mice (1.94 g to 2.46 g *i.e* 1.3 ± 0.05 fold increase) (data not shown). Therefore, these observations suggest that in male mice, the obesity is directly connected with the increase of liver TG. These data are in accordance with previous studies

which reported that, *in vitro*, low-concentrations of BPS are able to induce lipid accumulation in murine 3T3L1 preadipocyte cell line [33], but the results are more disputable in hepatic cell lines [34, 35]. However, BPS clearly increases glucose uptake in 3T3L1 preadipocytes and it induces a pro-inflammatory phenotype in the murine macrophage J774A1 lineage [34, 36]. *In vivo* and *ex vivo*, BPS affects steroid receptor expression and adipogenesis in adipose tissue and preadipocytes with a sex-specific effect in male sheep [37]. In liver of adult male mice fed with a standard diet, BPS did not affect normalized liver weight at doses ranging between 0 and 5000 µg/kg with the highest dose causing histological and pathophysiological damage [38]. Another recent study conducted on female mice adolescent offspring exposed to a low doses of BPS, showed an obesogenic effect of BPS associated with an increase in hepatic triglyceride and cholesterol content without variation of liver weight and with a hepatic glucose homeostasis disturbance [39]. BPS seems to contribute to liver damage associated to obesity by acting on adipose tissue and liver. In addition, a physiologically based pharmacokinetic modeling highlighted that BPS is the BPA substitute which has the most important enterohepatic recirculation leading to poorer urinary excretion [40]. BPS is also the BPA analog leading to the highest internal concentrations of unconjugated bisphenols [40]. Its metabolism differs between Humans and mice. In female mice, 50% of the administered BPS is excreted in the urine in 6 h in metabolized form (detoxification mainly in glucuro-conjugated form) [41] In Humans, 92% of BPS is eliminated in non-metabolized form in 48 h in men urine compared to 70% in women urine [42] Consequently, BPS adverse effects can be more critical in Humans than in mice. Hitherto, none of these studies attempted to describe the molecular effect of BPS by high throughput techniques that can provide an unbiased vision of the effects of this molecule.

At the molecular level, the transcriptomic analysis that we carried out, shows a decreased mRNA expression of complement components, especially C8b. This gene has

recently been identified as a strong novel candidate target for non-alcoholic fatty liver diseases (NAFLD) with a sexual dimorphism [43]. C8b is a target of multiple estrogen receptors (Esrα Esrrα, Esrrβ, Esrrγ) which are associated with NAFLD in female mice and affected by testosterone deficiency in male liver [43]. To support this regulatory role of sex hormones, Hsd3b4 and Hsd3b5, two genes involved in all classes of hormonal steroid biosynthesis are also down-regulated. The second down-regulated key gene Egr1 is involved in the metabolism of liver-adipose tissue axis, but data about Egr1 function in liver steatosis are contradictory. Egr1 deficient mice fed with a high fat diet are less susceptible to diet-induced obesity and obesity-associated disorders (insulin resistance, dyslipidemia and fatty liver) in relation to an increase of energy expenditure in the adipose tissue [44, 45]. In contrast, data from another study showed that increasing Egr1 levels in the liver ameliorates diet-induced fatty liver disease in mice, which is more consistent with our observations [46]. In addition, ApoA4 is strongly up-regulated in our experiment. Interestingly, ApoA4 participates in liver inflammatory reactions and is an early biomarker of liver fibrosis, which is a critical pathological response to chronic liver disease leading to impairment of liver function [47, 48]. As expected, many of the strongly up-regulated genes Cyp2b9, Cyp2b13 and Gstm3 are involved in the detoxification of environmental toxins and in the metabolism of xenobiotics, showing that the liver is actively reacting to the chronic exposure at this BPS low dose [49].

Expression alterations in our study occurred through perinatal exposure, suggesting durable epigenome change, in connection with the DOHAD concept [50]. In the present study, we analyzed in parallel the transcriptome and DNA methylation profile after BPS exposure in the liver. All dams were fed with a standard diet, limiting the risk of perinatal effects. Obviously, the risk that the HFD introduced in adulthood interfere with epigenetic changes related to perinatal exposure to BPS exists. Indeed, it has already been shown that the

DNA methylation pattern changes with developmental nutritional exposures [51]. Depending on their position, the differently methylated regions will have a variable influence on transcription. Given our knowledge of the mouse genome, we focused our study on the differentially methylated CpGs found on promoters or exons of genes.

We showed by RRBS that the obesogenic effect of BPS observed in the male mice was associated with DNA hypomethylation in autosomes and with hypermethylation in sex chromosomes. Overall, 6,552 fragments were found significantly altered, corresponding to 1,952 genes (Supplementary Table A3). Alterations in the DNA methylation of these genes constitute a signature of the effect of BPS on the liver, but do not necessarily translate into alterations in gene expression. Concerning the DNA methylations, we identified six genes related to the glucido-lipid metabolism in the liver with one or more hypermethylated CpGs regions in their promoter (Acsl4, Gpr40, Cel) or in their exons (Ppard, Abca6, Ces3a, Sgms2). Seven other genes involved in the same metabolic functions presented hypomethylated CpGs regions in their promoter (Soga1, Gpihbp1, Nr1d2, Mlxipl) or their exons (Rps6kb2, Esrrb, Thra, Cell death activator 3: Cidec). Furthermore, two genes involved in liver fibrosis have one or more hypomethylated CpGs regions on their exons (Hapln4, ApoA4).

In the present study, we show that the correlation between DNA methylation and mRNA expression is not always consistent with classical dogma, except when we consider highly deregulated genes. Currently, it is commonly described in the literature (for the liver, see for instance [52]). In the context of gene methylation and expression, several other mechanisms regulating gene expression may be involved, such as post-transcriptional modifications of histones and chromatin rearrangement, as well as aberrant miRNA regulation, remote effect of intron methylations, methylation on repeated sequence impacts and potential role of hydroxymethylation in regulating gene expression [53, 54, 55]]. All of these modifications participate in compression or relaxation processes of chromatin structures

494 that contribute to gene expression regulation [56]. Another potential explanation is linked to
495 the BPS-induced DNA hypomethylation. Indeed, transcribed regions often include functional
496 elements, such as alternative promoters, enhancers, transcription factor binding sites,
497 repetitive elements and enrichment of nucleosomes at intron-exon junctions. Most of these
498 genomic elements are suppressed or stabilized by DNA methylation and hypomethylation
499 may therefore result in their activation and interference with expression of the host gene.
500 However, the demethylation of these elements is only a necessary but not a sufficient
501 condition for them to regain their activities, because the presence of transcription factors
502 specific for these elements (such as highly tissue specific enhancers) is a requirement for their
503 reactivation. Likewise, not all transcription factors are sensitive to DNA methylation
504 occurring within their binding sites [57]. More specifically, in our experimental conditions
505 and from a metabolic point of view, the mice were fasting for 4 h at the time of sacrifice.
506 Hypothetically, this duration allows changes in mRNA expression in the liver (which
507 coordinates metabolic homeostasis by adapting to nutritional status) but not to modify the
508 DNA methylation profile. Indeed, mRNA transcription is a process more dynamic that DNA
509 methylation.

510 Obviously, the effect of BPS on the mRNA expression can be also independent of
511 DNA methylation. Others direct or indirect activity of BPS can occur. Even if the molecular
512 mechanism induced by the BPS are poorly described, many studies reported its endocrine
513 properties. BPS was described to exhibit an estrogenic and anti-androgenic activities by acting
514 directly on sex-hormone receptor [58, 59] or by decreasing basal testosterone secretion by
515 mouse and human fetal testes [60].

516 Most of the molecular mechanisms involved in an integrative approach of epigenetic
517 regulation mechanisms remain to be elucidated. Interestingly, three genes involved in lipid
518 metabolism and liver fibrosis are both upregulated and hypomethylated (Cidec, Hapln4 and

519 ApoA4). Cidec is involved in lipid accumulation in hepatic human and murine cell lines [61,
520 62, 63]. This result is in accordance with the increase of hepatic TG content observed in our
521 study. Hyaluronan and proteoglycan link protein 4 (Hapln4) is involved in formation of
522 extracellular matrix and ApoA4 may be implicated in the evolution of NAFLD in cirrhosis,
523 which is linked to the development of fibrosis [47, 48].

524 To our knowledge, no data are available on the effects of BPS on DNA-specific
525 methylations associated with metabolic disorders. For the first time, in this study, we have
526 demonstrated with an innovative RRBS approach that the obesogenic effect of BPS is
527 associated with global and specific changes in liver DNA methylation, and with the
528 deregulation of expression of genes involved in detoxification, lipid metabolism and protein
529 synthesis. The targets appear to be different from that of BPA although the physiological
530 consequences (obesity, hepatic lipid accumulation, hyperinsulinemia) are quite similar.

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534 **Acknowledgments**

535 This work was funded by a grant from the Endocrine Disruptor National Research
536 Program supported by the "Ministère de la transition écologique et solidaire". This work was
537 also funded by Ministère de l'Enseignement Supérieur et de la Recherche (doctoral fellowship
538 to AB) and AgrosupDijon (France). We are grateful to Mr. Guillaume Macquard, Genomic
539 platform of Cochin institute and lipidomic technical platform of Burgundy University for their
540 technical assistance.

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Graphical Abstract