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Sex-dependent gene expression after ochratoxin A insult in F344 rat kidney

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ABSTRACT

Ochratoxin A (OTA) is a potent rodent nephrocarcinogen; being males more sensitive than females. The objective was to study the response between sexes at gene expression level (whole genome transcriptomics) in kidneys of F344 rats treated with 0.21 or 0.50 mg/kg bw OTA for 21 days. DNA methylation analysis of selected genes was also studied (MALDI-TOF mass spectrometry). OTA-induced response was dose-dependent in males and females, although clearer in males. Females showed a higher number of altered genes than males but functional analysis revealed a higher number of significantly enriched toxicity lists in 0.21 mg/kg treated males. OTA modulated damage, signaling and metabolism related lists, as well as inflammation, proliferation and oxidative stress in both sexes. Eleven toxicity lists (damage, fibrosis, cell signaling and metabolism) were exclusively altered in males while renal safety biomarker and biogenesis of mitochondria lists were exclusively enriched in females. A high number of lists (39) were significantly enriched in both sexes. However, they contained many sex-biased OTA-modulated genes, mainly phase I and II, transporters and nuclear receptors, but also others related to cell proliferation/apoptosis. No biologically relevant changes were observed in the methylation of selected genes.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced as a secondary metabolite of different fungal species of *Aspergillus* and *Penicillium* genera. It can contaminate a great variety of vegetal products and enter to the food chain through raw or processed products due to its high heat stability (European Food Safety Authority (EFSA), 2006; Lee and Ryu, 2017).

Despite its nephrotoxic, hepatotoxic, immunosuppressive, neurotoxic and teratogenic effects, the main issue concerning OTA is its carcinogenic potential in kidney, the target organ (European Food Safety Authority (EFSA), 2006). OTA has been classified as reasonably anticipated to be a human carcinogen (NTP, 2016) and as probable human carcinogen (IARC, 1993) based on sufficient evidence from studies in experimental animals but inadequate in humans.

In F344 rats exposed by gavage to OTA for up two years kidney tumors were found in both, males and females, but the response was much more pronounced in males (NTP, 1989). Sex and strain differences in the incidence of kidney tumors were also described in lifespan studies in other rat strains (Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998), but more information is still needed in order to understand how the carcinogenesis process starts and the key events involved in the sex-dependent OTA response. In this sense, a recent study in male and female F344 rats treated daily for 7 or 21 days with similar doses to the one used in the abovementioned carcinogenicity studies, 0.21 and 0.50 mg/kg bw (body weight), has been published (Pastor et al., 2018a). In agreement with the fact that OTA is nephrotoxic in both sexes, similar mild toxic effects were found after short-term administration, but were more pronounced in males than in females.

Phenotypic effects such as body weight decrease and slight proteinuria, indicative of kidney damage, were only evident in males after 21 days of treatment. In addition, despite tubulonephrosis and collecting duct injury were observed in both sexes, a high severity of damage, as well as glomerulonephritis, was observed in 7-day OTA-treated male rats. After 21 days, both sexes tended to have similar histopathological renal alterations.

In a gene expression analysis of kidney tissues from the same rats, in which time modulation was evaluated, a clear different response was observed in males after 7 days of treatment, not only in relation to females, but also when both timepoints were compared in males (Pastor

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et al., 2018b). OTA-induced response was progressive and consistent in females over time. In contrast, male response was clearly different between both timepoints. After 7 days, although a higher number of genes were modified compared to females, fewer toxicity lists (from Ingenuity Pathway Analysis, IPA) were affected in male rats. Over time, the response tended to be more similar between both sexes in terms of number affected toxicity lists.

In general, genes related to damage, nuclear receptor signaling, metabolism, necrosis or cell death, cell cycle regulation and proliferation were altered in both sexes. Moreover, as previously observed at renal transporter level (Pastor et al. 2018a), males showed a later response of genes related with oxidative stress and inflammation compared to females. Although similar functions were altered in both sexes, in each category, some genes or isoforms were exclusively altered in one sex. It is known that several phase I and II enzymes, as well as transporters show sex-biased expression at basal level and most of the sex-dependent responses observed against toxicants are due to their role in activation or detoxification of these compounds (Trevisan et al., 2012). Short-term toxicogenomics studies in male rats (Arbillaga et al., 2008; Marin-Kuan et al., 2006; Stemmer et al., 2007), as well as results from Pastor et al. (2018b), also pointed to that, as some of most altered genes by OTA were related to the modulation of cytochromes (*Cyp2c11, Cyp2d1, Cyp2d5, Cyp24a1*, in males), aldo-keto reductases (*Akr1b7* and *Akr1c2* in females), alcohol dehydrogenase (*Adh6* in females), and transporters (*Slco1a1, Slc5*1b and *Slc22a22* in males). In addition, it is important to remark that most of these genes are regulated by nuclear receptors. Indeed, several studies have demonstrated that OTA might alter pregnane X receptor (PXR) and aryl hydrocarbon receptor (Ahr)-dependent pathways (Ayed-Boussema et al. 2012; Doricakova and Vrzal, 2015), hepatocyte nuclear factor 4 alpha (Hnf4α) (Pastor et al., 2018b; Marin-Kuan et al., 2006) and nuclear factor-erytroid 2-related factor (Nrf2) (Limonciel and Jennings, 2014). In addition, as it was described in Pastor et al. (2018b), several of these signaling pathways showed a clear sex-dependent response. Finally, these pathways could be relevant in others functions such as cell cycle progression or apoptosis, such as Ahr that has been proposed to be related with to some mechanisms of hepatocarcinogenesis (Souza et al., 2016).

On the other hand, the role of kidney transporters in OTA carcinogenesis has been studied by several authors, as renal transport has been hypothesized to be one of the key events that could explain sex- and species-dependent sensitivity to OTA (European Food Safety Authority (EFSA), 2006; Mally, 2012). In rats, after repeated administration for 7 or 21 days, gene expression of the main kidney transporters families showed different modulation pattern dependent on sex (Pastor et al., 2018a). Ochratoxin A tended to downregulate *Slc22* transporters, but female response tended to appear earlier than in males; some transporters were only downregulated in males, such as *Oat3* (*Slc22a8*, organic anion transporter) or *Oatp1* (*Slco1a1*, organic anion transporter polypeptide); and finally, *Bcrp* (breast cancer resistance protein) showed different sense of modulation in each sex. Moreover, it seems that the dose has an important impact in transporters expression. Zlender et al. (2009) showed in male rats treated for 10 days with different doses that OTA induced a dual modification pattern of *Oats* (Slc22) expression, being upregulated at low doses and downregulated at the high dose tested.

So, taking into account the high influence of time of exposure previously found in OTA gene expression response (Pastor et al., 2018b) and that little information is available about the impact of the dose in both sexes, the aim of the present study was to evaluate the gene expression response of both sexes F344 rats after daily oral administration of 0.21 and 0.50 mg/kg bw for 21 days. Moreover, DNA methylation analysis has been carried out for some selected genes at different doses and timepoints, in order to assess if gene expression was regulated by this mechanism.

2. Material and methods

2.1. Chemicals

Ochratoxin A was obtained in powder from Sigma-Aldrich (Steinheim, Germany), dissolved in $NaHCO_3$ (0.1 M pH 7.4) (Sigma-Aldrich; Steinheim, Germany). At beginning of the study, vehicle ($NaHCO_3$ 0.1M pH 7.4) was prepared. Then, powder OTA was dissolved in it (5 mg/mL). Thereafter, OTA solution was diluted in vehicle to a final concentration of 0.05 mg/mL and 0.021 mg/mL, for the 0.5 and 0.21 mg/kg bw doses, respectively. These final concentrations were prepared at the beginning of the study and stored in aliquots at −20 °C. Each aliquot was thawed daily before animal administration. Animals were gavaged with 1 mL/100 g of their respective solutions.

2.2. Samples

In compliance with the 3Rs for refining, reducing and replacing animals for research purposes, analyzed samples of the present study had been obtained in a previous *in vivo* study (Pastor et al., 2018a), in which OTA levels in plasma and tissues, general toxicity (histopathology and biochemistry) as well as gene expression (RT-qPCR) of renal transporters were evaluated. Thus, the RT-qPCR analysis carried out in the Pastor et al. (2018a) in the same samples is considered as a validation experiment of the microarray results obtained in the present study. Moreover, oxidative-stress related response (Enciso et al., 2018) as well as time-dependent transcriptomics (Pastor et al., 2018b) have also been evaluated in the same samples. The study was approved by the Ethics Committee on Animal Experimentation at the University of Navarra (report approval number: CEEA/019-12).

Briefly, kidneys from male and female Fischer 344 rats (F344/IcoCrl) gavaged daily for 21 days with OTA (0.21 or 0.50 mg/kg bw; low (LD) and high dose (HD) respectively) or vehicle ($NaHC0_3$) (n = 6 per treatment group and sex) were removed and longitudinally cut in two halves. Each half was then divided in four pieces and quickly frozen in liquid nitrogen and stored at −80 °C until use. RNA and DNA isolation was carried out using two different pieces of left kidneys.

2.3. RNA isolation

For gene expression analysis, four samples per treatment and sex were used. Total RNA was isolated from approximately 50 mg of frozen kidneys according to TRIzol® manufacturer's protocol (Invitrogen™, USA). For that purpose, samples were homogenized in TRIzol® (50 mg/mL) with a T25 Ultra-turrax Digital High Speed Homogenizer (IKA®, Germany). Extracted RNA (100 μg) was purified according to AllPrep DNA/RNA kit's protocol (Qiagen; Hilden, Germany) and dissolved in RNase-free water. The purity and quality of extracted RNA were evaluated firstly spectrophotometrically (SmartSpec™ Plus, Bio-Rad) by measuring the optical density at 260 nm (1 unit of absorbance corresponds to 44 μg of RNA)" and then, after purification, with Experion Bioanalyzer (Bio-Rad Laboratories, Hercules, CA, USA) using Experion™ STDSens RNA Chips (Bio-Rad). All the samples showed A260/A280 ratio between 1.8 and 2.0 and with an average RNA quality indicator (RQI) value of 8.7 ± 0.7 (standard deviation, SD).

2.4. Gene expression experiments (microarrays)

Gene expression analyses were performed by the Proteomics, Genomics and Bioinformatics Unit of Center for Applied Medical Research (CIMA) from the University of Navarra (Spain).

The sense cDNA was prepared from 300 ng of total RNA using the Ambion® WT Expression Kit. The sense strand cDNA was then fragmented and biotinylated with the Affymetrix GeneChip® WT Terminal Labeling Kit (PN 900671) (Affymetrix, Inc., Santa Clara, USA). Labeled sense cDNA was hybridized to GeneChip® Rat Gene 2.0 ST Array

(Affymetrix, Inc., Santa Clara, USA) according to the manufacturer protocols and using GeneChip® Hybridization, Wash and Stain Kit (Affymetrix, Inc., Santa Clara, USA). Genechips were scanned with the Affymetrix GeneChip® Scanner 3000.

2.5. Gene expression data analysis

Both background correction and normalization were done using RMA (Robust Multichip Average) algorithm (Irizarry et al., 2003). After quality study of samples and outlier detection, all samples were considered for further analysis. Then, a filtering process was performed to eliminate low expression probe sets. Applying the criterion of an expression value (log_2) greater than 5 in 2 samples in at least one of the experimental conditions, 27790 probe sets were selected for statistical analysis. R version 3.4.3 and Bioconductor 3.6 (Gentleman et al., 2005) were used for preprocessing and statistical analysis.

LIMMA (Linear Models for Microarray Data) package version 3.34.9 (Smyth, 2004) was used to find out the probe sets that showed significant differential expression between experimental conditions. To determine OTA treatment effect in each sex, treated versus control animals were compared per dose. A B statistic cut off of $B > 0$ was considered to select differentially expressed genes (DEG) in each contrast. The B-statistic (lods or B) is the log-odds that the gene is differentially expressed. That is, a B of zero corresponds to a 50-50 chance that the gene is differentially expressed (Smyth, 2004). Total number of DEG of each contrast was calculated taking into account the number of Affymetrix's annotated genes.

2.6. Functional analysis: toxicity lists

To determine the biological meaning of the transcriptomic changes, DEG ($B > 0$) were selected for the functional analysis which was carried out using Ingenuity Pathway Analysis (IPA, 2016: http://www.ingenuity.com/). For that purpose, gene names provided by Affymetrix ($B > 0$) were uploaded and default settings filtered by specie (rat) using a relaxed filtering stringency were applied. For functional analysis, Toxicity lists were selected in order to obtain more specific information about key events involved in OTA-induced response. Lists with –log (p-value) ≥ 1.30 (p-value ≤ 0.05) were considered as significantly modulated.

2.7. DNA isolation

For DNA methylation analysis, samples from 7 days control and treated animals of the previous trascriptomic study focused on determining OTA time effect were also included (Pastor et al., 2018b). Thus, six samples per timepoint (7 or 21 days), dose (vehicle, 0.21 or 0.50 mg/kg bw) and sex were used except for control males and LD females (n = 5, due to an animal death). DNA was isolated from thawed kidney samples (30 mg) according to Dnaeasy® Blood & Tissue Kit's protocol (Qiagen; Hilden, Germany) and finally resuspended in 200 μL elution buffer. In order to elude the highest amount of DNA, this volume was passed twice through the membrane of the column. Then, DNA was measured with NanoDrop® 1000 Spectrophotometer (Thermo Scientific; Delaware, USA) to confirm an acceptable extraction (A260/A280 ratio 1.6–1.9 and A260/A230 ratio 2.0–2.2). In addition, after samples dilution (1:500), the quantity and the integrity of dsDNA were determined with Quant-iT™ PicoGreen® assay (Invitrogen; California, USA) following manufacturer's instructions.

2.8. DNA methylation profile by MALDI-TOF mass spectrometry

DNA methylation analysis was performed by the laboratory of Epigenetics and Genotyping of the Central Unit for Research in Medicine of the University of Valencia (Spain).

Akr1b7 (aldo-keto reductase family 1, member B7; ID: 116463), *Cyp1a1* (cytochrome P450, family 1, subfamily a, polypeptide 1; ID: 24296), *Cyp2c11* (cytochrome P450, subfamily 2, polypeptide 11; ID: 29277), *Gstp1* (glutathione S-tranferase pi 1; ID: 24426) and *Slc22a7* (solute carrier family 22 member 7; ID: 89776) genes were selected for studying DNA methylation pattern.

For each gene, promoter region was studied by using Agena's EpiTyper approach (Agena, San Diego, CA, USA), which relies on base-specific cleavage followed by MALDI-TOF mass spectrometry.

For that purpose, a gDNA sequence, containing 2000 bp starting before the first exon begins and finishing 1200 pb after it ends, was analyzed with EpiDesigner software. Two or three amplicons (400–500 pb) were selected for each gene taking into account the number of CpG sites present, as well as, the presence of binding sites of different molecules or regulatory elements that could be involved in their expression. Therefore, specific primers were designed (Table 1, supplementary data) and purchased from IDT Technologies for each amplicon.

Bisulfite conversion of the target sequences was performed by Zymo's EZ-96 DNA Methylation Lighting Kit (Zymo Research, Irvine, CA, USA). The manufacturer's protocol was followed by using 1 μg of genomic DNA.

The bisulfite-treated genomic DNA was amplified by PCR. The PCRs were carried out in a 5 μL format with 10 ng/mL bisulfite-treated DNA, 0.2 units of *Taq*DNA polymerase (Sequenom), 1 × supplied *Taq* buffer, and 200 mM PCR primers. Amplification for the PCR was as follows: preactivation at 94 °C for 4 min, 45 cycles at 94 °C denaturation for 20 s, 56 °C annealing for 30 s, and 72 °C extension for 1 min, finishing with a 72 °C incubation for 3 min. Dephosphorylation of unincorporated dNTPs was performed by adding 1.7 mL of H_2O and 0.3 units of shrimp alkaline phosphatase (Sequenom), incubating at 37 °C for 40 min, and then for 5 min at 85 °C to deactivate the enzyme. Next, *in vivo* transcription and RNA cleavage was achieved by adding 2 μL of PCR product to 5 μL of transcription/cleavage reaction and incubating at 37 °C for 3 h. The transcription/cleavage reaction contains 27 units of T7 R&DNA polymerase, 0.64 × of T7 R&DNA polymerase buffer, 0.22 μL T Cleavage Mix, 3.14 mM DTT, 3.21 μL H_2O, and 0.09 mg/mL Rnase A. The reactions were additionally diluted with 20 mL of H_2O and conditioned with 6 mg of CLEAN Resin (Sequenom) for optimal mass-spectra analysis.

Later 10–15 nL of each sample were transferred onto the spectroCHIP array (Agena) by nanodispensation. Analysis with the Agena MALDI-TOF MS Compact Unit was performed after a 4-point calibration with oligonucleotides of different mass provided in the kit. The protocol described by van den Boom and Ehrich (2009) was used. Matched peak data were exported using EpiTyper software and analyzed. However, DNA methylation values of some CpGs could not be measured independently, which is the case of nearby CpGs and CpGs included in similar chemical fragments after the enzymatic cut. Thus, these CpGs were co-jointly quantified, reporting an average value.

2.9. Statistical analysis of DNA methylation results

DNA methylation results are reported as mean ± SD. Statistical comparisons between groups were performed by Mann-Whitney U test and differences were considered significant when p-value < 0.05. Moreover, 10% of variation in DNA methylation percentage was considered as a potential change in mRNA expression (Ozden et al., 2015). Statistical analyses were performed with SPSS 15.0 software.

3. Results

3.1. Gene expression analysis

Firstly, in order to determine the general pattern of expression, hierarchical clustering analysis (HCA) was performed with all the genes present on the GeneChip. As it can be observed in Fig. 1 (supplementary

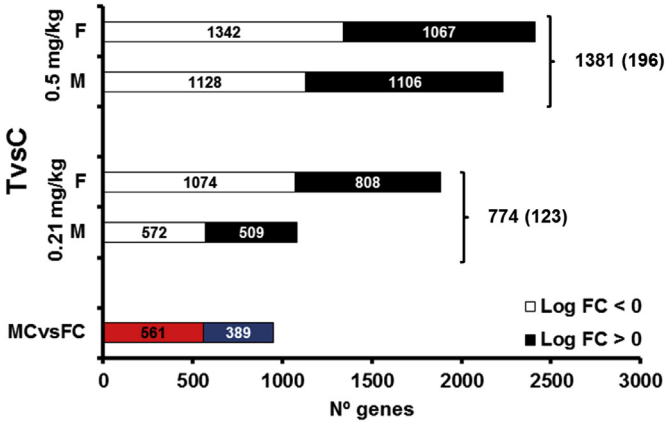

Fig. 1. Differentially expressed genes (DEG) in each contrast after 21 days of treatment with control vehicle ($NaHCO_3$) or OTA (0.21 or 0.50 mg OTA/kg bw). Total number of down- and upregulated annotated genes is represented in each bar: black and white for OTA treatment (T) versus control (C) comparison, red for basal female-biased genes and blue for basal male-biased genes. The number of common genes between males (M) and females (F) in each treatment are shown (joined by brackets). In parenthesis the number of genes that were also sex-biased at basal level. MCvsFC: Males control versus females control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

data), OTA effect was very clear after 21 days of treatment and two different clusters were obtained: control and treated animals. In addition, a clear sex-dependent expression profiles was detected in control animals. After OTA treatment, males and females tended to group in two different clusters (except for two males). In terms of OTA dose effect, this factor appeared to be more relevant in males than in females, as no clear grouping pattern was detected in treated females. In order to determine the number of DEG of each contrast, Affymetrix's annotated genes with B-value > 0 were considered significant and selected for functional analyses (Fig. 1).

Sex differences at basal level (male versus females control animals, MCvsFC) were already studied and discussed in Pastor et al. (2018b).

In relation to the effect of OTA, as shown in Fig. 1, females showed a higher response than males in terms of number of DEG at both doses, being the difference between both sexes much higher at the lower dose tested (a difference of 801 DEG between both sexes at 0.21 mg/kg bw versus a difference of 175 DEG at 0.5 mg/kg bw).

Studying the magnitude of the effect induced by OTA, only a few of the DEG had logFC higher than 1.5 or less than −1.5: in females, 84 and 136 genes, and in males, 33 and 136 genes, at LD and HD groups, respectively. In each sex, most genes were common to both doses (Males: 30; Females: 76 genes), but only 19 genes were common to all groups (Table 1). However, only few of the most modified genes were exclusive of each sex (Tables 2 and 3). Females showed higher number of sex-dependent induced genes than males (Males: 21 genes; Females: 32 genes), and almost all genes were common to both doses (30/32 genes). On the other hand, more than a half of all male-exclusive genes (12/21 genes) were modified in both dose groups, but in this sex, a high number of dose-dependent genes were determined (LD: 1 and HD: 8 genes). These results seem to point out that the effect of the dose might be more relevant in males than in females.

Finally, by comparing sexes at each dose, 774 genes were common to both sexes at the LD and 1381 at HD group (Fig. 1). It should be noted that from these DEGs common to both sexes, 123 and 196 genes of LD and HD groups respectively already showed sex-biased expression in control animals. In general, the number of genes involved in OTA-induced response that already showed a sex-biased expression at basal level was between 18% and 21% in each treatment group.

3.2. Functional analysis: toxicity lists

Ingenuity pathway analysis was carried out using DEG (B > 0) of all contrasts in order to determine toxicity lists which could be involved in OTA toxic effect. The criteria to consider these lists as significantly affected was –log (p-value) ≥ 1.3 which corresponds to a p-value ≤ 0.05. The search focused on rat-specific genes but the available information of all species was considered for determining the relation among then. Only rat-specific lists were considered when lists were defined for more than one species. Finally, lists were grouped in categories previously defined (according to Pastor et al., 2018b): damage, necrosis/cell death, proliferation, apoptosis, cell cycle regulation, signaling, metabolism, oxidative stress, fibrosis, inflammation and non-genotoxic biomarker panel (Table 4).

Despite the number of DEG (Fig. 1) was always higher in treated females than in males, a high number of significant altered lists in both male groups was observed (Table 4).

In total, 52 lists were altered after OTA treatment and more than half of them showed sex-biased response at basal level (29/52). Regarding lists significantly altered in all treated groups (29), the most repeated category was damage (7 lists), followed by oxidative stress response (4 lists), nuclear receptor signaling (4 lists), metabolism (3 lists), proliferation (3 lists), necrosis/cell death (2 lists), inflammation (3 lists), fibrosis (1 list) and cell cycle regulation (1 list) and non-genotoxic biomarker (1 list) (Table 4). The distribution of categories of toxicity lists induced by OTA treatment in males and females is presented in Fig. 2. Roughly, both sexes and doses showed similar response to OTA administration in terms of number and categories altered. However, males showed a higher response at LD with more lists involved in response, in particular nuclear receptor signaling lists, damage and metabolism.

Regarding gene expression modulation observed within the toxicity lists (Table 2, supplementary data), a clear pattern was observed. In damage, necrosis/cell death, proliferation, cell cycle regulation, fibrosis, inflammation and nongenotoxic biomarker panel lists, the main tendency in both sexes was gene expression upregulation. In contrast, downregulation was the main effect observed in genes related to fatty acid and xenobiotic metabolism, glutathione depletion, oxidative stress and some signaling lists such as *LPS/IL-1 Mediated Inhibition of RXR Function* or *FXR/RXR Activation*. However, some exceptions were found when nuclear receptor signaling lists were analyzed. RAR activation list showed a majority of upregulated DEGs in all groups. Females showed equal number of genes up- and downregulated in LD group and a tendency to downregulation in HD group in *Aryl Hydrocarbon Receptor Signaling* and *LXR/RXR Activation* lists, while in both dose males, genes were downregulated and upregulated in each list, respectively. In the case of *PXR/RXR Activation* list, a clear downregulation was observed in females while equal number of genes was modified in both senses in males. Finally, LD male group showed special expression pattern with respect to the rest of groups in *Mechanism of Gene Regulation by Peroxisome Proliferator via PPARα* and *TR/RXR Activation* lists. In the first list, a tendency to upregulation was observed in this group while similar number of genes was determined in the others. For *TR/RXR Activation* list, males LD group showed same number of DEGs in both directions, while gene expression tendency was upregulation in females and HD male groups.

3.3. DNA methylation study

Promoter DNA methylation of Akr1b7, Cyp1a1, Cyp2c11, Gstp1 and Slc22a7 genes was studied in order to determine if sex-dependent differences at basal level or/and OTA-induced expression changes were related to different methylation pattern. Some methylation changes in studied genes were detected but, as most of the changes were less than 10%, they were not considered to be biologically relevant (data not shown). Only Cyp2c11 and Slc22a7 showed some CpG site with

Table 1
DEG obtained in F344 rats after daily oral OTA treatment (0.21 or 0.50 mg/kg bw) that showed logFC higher than |1.5| and were commonly altered at both doses in males and females.

Gene Name	Gene Description	Males		Females	
		0.21	0.50	0.21	0.50
Cacng5	calcium channel, voltage-dependent, gamma subunit 5	−1.53	−4.79	−3.33	−3.69
Gng13	guanine nucleotide binding protein (G protein), gamma 13	−1.74	−2.17	−1.76	−2.73
LOC100910566	solute carrier family 7 member 13-like	−2.47	−5.59	−4.73	−5.88
Nat8b	N-acetyltransferase 8B	−1.55	−2.29	−2.13	−2.67
Rdh5	retinol dehydrogenase 5 (11-cis/9-cis)	−1.70	−1.97	−1.91	−2.30
Slc22a13	solute carrier family 22 (organic anion/urate transporter), member 13	−2.52	−2.16	−2.79	−3.22
Slc5a10	solute carrier family 5 (sodium/sugar cotransporter), member 10	−1.82	−2.28	−2.15	−3.38
Tmigd1	transmembrane and immunoglobulin domain containing 1	−1.76	−2.28	−2.10	−2.79
Havcr1	hepatitis A virus cellular receptor 1	4.97	5.66	5.97	5.88
Hist1h1t	histone cluster 1, H1t	1.57	1.77	1.56	1.87
LOC102549148	uncharacterized LOC102549148	2.55	3.30	2.92	2.62
LOC102549777	uncharacterized LOC102549777	3.29	4.10	3.53	3.82
LOC102552061	uncharacterized LOC102552061	1.84	2.49	2.55	2.14
LOC102556159	uncharacterized LOC102556159	2.54	3.29	2.90	2.62
LOC686967	similar to olfactory receptor 1442	2.47	2.69	1.70	2.16
Ly6g6c	lymphocyte antigen 6 complex, locus G6C	1.58	2.20	1.73	1.91
Olr338	olfactory receptor 338	2.70	2.91	1.93	2.09
Spp1	secreted phosphoprotein 1	1.80	2.41	3.04	3.08
Tbx10	T-box 10	2.19	1.77	1.95	2.17

methylation changes higher than 10% (Figures 2, 3, 4 and 5, supplementary data).

4. Discussion

The aim of the present study was to evaluate the impact of OTA dose at gene expression level in F344 rats of both sexes after 21 days of daily oral administration. The lowest dose (0.21 mg/kg bw) was selected for being the highest dose tested in the gold-standard carcinogenicity study (NTP, 1989) that induced tumors in rats (incidence of carcinomas: 30/50 in males versus 3/50 in females), while the dose of 0.50 mg/kg bw has been used in other short-term toxicity studies (Arbillaga et al., 2008; Corcuera et al., 2015, Vettorazzi et al., 2009, 2010, 2011) and is close to the 0.4 mg/kg bw dose used in other carcinogenicity studies (Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998).

Regarding OTA effect, in terms of number of DEG, a dose-dependent response was observed in both sexes. In both sexes almost all the genes altered at the lowest dose were also modified at the highest one. However, OTA-dependent response was stronger in males than in females as the number of DEG was almost duplicated (1081 versus 2234 genes) with dose.

4.1. DEG in both sexes

Few genes were commonly genes deregulated in both sexes (Table 1). Two of the most upregulated genes were *Havcr1* and *Spp1*. Both of them are well-established biomarkers of kidney injury (Kwekel et al., 2013) and have been reported to be modified by OTA treatment in male rats (Rached et al., 2008; Zhu et al., 2016). This is in agreement with the fact that OTA is nephrotoxic in both sexes (Pastor et al., 2018a, 2018b; NTP, 1989). In general, OTA modified in both sexes the expression of several genes involved in signal transduction (*Gng13, Olr338* and *Ly6g6c*), DNA transcription (*Tbx10*) and chromatin compaction (*Hist1h1*). Moreover, it also inhibited *Rdh5* expression, a microsomal

Table 2
DEG obtained in F344 rats after OTA treatment (0.21 or 0.50 mg/kg bw) with logFC higher than |1.5| that were exclusively modified in males.

Gene Name	Gene Description	Males	
		0.21	0.50
Cml2	camello-like 2	1.82	
Cml3	camello-like 3	−2.01	−2.15
Cyp2c11	cytochrome P450, subfamily 2, polypeptide 11	−3.48	−4.43
Cyp2d1	cytochrome P450, family 2, subfamily d, polypeptide 1	−1.31	−2.35
Cyp2d5	cytochrome P450, family 2, subfamily d, polypeptide 5	−1.16	−2.13
Dhrs7	dehydrogenase/reductase (SDR family) member 7	−1.36	−4.49
Gc	group specific component	−1.12	−2.07
LOC102550584	hornerin-like	−0.89	−3.18
Mlc1	megalencephalic leukoencephalopathy with subcortical cysts 1	−1.29	−1.57
RGD1564999	similar to isopentenyl-diphosphate delta isomerase 2	−2.44	−2.84
Slc51b	solute carrier family 51, beta subunit	−0.73	−2.02
Tmem236	transmembrane protein 236	−0.90	−1.71
S100g	S100 calcium binding protein G	1.15	1.58
Anxa13	annexin A13		−1.69
Cidec	cell death-inducing DFFA-like effector c		−1.65
Gucy1b2	guanylate cyclase 1, soluble, beta 2		−1.74
LOC100364391	dehydrogenase/reductase (SDR family) member 7-like		−2.27
Oosp1	oocyte secreted protein 1		−1.92
Slc22a22	solute carrier family 22 (organic cation transporter), member 22		−1.53
Slco1a1	solute carrier organic anion transporter family, member 1a1		−5.26
Cyp24a1	cytochrome P450, family 24, subfamily a, polypeptide 1		1.54

Table 3
DEG obtained in F344 rats after OTA treatment (0.21 or 0.50 mg/kg bw) with logFC higher than |1.5| that were exclusively modified in females.

Gene Name	Gene Description	Females	
		0.21	0.50
Akap17b	A kinase (PRKA) anchor protein 17B	−2.21	−2.50
Akr1b7	aldo-keto reductase family 1, member B7	−5.00	−5.37
Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A	−1.91	−1.96
Cnr1	cannabinoid receptor 1 (brain)	−1.33	−1.76
Cntnap4	contactin associated protein-like 4	−1.69	−2.05
Col17a1	collagen, type XVII, alpha 1	−0.96	−1.57
Col24a1	collagen, type XXIV, alpha 1	−1.20	−1.68
LOC102546376	disks large homolog 5-like	−2.23	−2.44
LOC102547093	uncharacterized LOC102547093	−2.35	−2.54
LOC102547212	disks large homolog 5-like	−2.30	−2.76
LOC102549465	disks large homolog 5-like	−2.18	−2.52
LOC102557499	uncharacterized LOC102557499	−2.66	−3.02
LOC498465	similar to RIKEN cDNA 1700001F09	−1.68	−1.84
LOC498470	similar to Spetex-2C protein	−1.55	−2.01
LOC680656	hypothetical protein LOC680656	−1.80	−1.76
Ly6i	lymphocyte antigen 6 complex, locus I	−2.17	−2.45
Mall	mal, T-cell differentiation protein-like	−1.55	−1.74
Nxpe2	neurexophilin and PC-esterase domain family, member 2	−3.26	−3.44
Plin1	perilipin 1	−1.61	−1.48
Slc17a9	solute carrier family 17 (vesicular nucleotide transporter), member 9	−1.67	−2.12
Spetex-2D	Spetex-2D protein	−2.07	−2.93
Spetex-2E	Spetex-2E protein	−2.24	−2.69
Spetex-2F	Spetex-2F protein	−1.78	−2.54
Spetex-2G	Spetex-2G protein	−2.28	−3.12
Spetex-2H	Spetex-2H protein	−2.42	−3.09
Alox15b	arachidonate 15-lipoxygenase, type B	1.60	1.20
Dppa3	developmental pluripotency-associated 3	1.92	1.28
Fkbp5	FK506 binding protein 5	1.42	2.03
Mybl1	myeloblastosis oncogene-like 1	2.49	2.34
Sectm1b	secreted and transmembrane 1B	2.07	1.76
Adh6	alcohol dehydrogenase 6 (class V)		−2.30
Akr1c2	aldo-keto reductase family 1, member C2		−2.10

NAD^+-dependent retinol dehydrogenase, involved in the biosynthesis of retinoids that regulate a wide variety of biological functions, such as cell differentiation, proliferation and apoptosis, and may activate or repress the transcription of multiple target genes by binding to nuclear retinoic acid receptors (RAR) and retinoid X receptors (RXR).

One of the most downregulated genes in both sexes was the calcium channel *Cacng5*. Although the specific role of calcium in OTA toxicity is still not clear, our findings agree with the alterations of genes involved in calcium homeostasis previously demonstrated in other gene expression studies carried out with OTA only in males (Arbillaga et al., 2008; Marin-Kuan et al., 2006).

In a previous study evaluating the effect of time in the sex-dependent gene expression response after OTA administration many genes related with metabolism and transport showed a sex-biased response (Pastor et al., 2018b). In the present analysis sex-biased response was also observed in these genes, however, it should be noted that genes involved in metabolism such as *Nat8b* and transport (*Slc5a10*, *Slc22a13*) are also robustly downregulated after OTA treatment in both sexes. The downregulation of *Sl22a13* and *Tmigd1*, an adhesion molecule, also downregulated by OTA in both sexes, have been related with apoptosis and cell survival functions, respectively. Indeed, Slc22a13 may have a tumor-specific apoptosis activity on renal cancer cells when is overexpressed, via stearoyl-Coa desaturase-1 (Scd-1) (AbuAli and Grimm, 2014; AbuAli et al., 2015). On the other hand, Arafa et al., (2015) demonstrated that Tmigd1 controls cell migration, cell morphology, and protects renal epithelial cells from oxidative cell injury. Moreover, its expression is significantly affected in both acute kidney injury and chronic kidney disease mouse models (Arafa et al., 2015),

Table 4
Toxicity lists significantly altered in male and female F344 rats after a daily oral treatment of OTA (0.21 or 0.50 mg/kg bw) for 21 days. Lists were considered significant when –log (*p*-value) ≥ 1.3 (*p*-value ≤ 0.05). In parenthesis: category in which each list has been grouped. They have been ordered according to these categories. In bold, toxicity lists related to kidney.

Toxicity list	-log(*p*-value)			
	Males		Females	
Dose (mg/kg)	**0.21**	**0.50**	**0.21**	**0.50**
Name of list (category [a]) Nº lists	46	41	35	38
Acute Renal Failure Panel (D)	13.30	17.30	14.50	11.7
Renal Proximal Tubule Toxicity Biomarker Panel (D)	11.10	12.80	7.93	8.60
Genes Downregulated in Response to Chronic Renal Failure (D)	3.00	5.06	5.58	4.85
Persistent Renal Ischemia-Reperfusion Injury (D)	2.47	3.92	3.84	2.97
Increases Renal Damage (D)	3.19	3.80	1.66	1.49
Increases Glomerular Injury (D)	2.17	3.35	3.00	2.19
Recovery from Ischemic Acute Renal Failure (D)	2.20	2.48	4.83	3.12
Genes associated with Chronic Allograft Nephropathy (D)	1.79	2.53	1.58	
Renal Glomerulus Panel (D)	1.31			
Renal Safety Biomarker Panel (PSTC) (D)				1.79
Hepatic Cholestasis (D)	2.71	1.59	1.44	
Increases Liver Damage (D)	1.39	1.89		
Cardiac Hypertrophy (D)		2.11		1.35
Biogenesis of Mitochondria (D)				1.72
Primary Glomerulonephritis Biomarker Panel (I)	1.80	3.45	1.92	2.34
Positive Acute Phase Response Proteins (I)	2.56	2.71	3.26	2.49
Irreversible Glomerulonephritis Biomarker Panel (I)	1.48	1.90	1.49	1.77
Increases Renal Nephritis (I)		1.43		
Increases Liver Hepatitis (I)	1.51		1.45	
Increases Renal Proliferation (P)	1.40	3.18	3.20	1.82
Increases Liver Hyperplasia/Hyperproliferation (P)	1.63	3.05	3.70	2.99
Liver Proliferation (P)	3.74	2.75	5.39	3.48
Increases Cardiac Proliferation (P)	1.45			
Cell Cycle: G2/M DNA Damage Checkpoint Regulation (C)	3.40	3.94	3.08	2.57
Cell Cycle: G1/S Checkpoint Regulation (C)		2.02		
NRF2-mediated Oxidative Stress Response (O)	5.12	5.83	4.97	4.93
Long-term Renal Injury Anti-oxidative Response Panel (O)	1.98	4.67	2.52	5.42
Oxidative Stress (O)	3.25	3.71	1.96	2.85
Long-term Renal Injury Pro-oxidative Response Panel (O)	1.68	3.16	2.60	2.12
Fatty Acid Metabolism (M)	7.91	9.12	4.26	5.12
Xenobiotic Metabolism Signaling (M)	3.68	4.04	5.31	5.70
Glutathione Depletion - Phase II Reactions (M)	2.71	2.70	3.16	2.52
Cytochrome P450 Panel - Substrate is a Fatty Acid (M)	1.68	1.50		1.40
Glutathione Depletion - CYP Induction and Reactive Metabolites (M)	1.94		2.11	1.73
Cytochrome P450 Panel - Substrate is a Xenobiotic (M)	1.42	1.35		
Aryl Hydrocarbon Receptor Signaling (S)	6.35	6.03	3.48	2.46
LPS/IL-1 Mediated Inhibition of RXR Function (S)	6.65	6.02	3.74	3.53
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα (S)	2.23	2.67	2.06	2.64
LXR/RXR Activation (S)	4.73	2.39	2.60	2.34
FXR/RXR Activation (S)	2.15		1.79	1.61
PXR/RXR Activation (S)	2.42	2.37		1.71
TR/RXR Activation (S)	1.47			2.70
RAR Activation (S)	2.23			
VDR/RXR Activation (S)	1.61			
Hepatic Stellate Cell Activation (S)	1.44	1.48		

(*continued on next page*)

Table 4 (*continued*)

Toxicity list		-log(*p*-value)			
		Males		Females	
	Dose (mg/kg)	**0.21**	**0.50**	**0.21**	**0.50**
Name of list (category [a])	Nº lists	46	41	35	38
CAR/RXR Activation (S)		2.18	1.44		
Renal Necrosis/Cell Death (N)		5.88	8.06	7.92	5.45
Liver Necrosis/Cell Death (N)		5.00	8.02	6.86	7.55
Cardiac Necrosis/Cell Death (N)		2.39		2.09	2.49
Hepatic Fibrosis (F)		2.68	5.83	3.66	3.01
Cardiac Fibrosis (F)			1.76		
Nongenotoxic Hepatocarcinogenicity Biomarker Panel (NGTX)		1.49	2.61	3.11	2.41

[a] Categories are indicated in parenthesis: apoptosis (A); cell cycle (C); damage (D); fibrosis (F); inflammation (I); necrosis/cell death (N); non-genotoxic biomarker (NGTX); metabolism (M); oxidative stress (O); proliferation (P); signaling (S).

which is in agreement with our results and the fact that OTA has been shown to damage kidneys of both males and females (Pastor et al., 2018a). Overall, these genes could be considered as robustly affected after a short-term OTA treatment in F344 rats regardless of sex. Indeed, all these genes, except *Spp1* and *Cacng5* in males, were also significantly modulated and in the same direction in both sexes even after 7 days of OTA exposure (Pastor et al., 2018b).

4.2. DEG in males

A high number of DEG exclusively modulated in males (Table 2), were affected at both doses and were mainly related with metabolism (*Cyp2c11, Cyp2d1, Cyp2d5, Dhrs7*), calcium homeostasis (*Gc, S*100g) and transport (*Slc*51b). From these male-biased DEG, only *Cyp2c11, Cyp2d1* and *S*100g were also significantly altered after 7 days of OTA exposure (Pastor et al., 2018b). Surprisingly, camello-like 2 (*Cml2*), considered as a probable N-acetyltransferase, was only significantly upregulated at the lowest dose tested at 21 days and at 7 days (Pastor et al., 2018b) but not at the highest dose tested after 21 days of exposure.

Moreover, some genes were exclusively altered in males by OTA at the higher time of exposure (21 days) and at the higher dose tested (0.50 mg/kg bw) such as *Anxa 13*, *Cyp24a1* and *Slco1a1*. Although other annexins (*Anx*) such as *Anxa1, Anxa2, Anxa3* and *Anxa5* were found to be upregulated *in vivo* in a similar experimental design in males (Arbillaga et al., 2008) and in males and females of our dataset (Table 2), *Anxa13* was downregulated only in males at the highest dose tested. Annexins are cytosolic Ca^{2+} binding protein involved in cytoskeleton organization that have been associated with poor cancer prognosis in some tumors when upregulated (Hayes et al., 2004; Sharma and Sharma, 2007;; Jiang et al., 2017).

One of the most upregulated male-biased genes at the highest dose, *Cyp24a1*, involved in calcium homeostasis and vitamin D endocrine system, has been proposed as a candidate oncogene for colorectal tumorigenesis (Horváth et al., 2010). On the other hand, *Slco1a1* was highly downregulated only in males at the highest dose. This gene, also known as *Oatp1*, was one of the genes showing stronger sexual differences at basal level (Pastor et al., 2018b), and has been validated by RT-qPCR in the same samples (Pastor et al., 2018a) and hypothesized to be involved in a higher exposure to OTA in male kidney (Pastor et al., 2016).

4.3. DEG in females

In relation to genes exclusively modified by OTA in females (Table 3), almost all the highly expressed genes (logFC higher than 1.5 or less than −1.5) were differentially expressed at both doses, except *Adh6* and *Akr1c2* that were only significantly downregulated at the highest dose tested. Unfortunately, although known to be involved in many bioactivation/detoxification processes, their role in OTA metabolism have not been explored yet. Indeed, another aldo-keto reductase, *Akr1b7*, was the most downregulated gene in the female dataset and was also highly downregulated even after shorter times of exposure and presented a clear female-biased expression at basal level (Pastor et al., 2018a). Thus exploring the function of these enzymes might be a key aspect for understanding the sex-dependent response of OTA and other xenobiotics. Other gene highly downregulated in females at both doses after 21 days of exposure, as well as after 7 days of exposure (Pastor et al., 2018b), are the cytokine *Cmtm2a* and glycosylphosphatidylinositol-linked protein *Ly61*. On the other hand, the two most upregulated genes in females were *Mybl1* and *Sectm1b*, both also upregulated at shorter exposure times (Pastor et al., 2018b). The proto-oncogene *Mybl1*, a gene involved in cell proliferation and pathogenesis of cancer, has been reported to be induced by Bcl6 regulated by Ahr/Arnt (Ding et al., 2015) and by Igf-1 (Mitsui et al., 2013); and it has been described to be involved in some rat strain differences in the responsiveness to estrogen (Mitsui et al., 2013). On the other hand, the secreted and transmembrane 1B protein (Sectm1b) has been described to be upregulated in several tumors and to be involved in the immune response in cancer (Huyton et al., 2011) and may act as a chemoattractant for monocytes to modulate the tumor microenvironment

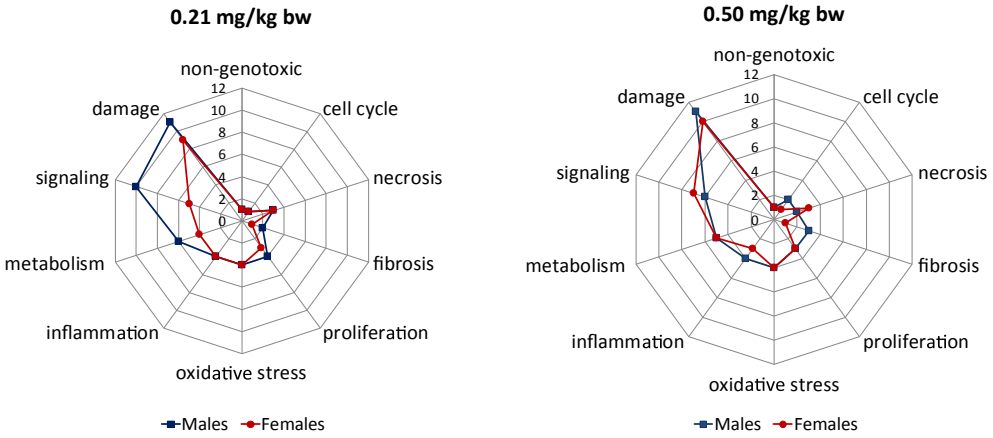

Fig. 2. Number of toxicity lists included in each category that were significantly altered after a daily oral administration of OTA (0.21 or 0.50 mg/kg bw) for 21 days in male and female F344 rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Wang et al., 2014).

The role of these two genes in the sex-dependent response to OTA should be further explored as OTA has been shown to alter some of the sexual hormones in the rats used in the present study (Pastor et al., 2018a), as well as females seemed to have an early inflammatory response different from males (Pastor et al., 2018b).

Overall, the analysis of DEG in the present study and in Pastor et al. (2018b) points to the fact that males have a different early response (in terms of both, time and dose), while females' response seems to be more consistent over time and doses. Moreover, the sex-dependent incidence of tumors observed after OTA exposure (NTP, 1989) might be explained by the sex-biased deregulation in genes with metabolic, cell survival and immune functions.

4.4. Functional analysis: toxicity lists

Regarding functional analysis, males showed more toxicity lists altered than females (Males: 46 and 41; Females: 35 and 38 lists, in each dose respectively), and interestingly, LD male group showed the highest number of lists altered, despite having the lowest number of DEG (Fig. 1). Most of the toxicity lists induced by OTA treatment were significantly altered in both sexes (39 lists) and, as observed at gene level, many of the lists altered after the lowest dose tested (0.21 mg/kg bw) were also altered at 0.50 mg/kg bw. As shown in Table 4 and Fig. 2, the most affected categories were damage, signaling and metabolism. However, inflammation, proliferation and oxidative stress related lists also showed a role in OTA response. It should be pointed that, at the gene level, many of these common toxicity lists showed sex-dependent OTA-induced genes, indicating a possible different regulation of same pathways between sexes. Moreover, some lists were exclusively modified in one sex (11 in males, and 2 in females). In general, male-unique toxicity lists were mainly related to damage, cell signaling and metabolism. Interestingly, it should be noted that LD male group showed the highest number of signaling toxicity lists altered while *Cell Cycle: G1/S Checkpoint* was only significantly enriched in males at the highest dose tested. Regarding female-specific toxicity lists, only *Renal Safety Biomarker* and *Biogenesis of mitochondria* were exclusively altered in the HD tested.

In agreement with a similar whole genome study carried out in male and female F344 rats treated for 7 or 21 days with 0.50 mg/kg bw (Pastor et al., 2018b), lists related with damage, proliferation, necrosis, inflammation, fibrosis or cell cycle contained mainly upregulated genes. On the contrary, metabolism and glutathione depletion and oxidative stress related genes were downregulated. Interestingly, some genes involved in nuclear receptor signaling lists showed a sex-dependent pattern.

4.4.1. Kidney damage

As expected for a nephrotoxin, many kidney damage response related lists were significantly affected in both sexes with a great deal of commonly deregulated genes. However, males tended to show a dose-dependent increase in the response [in terms of -log(*p*-value)] while in females the response tended to decrease or to be more similar between doses. In agreement with this, fibrosis related lists were almost exclusively altered in males also in a dose-dependent manner. This data match the histopathological findings obtained from the same samples (Pastor et al., 2018a) in which the tubulonephrosis observed in both sexes tended to a slight dose-dependent decrease in females. Moreover, the higher glomerulophritis observed at phenotypic (Pastor et al., 2018a) and gene expression level (Pastor et al., 2018b) in male rats compared to females after 7 and 21 days of OTA treatment (0.50 mg/kg bw), was similar between sexes at the dose of 0.21 mg/kg bw for 21 days, although to a lower extent than the one observed at 7 days in males. This might correlate with the fact that lists such as *Primary Glomerulonephritis Panel Biomarker Panel* and *Increases Glomerular injury* were affected in both sexes. However, the higher glomerulonephritis observed at 7 days in males might correlate with *Reversible Glomerulophritis Biomarker Panel* toxicity list that only appeared as significantly deregulated in this group (Pastor et al., 2018b) and not in any of the groups after 21 days.

In general, damage related lists included some important kidney damage biomarkers (check Table 2, supplementary data for specific information of each gene) that have been also modified in others OTA *in vivo* studies (Qi et al., 2014; Zhu et al., 2016). OTA increased the expression of *Kim-1* (kidney injury molecule-1 or hepatitis A virus cellular receptor 1, Havcr1), *Clu* (clusterine), *Lcn2* (lipocalin 2) and *Spp1* (secreted phosphoprotein 1 or osteopontin, Opn) in both sexes. These biomarkers, except *Lcn2*, were modified in all treated groups and showed a dose-dependent increase in males while in females the response tended to diminish slightly with dose. In general, modification levels of these genes tended to be similar in both sexes, but *Spp1* expression was considerably higher in females than in males at both doses. Finally, two lists related with damage that were only significantly altered in females at HD were *Renal Safety Biomarker Panel* and *Biogenesis of mitochondria*. The main sex-differences were due to the fact that the transmembrane protein, *Letm1*, and trefoil factor 3 (*Tff3*) were downregulated, while the Pparg-coactivator (*Ppargc1a*) and the beta-2-microglobulin (*B2m*) were induced only in females.

This, together with the fact that in a previous study fewer altered toxicity lists appeared in males after 7 days of OTA exposure (Pastor et al., 2018b), could indicate that response to chronic damage in males appears later but might come to be more stable over time or increases with dose.

4.4.2. Oxidative stress

It is known that OTA induces oxidative stress but, its specific role in the OTA carcinogenesis process is unclear. Our results showed the modification of four lists related with this effect in both sexes. Unlike, the strong time-effect previously observed (Pastor et al., 2018b) where females seemed to respond earlier and stronger to oxidative stress than males, the influence of dose seems to be less critical. The *Anti-oxidative and Pro-oxidative Response* panels were indeed, very similarly affected in both sexes although the latter seemed to have a stronger dose-dependent increase in males. On the other hand, the *Oxidative Stress* list appeared as strongly deregulated in males, with some molecules exclusively altered in males at the HD (*Cyp2e1, Txnrd2* and *Prdx1* were downregulated while *Fos* was upregulated) or in females (*Sod3, Gpx4* and *Nqo1* were downregulated). As also observed previously (Pastor et al., 2018a), although showing similar -log(*p*-value) in all groups, the most significant sex-dependent differences could be observed in the *Nrf2-mediated Oxidative Stress Response* list as some genes were exclusively modified in one sex (check Table 2 supplementay data for details). Briefly, OTA affected in both sexes: aldo-keto reductases (*Akr7a2, Akr7a3*), glutamate-cysteine ligases (*Gclc*, *Gclm*), several glutathione S-transferases, both citosolic (*Gsta1, Gstk1, Gstm7, Gsto1, Gsto2, Gstp1, Gstt2*), and microsomal (*Mgst1*), flavin containing monooxygenases (*Fmo1, Fmo3, Fmo4, Fmo5*), Cyp450 isoforms and some DnaJ heat shock proteins (*Dnaja3, Dnajb12, Dnajb2*). Interestingly, some isoforms showed a sex-dependent modification. That was the case of *Sod1* (superoxide dismutase) which was downregulated in both sexes but, *Sod3* was only decreased in females. More evident was the case of Nqo1 and Nqo2 (NAD(P)H-quinone oxidoreductase), two cytosolic flavoenzymes that catalyse the two-electron reduction of quinones to hydroquinones (Vasiliou et al., 2006). *Nqo1* showed a female-dependent downregulation while *Nqo2* expression only decreased in males. As OTA has been proposed to undergo an oxidative activation to a quinone/hydroquinone which in turn might cause DNA damage (Tozlovanu et al., 2012), the role of the Nqo isoforms might be interesting to further explore. In addition, some genes encoding for enzymes with redox activity were only downregulated in females (*Gpx4*) and others in males (*Prdx1, Txnrd2* and *Txn1*). Finally, it is important to remark that hypoxia inducible factor 3 alpha (*Hif3α*) was only

downregulated in males. Hif3α leads the induction of genes with cytoprotective effects to combat the deleterious effects of hypoxia. In addition, Hif3α is a key enzyme of angiogenesis, essential in the growth and metastasis of solid tumors (Li et al., 2013). Thus, our results pointed out to an important downregulation of antioxidative defenses, in agreement with previous studies (Pastor et al., 2018b; Kamp et al., 2005; Luhe et al., 2003; Marin-Kuan et al., 2006; Vettorazzi et al., 2013). As proposed by Limonciel and Jennings (2014), the Nfr2 pathway might play an important role in OTA response and, according to our results, might also have a role in the sex-specific response of the mycotoxin.

4.4.3. Nuclear receptor signaling

The most important sex differences observed were at nuclear receptor signaling pathways level. These pathways might indeed be involved in regulation of genes related to metabolism, cell death and proliferation.

Most of the lists included in this category were modified in both sexes (Table 4), but some of them were only significantly modified in males (*RAR Activation*, *VDR/RXR Activation* and *CAR/RXR Activation*). The analysis of genes from lists commonly altered in both sexes showed clear sex-dependent differences (for details, check Table 2, supplementary data). For example, *LXR/RXR Activation* list contained mainly genes solely upregulated in males (*Mylip, Abca1, Scd, Cd14, Lcat*) while downregulation of some sex-biased genes was observed in females (*Apob, Fdft1, Lpl, Nr1h3, Serpinf1, Il33, Tf*). Indeed, *Nr1h3*, which is indeed the nuclear receptor LXR (liver X receptor), was significantly inhibited in females. Also, *Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα* showed a clear influence of dose in males: upregulation was the main effect observed at LD while equal number of genes in both directions was observed at HD, as observed in females at both doses. Similary, *TX/RXR Activation* showed equal number of up- and downregulated genes at LD male group, and a clear upregulation in the rest of groups. Other lists, such as *Aryl Hydrocarbon Receptor Signaling, LPS/IL-mediated Inhibition of RXR Function* or *FXR/RXR Activation* showed a clear downregulation of the containing genes in both sexes and doses while opposite effect was observed in *RAR Activation* and *VDR/RXR Activation*. Finally, equal number of genes in both senses was observed in *PXR/RXR Activation* list in both sexes. The sense of these genes is in agreement with the general pattern previously described: genes involved in metabolic process (phase I, II and transporters) were downregulated while those that were related with damage, cell cycle or proliferation were upregulated. Overall, a great deal of genes involved in metabolic processes and nuclear receptor signaling were exclusively altered in one sex (Table 5).

Moreover, it is important to remark that some nuclear receptors were directly modified by OTA treatment (Table 2, supplementary data and Table 5), and this may explain the sex differences observed in the regulation of several genes. Ahr was downregulated in both sexes, although in males this decreased was dose-dependent. *Hnf4α* was downregulated in males at high dose and in females at low dose. And finally, *Nr1h3* (LXR), *Nr1i2* (PXR) and *Hnf1α* expression was only decreased in females. In agreement with our results, other authors have demonstrated a deregulation of these genes by OTA (Doricakova and Vrzal, 2015; Ayed-Boussema et al., 2012; Marin-Kuan et al., 2006). Although few studies have been focused in determining the role of nuclear receptors in OTA carcinogenesis, it seems that they might have an important impact in carcinogenesis process taking into account the wide variety functions that are under their control. Moreover, the early response of the signaling pathways of these nuclear receptors might also be behind the different sex-dependent responses to toxic insults.

4.4.4. Metabolism

The role of metabolism in OTA toxicity has been evaluated in several studies (Pfohl-Leszkowicz and Manderville, 2012; Ringot et al., 2006) and sex-dependent differences have been observed previously at gene expression level (Pastor et al., 2018b). Again, many of the sex differences were found in phase I, II and III metabolic processes (Table 5). An important OTA dose-dependent effect was observed in *Xenobiotic Metabolism* list. This list was strongly affected in both sexes but, the general response was higher in females. Moreover, this list is directly related with *Cytochrome P450 Panel - Substrate is a Xenobiotic* which appeared as deregulated only in males, and contains the strongly male-biased downregulated *Cyp2c11, Cyp2d1, Cyp2d5* (Table 2) and *Cyp2e1* and upregulated *Cyp3a9*. On the other hand, *Glutathione Depletion* related lists were similarly modified in both sexes.

4.4.5. Cell death, apoptosis and proliferation functions

Regarding cell death, apoptosis and proliferation functions, despite no specific apoptosis related lists were altered after 21 days of OTA exposure; few genes well-described to be involved in this function were modified after OTA treatment. That was the case of three genes of Bcl2 family (*Bak1, Bax* and *Bok*) that were upregulated after OTA treatment (check Table 2 supplementary data for details). All of them have pro-apoptotic activity. However, they showed sex-dependent modulation. *Bax* was upregulated in both sexes in a dose-dependent manner. *Bak1* increase was also dose-dependent in males, being only modified at high dose while similar levels were observed in both doses in females. Finally, *Bok* was only altered in females. So, it seems that pro-apoptosis activity (related with these three genes) is dose-dependent in males while a higher response was observed in females independently of the dose. On the other hand, important cell proliferation related genes were modified after OTA treatment. This might be a key aspect as one of the mechanisms proposed for OTA carcinogenicity is the generation of tumors secondary to chronic renal toxicity and compensatory cell proliferation (WHO, 2008). In our study, the protooncogenes *Jun* or *Myc*, were upregulated in both sexes in a dose-dependent manner. On the contrary, *Fos*, another important protooncogene, was only upregulated in males at high dose. And finally, a dose-dependent increase of *Junb*, in females, and *Jund*, in males, was observed.

Other important molecule involved in several proliferation-related lists and that was only significantly downregulated in males was estrogen nuclear receptor 1 (*Esr1*). Esr1 is a nuclear receptor known to have antiproliferative effects by activating pro-apoptotic signaling through the p38/Mapk pathway (Caiazza et al., 2015). This might also be related to the fact, that *Cell Cycle: G1/S Checkpoint Regulation* list was only significantly altered in males at HD. Even several molecules were regulated equally in both sexes, the proliferation associated protein (*Pa2g4*, also known as *Ebp1*), the cyclin kinase 6 (*Cdk6*) and transforming growth factor beta 1 (*Tgfb1*) were significantly upregulated and S-phase kinase associated protein 1 (*Skp1*) was downregulated in males and not in females., Moreover, molecules included in *Cell cycle: G2/M DNA Damage Checkpoint Regulation* list such as the Tp53 arrest mediator, Rprm; the checkpoint kinases, Weei and the activation protein, *Ywhae*, were only upregulated at HD in males while the cell cycle kinases, *Bora, Cdk1* and *Cks1b/2* were only upregulated in females, As OTA has been described to induce karyomegalia in both sexes (Pastor et al., 2018a, 2018b), a process indicative of cell division disruption, all these results might indicate a different cell cycle progression that might explain the different sensitivity to OTA tumorigenesis of male rats. Even if the role of the Mapk and ochratoxin have reported by several authors (Dai et al., 2002; Marin-Kuan et al., 2008; Stemmer et al., 2007) and that they are known to influence cell cycle (Meloche and Pouysségur, 2007), it appears clear that exploring the different sex-dependent response might shed some light to OTA mechanism of action.

4.5. DNA methylation

OTA effect has been related with several epigenetic modifications (for a review check Zhu et al., 2017) such as DNA methylation (Li et al., 2015; Ozden et al., 2015) or miRNA (Dai et al., 2014) that could be linked to carcinogenic processes. Although OTA did not cause changes

Table 5
Sex-specific genes altered in male and female F344 rats treated with a daily oral of OTA for 7 days (0.50 mg/kg) (data from Pastor et al., 2018b) or 21 days (0.21 or 0.50 mg/kg bw) included in significantly altered toxicity lists (-log(*p*-value) ≥ 1.3). Only genes modified exclusively in one sex are presented (in bold, genes common to both doses). For details, check Table 2 supplementary data.

Sex	Modulation	Genes		
Time (days)		7	7 & 21	21
Phase I				
M	*Up*	Aldh3a2, Aldh8a1, Hsd17b2, Dhdh, Ephx1, Gstm4	**Nqo2**	Cyp2c23, Cyp2j3, Cyp3a9, Mgmt
	Down		**Cyp2c11**, **Cyp2d1**	**Aldh1a1**, Aldh1b1, Aldh7a1, **Cycs**, **Cyp2d5**, Cyp2e1, Cyp4f6, Pdrx1, **Txnrd2**, Txn1
F	*Up*			**Glrx3**, **Lox**
	Down		**Sod3**	**Adh1**, Adh6, **Adhfe1**, **Akr1c1**, Akr1c2, Aldh4a1, **Cyp1a1**, **Cyp4b1**, **Abhd6**, Nqo1, Gpx4
Phase II				
M	*Up*			Acsl5, Scd
	Down			Sult1b1, Acad11, Acad11, Acadm, Acadsb, Hadhb, Ivd
F	*Up*			Chst14, **Sult1a1**, Acsl3
	Down			**Acaa2**
Transporters				
M	*Up*	Slc16a2, Slc40a1, Slc51a, Slc5a2		**Abca1**, Abcc5, Tap1, Slc20a1,
	Down	Ucp1		Abcc8, **Abcg2**, Slc25a5, **Slc27a2**, **Slc51b**, **Slc6a4**, Slco1a1, **Gc**
F	*Up*			**Abcg4**
	Down			**Abcc3**, Slc7a9
Nuclear Receptors				
M	*Up*			Nfic, Nfkbia, Vdr
	Down			**Esr1**, Hif3α
F	*Up*			Ncoa3, Ncoa6, Ppargc1a, **Pprc1**
	Down			**Cnr1**, **Hnf1α**, **Nfatc4**, **Nr1h3**, **Nr1i2**, **Nr3c2**, Oxtr
Growth factors				
M	*Up*	Igfbp3, Igfbp5, Tgfb1i1		Egr1, Hbegf, Pgf, Tgfb1,
	Down			Fgfr3
F	*Up*			**Frs2**, **Hgf**, **Pdgfra**, Pdgfra
	Down			Fgfr4, **Igf1**

F: females; M: males.

in genome-wide methylation, it produced DNA methylation alterations in genes involved in phosphate metabolic process, protein kinase activity and mTOR cell signaling after 90 days of treatment in F344 rats (Ozden et al., 2015). Similarly, 13-weeks study showed that, by *E-cadherin* and *N-cadherin* methylation silencing, OTA disrupts Wnt and Pi3K/Akt pathways which are linked to tumorigenesis (Li et al., 2015). Thus, taking into account the impact of OTA in the expression of several phase I, phase II and transporters observed in the present study, we hypothesized that this modulation could be due to changes in the methylation profile. The selection of *Cyp2c11* (Males), *Akr1b7*, *Cyp1a1* (Females) was based on the fact that were only downregulated in one sex but not in the other, while *Gstp1* and *Slc22a7*, were downregulated in both sexes but showed slight different time-dependent responses. Moreover, *Akr1b7, Cyp2c11* and *Slc22a7* belonged to the most downregulated genes after OTA treatment and already showed important sex differences at basal level (Males > Females: *Cyp2c11*; Males < Females: *Akr1b7, Slc22a7*) (Pastor et al., 2018b). Moreover, Akr1b7 is a NAD(P)H-linked oxidoreductase that plays an important role in the detoxification of aldehydes and ketones from endogenous and exogenous compounds and in the detoxification of lipid peroxidation (Liu et al., 2009). Cyp1a1 and Cyp2c11 have been related to OTA metabolism (Ayed-Boussema et al., 2012). Gstp1 may be involved in the conjugation of the OTA radical with glutathione, contributing in its toxification/detoxification (Limonciel and Jennings, 2014). On the other hand, Slc22a7 is an apical renal transporter involved in OTA excretion/reabsorption that has been hypothesized to contribute to the high kidney damage produced by OTA in male F344 rats (Pastor et al., 2016). Finally, these genes were also selected as they have been described to be modulated by nuclear receptor regulation that might play important role in OTA mode of action. Nrf2 might regulate *Akr1b7* and *Gstp1* (Limonciel and Jennings, 2014), Hnf4α controls *Cyp2c11* (Velenosi et al., 2014) and *Slc22a7* expression (Hagos et al., 2014) and *Cyp1a1* is under control of Ahr (Ayed-Boussema et al., 2012). However, our results indicate that promoter DNA modifications did not explain gene expression differences observed at basal level or after OTA treatment in our dataset.

5. Conclusion

Overall, our results showed that OTA-induced response was dose-dependent in males and females, although the effect of dose seems to be stronger in males. Thus, taking into account the present study and a previous study in which the time effect was analyzed (Pastor et al., 2018b), it seems that males have a different early response (in terms of both, time and dose), while females' response seems to be more consistent over time and doses. In addition, although when both sexes reached similar response (in terms of toxicity lists), each sex showed exclusively altered genes, mainly of phase I, II and transporters, but also others related to cell proliferation/apoptosis. This sex-specific response might be mediated by nuclear receptors and might in turn affect both the oxidative stress and proliferative sex-biased response towards OTA insult. Further studies should be done in this issue to understand if they

could contribute to the different progression to carcinogenesis.

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Appendix A. Supplementary data

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Transparency document

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