

# Accepted Manuscript

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

Ariane Vettorazzi, Laura Pastor, Elizabeth Guruceaga, Adela López de Cerain

PII: S0278-6915(18)30796-8

DOI: <https://doi.org/10.1016/j.fct.2018.10.057>

Reference: FCT 10162

To appear in: *Food and Chemical Toxicology*

Received Date: 9 September 2018

Revised Date: 19 October 2018

Accepted Date: 24 October 2018

Please cite this article as: Vettorazzi, A., Pastor, L., Guruceaga, E., López de Cerain, A., Sex-dependent gene expression after ochratoxin A insult in F344 rats kidney, *Food and Chemical Toxicology* (2018), doi: <https://doi.org/10.1016/j.fct.2018.10.057>.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



## Title Page

**Sex-dependent gene expression after ochratoxin A insult  
in F344 rats kidney****Vettorazzi, Ariane<sup>\*1,2, #</sup>, Pastor Laura<sup>\*1</sup>, Guruceaga Elizabeth<sup>2,3</sup>, López de Cerain Adela<sup>1,2</sup>**

*\* both authors contributed equally to the study*

<sup>1</sup>University of Navarra, Department of Pharmacology and Toxicology, Faculty of Pharmacy and Nutrition, E-31008, Pamplona, Spain. E-mails: Laura Pastor: lpastor@alumni.unav.es. Ariane Vettorazzi: avettora@unav.es. Adela López de Cerain: acerain@unav.es

<sup>2</sup>IdiSNA, Navarra Institute for Health Research, E-31008, Pamplona, Spain

<sup>3</sup>Bioinformatics Platform, Center for Applied Medical Research (CIMA), University of Navarra, E-31008, Pamplona, Spain. E-mail: eguruce@unav.es

# Corresponding author: Ariane Vettorazzi. University of Navarra, Department of Pharmacology and Toxicology, Faculty of Pharmacy and Nutrition, CIFA Building, c/Irunlarrea 1, 31008, Pamplona, Spain. E-mail: avettora@unav.es. Phone: +34 948425600 ext 803324

**Highlights**

1. Whole genome gene expression and DNA methylation in kidneys of F344 rats treated with ochratoxin A for 21 days was studied.
2. Ochratoxin-induced gene expression response was dose-dependent in males and females, although clearer in males.
3. OTA modulated damage, signalling, metabolism inflammation, proliferation and oxidative stress related lists in both sexes.

4. 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

5. Main sex differences were in genes related with metabolism (phase I and II), transport, nuclear receptors and cell proliferation/apoptosis.

ACCEPTED MANUSCRIPT

**Abstract and keywords (200 words)**

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.

*Keywords: Ochratoxin A (OTA), sex differences, gene expression, metabolism, nuclear receptors, methylation*

## 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 (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 (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-Leskowicz *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 *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*, *Slc51b* 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 (Hnf4 $\alpha$ ) (Pastor *et al.*, 2018b; Marin-Kuan *et al.*, 2006) and nuclear factor-erythroid 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 (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  $\text{NaHCO}_3$  (0.1 M pH 7.4) (Sigma-Aldrich; Steinheim, Germany). At beginning of the study, vehicle ( $\text{NaHCO}_3$  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  $-20^\circ\text{C}$ . Each aliquot was thawed daily before animal administration. Animals were gavaged with 1mL/100g 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 ( $\text{NaHCO}_3$ ) (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<sup>®</sup> manufacturer's protocol (Invitrogen<sup>™</sup>, USA). For that purpose, samples were homogenized in TRIzol<sup>®</sup> (50 mg/mL) with a T25 Ultra-turrax Digital High Speed Homogenizer (IKA<sup>®</sup>, 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<sup>™</sup> 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<sup>™</sup> STDSens RNA Chips (Bio-Rad). All the samples showed A260/A280 ratio between 1.8-2.0 and with an average RNA quality indicator (RQI) value of  $8.7 \pm 0.7$  (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<sup>®</sup> WT Expression Kit. The sense strand cDNA was then fragmented and biotinylated with the Affymetrix GeneChip<sup>®</sup> WT Terminal Labeling Kit (PN 900671) (Affymetrix, Inc., Santa Clara, USA). Labeled sense cDNA was hybridized to GeneChip<sup>®</sup> Rat Gene 2.0 ST Array (Affymetrix, Inc., Santa Clara, USA) according to the manufacturer protocols and using GeneChip<sup>®</sup> Hybridization, Wash and Stain Kit (Affymetrix, Inc., Santa Clara, USA). Genechips were scanned with the Affymetrix GeneChip<sup>®</sup> 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, <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  $-\log(p\text{-value}) \geq 1.30$  ( $p\text{-value} \leq 0.05$ ) were considered as significantly modulated.

## 2.7 DNA isolation

For DNA methylation analysis, samples from 7 days control and treated animals of the previous transcriptomic 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-transferase 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), 1x 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<sub>2</sub>O 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, 0.64x of T7 R&DNA polymerase buffer, 0.22 µL T Cleavage Mix, 3.14 mM DTT, 3.21 µL H<sub>2</sub>O, and 0.09 mg/mL Rnase A. The reactions were additionally diluted with 20 mL of H<sub>2</sub>O 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

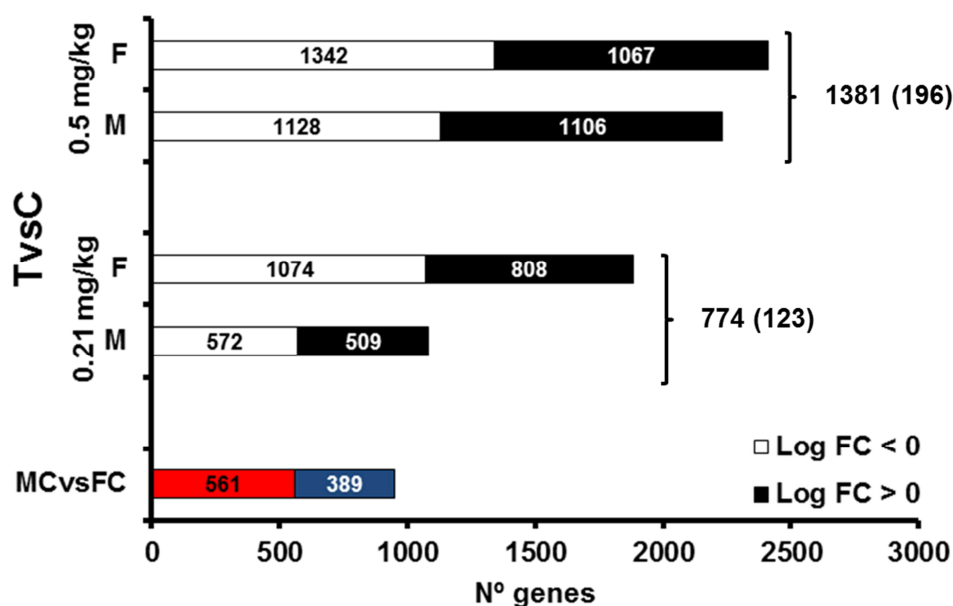
DNA methylation results are reported as mean  $\pm$  SD. Statistical comparisons between groups were performed by Mann-Whitney *U* test and differences were considered significant when *p*-value < 0.05. Moreover, 10% of variation in DNA methylation percentage was considered as a potential change in mRNA 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 figure 1 (supplementary 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 (Figure 1)



**Figure 1** Differentially expressed genes (DEG) in each contrast after 21 days of treatment with control vehicle ( $\text{NaHCO}_3$ ) 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.

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 figure 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.

**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

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

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

Finally, by comparing sexes at each dose, 774 genes were common to both sexes at the LD and 1381 at HD group (Figure 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.



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

### 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  $-\log(p\text{-value}) \geq 1.3$  which corresponds to a  $p\text{-value} \leq 0.05$ . The search focused on rat-specific genes but the available information of all species was considered for determining the relation among them. 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)

**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  $-\log(p\text{-value}) \geq 1.3$  ( $p\text{-value} \leq 0.05$ ). 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.

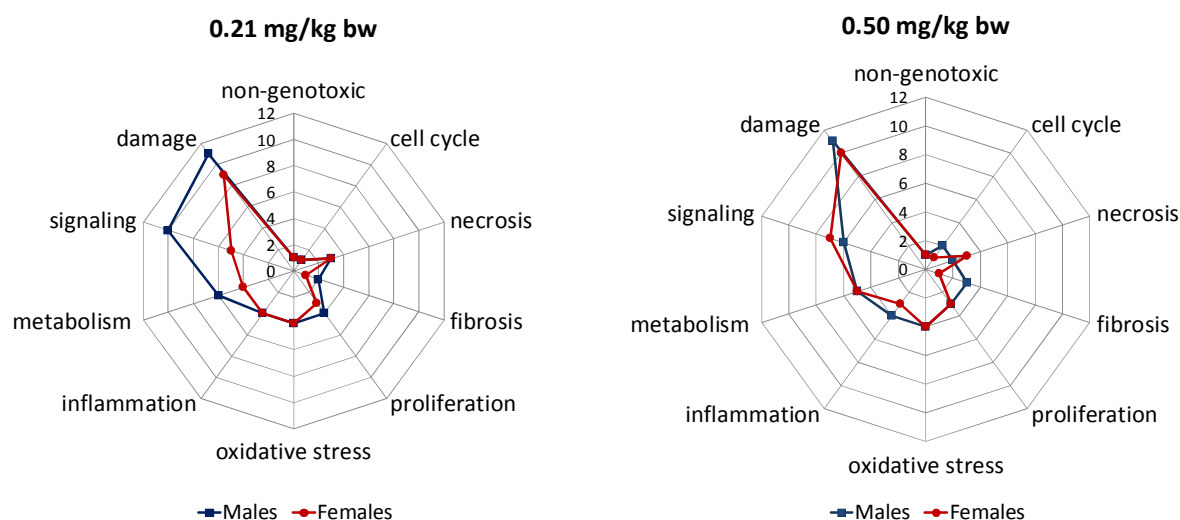
Toxicity list	Dose (mg/kg)	-log(p-value)			
		Males		Females	
Name of list (category <sup>1</sup> )	Nº lists	0.21	0.50	0.21	0.50
<b>Acute Renal Failure Panel (D)</b>		13.30	17.30	14.50	11.7
<b>Renal Proximal Tubule Toxicity Biomarker Panel (D)</b>		11.10	12.80	7.93	8.60
<b>Genes Downregulated in Response to Chronic Renal Failure (D)</b>		3.00	5.06	5.58	4.85
<b>Persistent Renal Ischemia-Reperfusion Injury (D)</b>		2.47	3.92	3.84	2.97
<b>Increases Renal Damage (D)</b>		3.19	3.80	1.66	1.49
<b>Increases Glomerular Injury (D)</b>		2.17	3.35	3.00	2.19
<b>Recovery from Ischemic Acute Renal Failure (D)</b>		2.20	2.48	4.83	3.12
<b>Genes associated with Chronic Allograft Nephropathy (D)</b>		1.79	2.53	1.58	
<b>Renal Glomerulus Panel (D)</b>		1.31			
<b>Renal Safety Biomarker Panel (PSTC) (D)</b>					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
<b>Primary Glomerulonephritis Biomarker Panel (I)</b>		1.80	3.45	1.92	2.34
Positive Acute Phase Response Proteins (I)		2.56	2.71	3.26	2.49
<b>Irreversible Glomerulonephritis Biomarker Panel (I)</b>		1.48	1.90	1.49	1.77
<b>Increases Renal Nephritis (I)</b>			1.43		
Increases Liver Hepatitis (I)		1.51		1.45	
<b>Increases Renal Proliferation (P)</b>		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
<b>Long-term Renal Injury Anti-oxidative Response Panel (O)</b>		1.98	4.67	2.52	5.42
Oxidative Stress (O)		3.25	3.71	1.96	2.85
<b>Long-term Renal Injury Pro-oxidative Response Panel (O)</b>		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 PPAR $\alpha$ (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		
CAR/RXR Activation (S)	2.18	1.44		
<b>Renal Necrosis/Cell Death (N)</b>	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

<sup>1</sup>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).

Despite the number of DEG (Figure 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) (in bold, table 4). 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). The distribution of categories of toxicity lists induced by OTA treatment in males and females is presented in figure 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.



**Figure 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.

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 analysed. 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 PPAR $\alpha$*  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 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 and 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 NAD<sup>+</sup>-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 *et al.*, 2014 and 2015). On the other hand, Arafa and

collaborators (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), 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*, *S100g*) and transport (*Slc51b*). From these male-biased DEG, only *Cyp2c11*, *Cyp2d1* and *S100g* 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<sup>2+</sup> 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.* 2018a). 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 (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 (figure 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 list 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 figure 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 (4 lists) 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  $-\log(p\text{-value})$ ] 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 glomerulonephritis 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 Glomerulonephritis 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  $-\log(p\text{-value})$  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 supplementary data for details). Briefly, OTA affected in both sexes: aldo-keto reductases (*Akr7a2*, *Akr7a3*), glutamate-cysteine ligases (*Gclc*, *Gclm*), several glutathione S-transferases, both cytosolic (*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.*, 2006), the role of the Noq 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 (*Hif3 $\alpha$* ) was only downregulated in males. *Hif3 $\alpha$*  leads the induction of genes with cytoprotective effects to combat the deleterious effects of hypoxia. In addition, *Hif3 $\alpha$*  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*, *I133*, *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 PPAR $\alpha$*  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. Similarly, *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).

**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 ( $-\log(p\text{-value}) \geq 1.3$ ). 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 & 21	21
<b>Phase I</b>				
M	Up	Aldh3a2, Aldh8a1, Hsd17b2, Dhdh, Ephx1, Gstm4	<b>Nqo2</b>	Cyp2c23, Cyp2j3, Cyp3a9, Mgmt
	Down		<b>Cyp2c11, Cyp2d1</b>	<b>Aldh1a1</b> , Aldh1b1, Aldh7a1, <b>Cyca</b> , <b>Cyp2d5</b> , Cyp2e1, Cyp4f6, Pdrx1, <b>Txnrd2</b> , Txn1
F	Up			<b>Glrx3, Lox</b>
	Down		<b>Sod3</b>	<b>Adh1</b> , Adh6, <b>Adhfe1</b> , <b>Akr1c1</b> , Akr1c2, Aldh4a1, <b>Cyp1a1</b> , <b>Cyp4b1</b> , <b>Abhd6</b> , Nqo1, Gpx4
<b>Phase II</b>				
M	Up			Acs15, Scd
	Down			Sult1b1, Acad11, Acad11, Acadm, Acadsb, Hadhb, Ivd
F	Up			Chst14, <b>Sult1a1</b> , Acs13
	Down			<b>Acaa2</b>
<b>Transporters</b>				
M	Up	Slc16a2, Slc40a1, Slc51a, Slc5a2		<b>Abca1</b> , Abcc5, Tap1, Slc20a1,
	Down	Ucp1		Abcc8, <b>Abcg2</b> , Slc25a5, <b>Slc27a2</b> , <b>Slc51b</b> , <b>Slc6a4</b> , Slc01a1, <b>Gc</b>
F	Up			<b>Abcg4</b>
	Down			<b>Abcc3</b> , Slc7a9
<b>Nuclear Receptors</b>				
M	Up			Nfic, Nfkb1a, Vdr
	Down			<b>Esr1</b> , Hif3 $\alpha$
F	Up			Ncoa3, Ncoa6, Ppargc1a, <b>Pprc1</b>
	Down			<b>Cnr1</b> , <b>Hnf1<math>\alpha</math></b> , <b>Nfatc4</b> , <b>Nr1h3</b> , <b>Nr1i2</b> , <b>Nr3c2</b> , Oxt
<b>Growth factors</b>				
M	Up	Igfbp3, Igfbp5, Tgfb1i1		Egr1, Hbegf, Pgf, Tgfb1,
	Down			Fgfr3
F	Up			<b>Frs2</b> , <b>Hgf</b> , <b>Pdgfra</b> , Pdgfra
	Down			Fgfr4, <b>Igf1</b>

F: females; M: males

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. *Hnf4 $\alpha$*  was downregulated in males at high dose and in females at low dose. And finally, *Nr1h3* (LXR), *Nr1i2* (PXR) and *Hnf1 $\alpha$*  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* (tables 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 non-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 tumours 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 and 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.*, 2014; Marin-Kuan *et al.*, 2008; Stemmer *et al.*, 2007) and that they are known to influence cell cycle (Meloche and Pouyssegur, 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 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), *Hnf4 $\alpha$*  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.

## 4.6 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 analysed (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.

## Acknowledgements

The authors thank Fermin Milagro from the University of Navarra and Enrique Buso from the University of Valencia for their kind help in the methylation studies.

## Funding

Funding Information: This work was supported by the University of Navarra through the PIUNA Project "Efecto cancerígeno de la ocratoxina A: influencia del sexo en el mecanismo de acción" [PIUNA 2012]. L.P thanks the "Asociación de Amigos" (University of Navarra) for the predoctoral grant received.

## References

- AbuAli, G., Chaisaklert, W., Stelloo, E., Pazarentzos, E., Hwang, M. S., Qize, D., Harding, S. V., Al-Rubaish, A., Alzahrani, A. J., Al-Alj, A., Sanders, T. A., Aboagye, E. O., Grimm, S. (2015). The anticancer gene ORCTL3 targets stearyl-CoA desaturase-1 for tumour-specific apoptosis. *Oncogene*. **34**: 1718-1728. doi: 10.1038/onc.2014.93.
- AbuAli, G., Grimm, S. (2014). Isolation and characterization of the anticancer gene organic cation transporter like-3 (ORCTL3). *Adv. Exp. Med. Biol.* **818**: 213-227. doi: 10.1007/978-1-4471-6458-6\_11.
- Arafa, E., Bondzie, P. A., Rezazadeh, K., Meyer, R. D., Hartsough, E., Henderson, J. M., Schwartz, J. H., Chitalia, V., Rahimi, N. (2015). TMIGD1 is a novel adhesion molecule that protects epithelial cells from oxidative cell injury. *Am. J. Pathol.* **185**: 2757-2767. doi: 10.1016/j.ajpath.2015.06.006.
- Arbillaga, L., Vettorazzi, A., Gil, A. G., van Delft, J. H., Garcia-Jalon, J. A., Lopez de Cerain, A. (2008). Gene expression changes induced by ochratoxin A in renal and hepatic tissues of male F344 rat after oral repeated administration. *Toxicol. Appl. Pharmacol.* **230**: 197-207. doi: 10.1016/j.taap.2008.02.018.
- Ayed-Boussema, I., Pascussi, J. M., Zaied, C., Maurel, P., Bacha, H., Hassen, W. (2012). Ochratoxin A induces CYP3A4, 2B6, 3A5, 2C9, 1A1, and CYP1A2 gene expression in primary cultured human hepatocytes: a possible activation of nuclear receptors. *Drug Chem. Toxicol.* **35**: 71-80. doi: 10.3109/01480545.2011.589438.
- Caiazza, F., Ryan, E. J., Doherty, G., Winter, D. C., Sheahan, K. (2006). Estrogen receptors and their implications in colorectal carcinogenesis. *Front. Oncol.* **5**: 1-19. doi: 10.3389/fonc.2015.00019.
- Castegnaro, M., Mohr, U., Pfohl-Leszkowicz, A., Estève, J., Steinmann, J., Tillmann, T., Michelon, J., Bartsch, H. (1998). Sex- and strain-specific induction of renal tumors by ochratoxin A in rats

correlates with DNA adduction. *Int. J. Cancer.* **77**: 70-75. doi: 10.1002/(SICI)1097-0215(19980703)77:1<70::AID-IJC12>3.0.CO;2-D.

Corcuera, L. A., Vettorazzi, A., Arbillaga, L., Pérez, N., Gil, A. G., Azqueta, A., González-Peñas, E., García-Jalón, J. A., López de Ceráin, A. (2015). Genotoxicity of aflatoxin B1 and ochratoxin A after simultaneous application of the *in vivo* micronucleus and comet assay. *Food Chem. Toxicol.* **76**: 116-124. doi: 10.1016/j.fct.2014.12.003.

Dai, Q., Zhao, J., Qi, X., Xu, W., He, X., Guo, M., Dweep, H., Cheng, W.H., Luo, Y., Xia, K., Gretz, N., Huang, K. (2014). MicroRNA profiling of rats with ochratoxin A nephrotoxicity. *BMC Genomics* **15**: 333. doi: 10.1186/1471-2164-15-333.

Dai, J., Park, G., Wright, M., Adams, M., Akman, S., Manderville, R., (2002). Detection and characterization of a glutathione conjugate of ochratoxin A. *Chem. Res. Toxicol.* **15**, 1581–1588.

Ding, J., Dirks, W. G., Ehrentraut, S., Geffers, R., MacLeod, R. A., Nagel, S., Pommerenke, C., Romani, J., Scherr, M., Vaas, L. A., Zaborski, M., Drexler, H. G., Quentmeier, H. (2015). BCL6-regulated by AhR/ARNT and wild-type MED2B-drives expression of germinal center markers MYBL1 and LMO2. *Haematologica.* **100**: 801-809. doi: 10.3324/haematol.2014.120048.

Doricakova, A., Vrzal, R. (2015). A food contaminant ochratoxin A suppresses pregnane X receptor (PXR)-mediated CYP3A4 induction in primary cultures of human hepatocytes. *Toxicology.* **337**: 72-78. doi: 10.1016/j.tox.2015.08.012.

Enciso, J.M., López de Ceráin, A., Pastor, L., Azqueta, A., Vettorazzi, A (2018). Is oxidative stress involved in the sex-dependent response to ochratoxin A renal toxicity? *Food Chem Toxicol.* **116(Pt B)**: 379-387. doi: 10.1016/j.fct.2018.04.050

European Food Safety Authority (EFSA) (2006). Contaminants in the food chain on a request from the Commission related to ochratoxin A (OTA) in food. *EFSA J.* **365**: 1-56. doi: 10.2903/j.efsa.2006.365.

Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber (ed.) (2005). Bioinformatics and computational biology solutions using R and Bioconductor. Springer, New York, NY.

Hagos, Y., Wegner, w., Kuehne, A., Floerl, S., Marada, V. V., Burckhardt, G., Henjakovic, M. (2014). HNF4 $\alpha$  induced chemosensitivity to oxaliplatin and 5-FU mediated by OCT1 and CNT3 in renal cell carcinoma. *J. Phar. Sci.* **103**: 3326-3334. doi: 10.1002/jps.24128.

Hayes, M. J., Rescher, U., Gerke, V., Moss, S. E. (2004). Annexin-actin interactions. *Traffic.* **5**: 571–576. doi: 10.1111/j.1600-0854.2004.00210.x.

Horváth, H. C., Lakatos, P., Kósa, J. P., Bácsi, K., Borka, K., Bises, G., Nittke, T., Hershberger, P. A., Speer, G., Kállay, E. (2010). The candidate oncogene CYP24A1: a potential biomarker for colorectal tumorigenesis. *J Histochem. Cytochem.* **58**: 277-285. doi: 10.1369/jhc.2009.954339.

Huyton, T., Göttmann, W., Bade-Döding, C., Paine, A., Blasczyk, R. (2011). The T/NK cell costimulatory molecule SECTM1 is an IFN “early response gene” that is negatively regulated by LPS in human monocytic cells. *Biochim. Biophys. Acta.* **1810**: 1294-1301. doi: 10.1016/j.bbagen.2011.06.020.

Ingenuity® Pathways Analysis (IPA) (2016). [Internet]. Available from: [www.ingenuity.com](http://www.ingenuity.com); accessed: December of 2016.

International Agency for Research in Cancer (IARC) (1993). IARC Monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monographs.* **56**: 489-521.

Irizarry, R. A., Bolstad, B. M., Collin, F., Cope L. M., Hobbs, B., Speed, T. P. (2003). Summaries of affymetrix genechip probe level data. *Nucleic Acids Res.* **31**: 15-33.

Jiang, G., Wang, P., Wang, W., Li, W., Dai, L., Chen, K. (2017). Annexin A13 promotes tumor cell invasion *in vitro* and is associated with metastasis in human colorectal cancer. *Oncotarget*. **8**: 21663-21673. doi: 10.18632/oncotarget.15523.

Kamp, H. G., Eisenbrand, G., Janzowski, C., Kiossev, J., Latendresse, J. R., Schlatter, J., Turesky, R. J. (2005). Ochratoxin A induces oxidative DNA damage in liver and kidney after oral dosing to rats. *Mol. Nutr. Food Res.* **49**: 1160-1167. doi: 10.1002/mnfr.200500124.

Kwekel, J. C., Desai, V. G., Moland, C. L., Vijay, V., Fuscoe, J. C. (2013). Sex differences in kidney gene expression during the life cycle of F344 rats. *Biol. Sex. Differ.* **4**: 1-14. doi: 10.1186/2042-6410-4-14.

Lee, H.J. and Ryu, D. (2017) Worldwide Occurrence of Mycotoxins in Cereals and Cereal-Derived Food Products: Public Health Perspectives of Their Co-occurrence. *J Agric Food Chem.* **65**(33):7034-7051. doi: 10.1021/acs.jafc.6b04847.

Li, X., Gao, J., Huang, K., Qi, X., Dai, Q., Mei, X., Xu, W. (2015). Dynamic changes of global DNA methylation and hypermethylation of cell adhesion-related genes in rat kidneys in response to Ochratoxin A. *World Mycotoxin J.* **8**: 465-476. doi: 10.3920/WMJ2014.1795.

Li, G. Y., Jung, K. H., Lee, H., Son, M. K., Seo, J., Hong, S. W., Jeong, Y., Hong, S., Hong, S. S. (2013). A novel imidazopyridine derivate, HS-106, induces apoptosis of breast cancer cells and represses angiogenesis by targeting the PI3K/mTOR pathway. *Cancer Lett.* **329**: 59-67. doi: 10.1016/j.canlet.2012.10.013.

Li, T., Han, W., Yang, T., Ding, P., Rui, M., Liu, D., Wang, Y., Ma, D. (2006). Molecular cloning and identification of mouse Cklfsf2a and Cklfsf2b, two homologues of human CKLFSF2. *Int. J. Biochem. Cell Biol.* **38**: 420-429. doi: 10.1016/j.biocel.2005.10.003.

Limonciel, A., Jennings, P. (2014). A review of the evidence that ochratoxin A is an Nrf2 inhibitor: implications for nephrotoxicity and renal carcinogenicity. *Toxins (Basel)*. **6**: 371-379. doi: 10.3390/toxins6010371.

Liu, M. J., Takahashi, Y., Wada, T., He, J., Gao, J., Tian, Y., Li, S., Xie, W. (2009). The aldo-keto reductase Akr1b7 gene is a common transcriptional target of xenobiotic receptors pregnane X receptor and constitutive androstane receptor. *Mol. Pharmacol.* **76**: 604-611. doi: 10.1124/mol.109.057455.

Luhe, A., Hildebrand, H., Bach, U., Dingermann, T., Ahr, H. J. (2003). A new approach to studying ochratoxin A (OTA)-induced nephrotoxicity: expression profiling *in vivo* and *in vitro* employing cDNA microarrays. *Toxicol. Sci.* **73**: 315-328. doi: 10.1093/toxsci/kfg073.

Mally, A. (2012). Ochratoxin A and mitotix disruption: mode of action analysis of renal tumor formation by ochratoxin A. *Toxicol. Sci.* **127**: 315-330. doi: 10.1093/toxsci/kfs105.

Marin-Kuan, M., Cavin, C., Delatour, T., Schilter, B. (2008). Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. *Toxicon.* **52**: 195-202. doi: 10.1016/j.toxicon.2008.04.166.

Marin-Kuan, M., Nestler, S., Verguet, C., Bezencon, C., Piguet, D., Mansourian, R., Holzwarth, J., Grigorov, M., Delatour, T., Mantle, P., Cavin, C., Schilter, B. (2006). A toxicogenomics approach to identify new plausible epigenetic mechanisms of ochratoxin a carcinogenicity in rat. *Toxicol. Sci.* **89**: 120-134. doi: 10.1093/toxsci/kfj017.

Meloche, S., Pouvsségur, J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1 to S phase transition. *Oncogene.* **26**: 3227-3239. doi: 10.1038/sj.onc.1210414.



Mitsui, T., Ishida, M., Izawa, M., Arita, J. (2013). Differences between rat strains in the development of PRL-secreting pituitary tumors with long-term estrogen treatment: In vitro insulin-like growth factor-1-induced lactotroph proliferation and gene expression are affected in Wistar-Kyoto rats with low estrogen-susceptibility. *Endocr. J.* **60**: 1251-1259. doi: 10.1507/endocrj.EJ13-0245.

National Toxicology Program (NTP) (2016). Report on Carcinogens, Fourteenth Edition. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service.

National Toxicology Program (NTP) (1989). Toxicology and carcinogenesis studies of ochratoxin A (CAS No. 303-47-9) in F344/N Rats (Gavage studies). *Natl. Toxicol. Program. Tech. Rep. Ser.* **358**: 1-142.

Ozden, S., Turgut Kara, N., Sezerman, O. U., Durasi, I. M., Chen, T., Demirel, G., Alpertunga, B., Chipman, J. K., Mally, A. (2015). Assessment of global and gene-specific DNA methylation in rat liver and kidney in response to non-genotoxic carcinogen exposure. *Toxicol. Appl. Pharmacol.* **289**: 203-212. doi: 10.1016/j.taap.2015.09.023.

Pastor, L., Vettorazzi, A., Enciso, J.M., González-Peñas, E., García-Jalón, J.A, Monreal, J.I, López de Cerain A (2018a). Sex differences in ochratoxin a toxicity in F344 rats after 7 and 21 days of daily oral administration. *Food Chem Toxicol.* **111**: 363-373. doi: 10.1016/j.fct.2017.11.003.

Pastor, L., Vettorazzi, A., Guruceaga, E., López de Cerain, A. (2018b). Time modulation of sex-dependent gene expression response to ochratoxin A insult in F344 rats. *Manuscript submitted to Toxicological Sciences.*

Pastor, L., Vettorazzi, A., Campión, J., Cordero, P., López de Cerain, A. (2016) Gene expression kinetics of renal transporters induced by ochratoxin A in male and female F344 rats. *Food Chem Toxicol.* **98(Pt B)**:169-178. doi:10.1016/j.fct.2016.10.019.

Pfohl-Leszkowicz, A., Manderville, R.A. (2012). An update on direct genotoxicity as a molecular mechanism of ochratoxin a carcinogenicity. *Chem Res Toxicol.* **25**: 252-262. doi: 10.1021/tx200430f. Review.

Pfohl-Leszkowicz, A., Pinelli, E., Bartsch, H., Mohr, U., Castegnaro, M. (1998). Sex- and strain-specific expression of cytochrome P450s in ochratoxin A-induced genotoxicity and carcinogenicity in rats. *Mol. Carcinog.* **23**: 76-85. doi:10.1002/(SICI)1098-2744(199810)23:2<76::AID-MC4>3.0.CO2-B.

Qi, X., Yu, T., Zhu, L., Gao, J., He, X., Huang, K., Luo, Y., Xu, W (2014). Ochratoxin A induces rat renal carcinogenicity with limited induction of oxidative stress responses. *Toxicol. Appl. Pharmacol.* **280**: 543-549. doi: 10.1016/j.taap.2014.08.030.

Rached, E., Hoffmann, D., Blumbach, K., Weber, K., Dekant, W., and Mally, A. (2008). Evaluation of putative biomarkers of nephrotoxicity after exposure to ochratoxin a in vivo and in vitro. *Toxicol. Sci.* **103**: 371-381. doi: 10.1093/toxsci/kfn040.

Ringot, D., Chango, A., Schneider, Y.J., Larondelle, Y. (2006). Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chem Biol Interact.* **159(1)**: 18-46. Review

Sharma, M. C., Sharma, M. (2007). The role of annexin II in angiogenesis and tumour progressin: a potential therapeutic target. *Curr. Pharm. Des.* **13**: 3568–3675.

Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**: Article 3. doi: 10.2202/1544-6115.1027.

Souza, T., Jennen, D., van Delft, J., van Herwijnen, M., Kyratoupolos, S., Kleinjans, J. New insights into BaP-induced toxicity: role of major metabolites in transcriptomics and contribution to hepatocarcinogenesis. *Arch. Toxicol.* **90**: 1449-1458. doi: 10.1007/s00204-015-1572-z.

Stemmer, K., Ellinger-Ziegelbauer, H., Ahr, H. J., Dietrich, D. R. (2007). Carcinogen-specific gene expression profiles in short-term treated Eker and wild-type rats indicative of pathways involved in renal tumorigenesis. *Cancer Res.* **67**: 4052-4068. doi: 10.1158/0008-5472.CAN-06-3587.

Tozlovanu, M., Canadas, D., Pfohl-Leskowicz, A., Frenette, C., Paugh, R. J., Manderville, R. A. (2012). Glutathione conjugates of ochratoxin A as biomarkers of exposure. *Arh. Hig. Rada. Toksikol.* **63**: 417-427. doi: 10.2478/10004-1254-63-2012-2202.

Trevisan, A., Chiara, F., Mongillo, M., Quintieri, L., Cristofori, P. (2012). Sex-related differences in renal toxicodynamics in rodents. *Expert Opin. Drug Metab. Toxicol.* **8**: 1173-1188. doi: 10.1517/17425255.2012.698262.

van den Boom D., Ehrich M. (2009) Mass Spectrometric Analysis of Cytosine Methylation by Base-Specific Cleavage and Primer Extension Methods. In: Tost J. (eds) DNA Methylation. Methods in Molecular Biology, vol 507. Humana Press DOI.

Vasiliou, V., Ross, D., Nebert, D. W. (2006). Update of the NAD(P)H:quinone oxidoreductase (NQO) gene family. *Hum. Genomics.* **2**: 329-335. doi: 10.1186/1479-7364-2-5-329.

Velenosi, T. J., Feere, D. A., Sohi, G., Hardy, D. B., Urguhart, B. L. (2014). Decreased nuclear receptor activity and epigenetic modulation associates with downregulation of hepatic drug-metabolizing enzymes in chronic disease. *FASEB J.* **28**: 5388-5397. doi: 10.1096/fj.14-258780.

Vettorazzi, A., van Delft, J., López de Cerain, A. (2013). A review on ochratoxin A transcriptomic studies. *Food Chem Toxicol.* **59**: 766-83. doi: 10.1016/j.fct.2013.05.043. Review.

Vettorazzi, A., de Trocóniz, I. F., González-Peñas, E., Arbillaga, L., Corcuera, L., Gil, A. G., López de Cerain, A. (2011). Kidney and liver distribution of ochratoxin A in male and female F344 rats. *Food Chem. Toxicol.* **49**: 1935-1942. doi: 10.1016/j.fct.2011.04.021.

Vettorazzi, A., Trocóniza, I. F., Gonzalez-Peñas, E., Corcuera, L. A., Arbillaga, L., Gil, A. G., Nagy, J. M., Mantle, P. G., de Cerain, A. L. (2010). Effects of fasting and gender on ochratoxin A toxicokinetics in F344 rats. *Food Chem. Toxicol.* **48**: 3159-3166. doi: 10.1016/j.fct.2010.08.012.

Vettorazzi, A., Gonzalez-Peñas, E., Trocóniz, I. F., Arbillaga, L., Corcuera, L. A., Gil, A. G., de Cerain, A. L. (2009). A different kinetic profile of ochratoxin A in mature male rats. *Food Chem. Toxicol.* **47**: 1921-1927. doi: 10.1016/j.fct.2009.05.003.

Wang, T., Ge, Y., Xiao, M., Lopez-Coral, A., Li, L., Roesch, A., Huang, C., Alexander, P., Vogt, T., Xu, X., Hwang, W. T., Lieu, M., Belser, E., Liu, R., Somasundaram, R., Herlyn, M., Kaufman, R. E. (2014). SECTM1 produced by tumor cells attracts human monocytes via CD7-mediated activation of the PI3K pathway. *J. Invest. Dermatol.* **134**: 1108-1118. doi: 10.1038/jid.2013.437.

WHO (World Health Organisation), 2008. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 59

World Health Organisation (WHO) (2008). Safety evaluation of certain food additives and contaminants. *WHO Food Additives Series.* **59**: 357-429.

Zhu, L., Zhang, B., Dai, Y., Li, H., Xu, W. (2017) A Review: Epigenetic Mechanism in ochratoxin A toxicity studies. *Toxins* .9(4). pii: E113. doi: 10.3390/toxins9040113. Review.

Zhu, L., Yu, T., Qi, X., Gao, J., Huang, K., He, X., Luo, H., Xu, W. (2016). Limited link between oxidative stress and ochratoxin A-induced renal injury in an acute toxicity rat model. *Toxins (Basel).* **8**: 373-385. doi:10.3390/toxins8120373.

Zlender, V., Breljak, D., Ljubojevic, M., Flajs, D., Balen, D., Brzica, H., Domijan, A. M., Peraica, M., Fuchs, R., Anzai, N., Sabolic, I. (2009). Low doses of ochratoxin A upregulate the protein expression of organic anion transporters Oat1, Oat2, Oat3 and Oat5 in rat kidney cortex. *Toxicol. Appl. Pharmacol.* **239**: 284-296. doi: 10.1016/j.taap.2009.06.008.

**Supplementary data**

ACCEPTED MANUSCRIPT

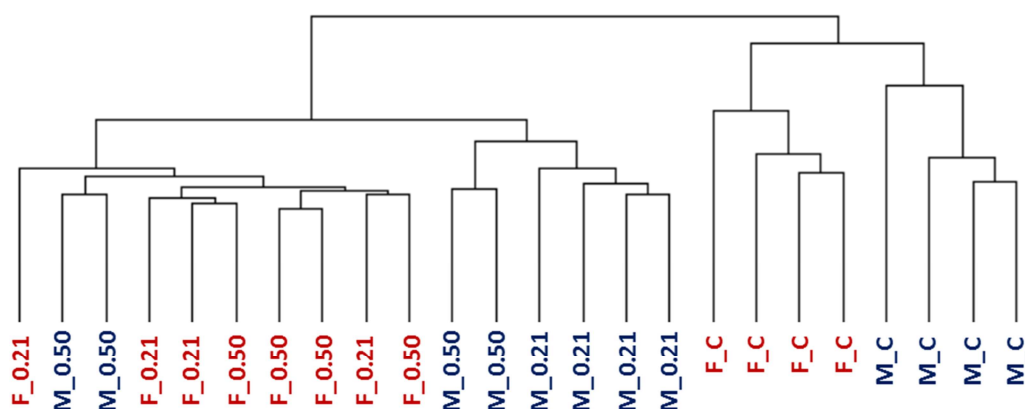
**Table 1 Supplementary data. DNA methylation study.** The table contains: amplicons selected for each gene after analysing their gDNA sequence (-2000 bases before and 1500 bases after the first exon) with EpiDesigner software; percentage of CpG in each amplicon; sequence of selected primers; and number of CpG detected by MALDI-TOF spectrometry.

Gene Symbol	CpG islands				Primer sequence		Amplicon size (bp)	Total N° of CpG sites	N° of analyzed CpG sites (Real N°)	N° of analyzed CpG sites		
	Start	End	GC	OE	Primer	Start				Sequence	Single sites	Composite sites
<b>Akr1b7</b>					Left	491	TAGGTGTTAGTGGGATTTTAGAGGA	365	7	4	4	1
					Right	855	ACAAAAATCCCTTCTCTATTCCAT					
					Left	2315	AAGGTGAGTGTTTAGTTTTGGGATT	393	7	7	3	2
					Right	2707	CCCAAACCACTCAAATATTAACAAA					
<b>Cyp1a1</b>	709	996	65.28	0.64	Left	583	TGGGAGTATTTTTAGGGTTAGAGAGTA	397	20	18 (16)	9	5
					Right	979	CCCCACCTAACTAAAAACAAAATA					
	1104	1179	59.21	0.88	Left	1000	TTGTTTTTAGAGGATGGAGTAGGT	392	16	13	8	4
	1230	1363	55.97	0.76	Right	1391	AAAAATCACAAAACAAAAAACCAA					
<b>Cyp2c11</b>	346	610	54.72	346	Left	156	TTTTTATAGAAGTTTAGGAGGTATAGTTT	397	11	11 (10)	7	2
					Right	552	TCTAACTCCTCTCAAATAATAAATACTCA					
					Left	2987	TTAGTTAAGTGGGGTTTAAATTTGG	396	4	3 (2)	4	
					Right	3382	CCCAATATCTCTAACCACTACACAC					
<b>Gstp1</b>	1716	1766	54.90	0.69	Left	1424	TTGGGAGTGTTTTAATTATGAGTT	399	6	6	6	
					Right	1822	AAAAATATATTCAACTCCCTTTCCAC					
	1867	2197	62.54	0.65	Left	1787	TTAAAATAAAGTGGAAAGGGAGTTG	386	20	17	12	3
					Right	2172	CAAACTCCCAACTCTTAATACAA					
<b>Slc22a7</b>	467	629	60.74	0.69	Left	302	GTTGTTGAGGGTAGGAATTGTGT	363	13	13 (7)	13	
					Right	664	CACTTCCAAAAAACTCAAAAAAA					
	1587	1678	55.43	0.71	Left	1364	AGGGGTAGATTGGATTGTAGAAGAA	369	6	5	6	
					Right	1732	AAAACCCCAACTACCATAAAAAAC					
	3392	3537	67.12	0.62	Left	3219	GTGTTGGGTTTGATGTTTGTAGTTT	383	16	13 (12)	11	2
					Right	3601	CCACTCTACCAAATACTAATAACCT					

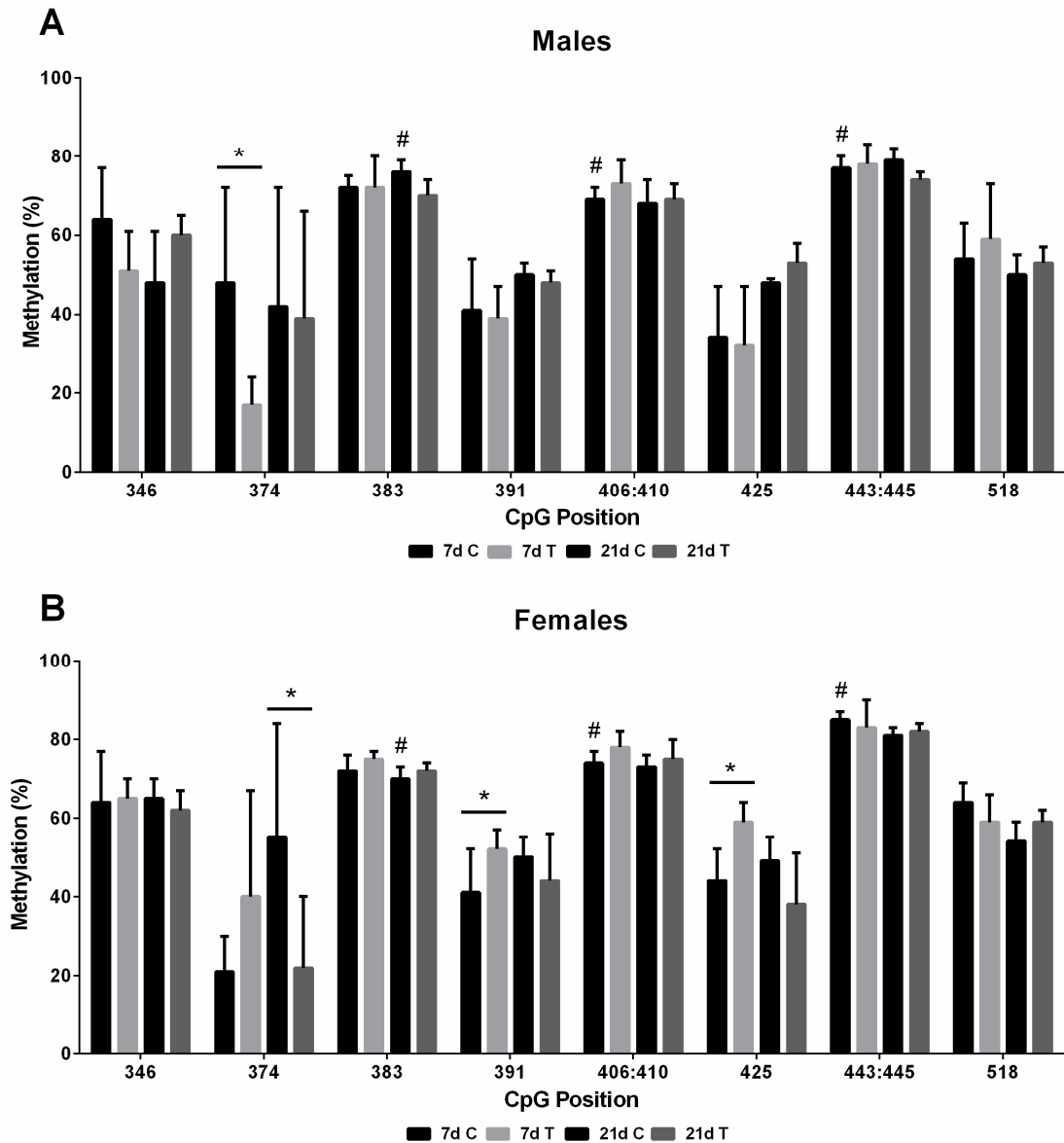
**Table 2. Supplementary data List of LogFC of DEG (B > 0) present in significantly altered toxicity lists (-log(p-value) ≥ 1.3**

The number of sex-specific of each group is presented in brackets.

Information provided in electronic support (xls. File). Toxicity lists are shown in alphabetical order in different spreadsheets: 1) significant lists commonly altered in all groups; 2) lists significantly altered in three groups; 3) dose-dependent lists; 4) male- specific lists; and 5) female-specific lists

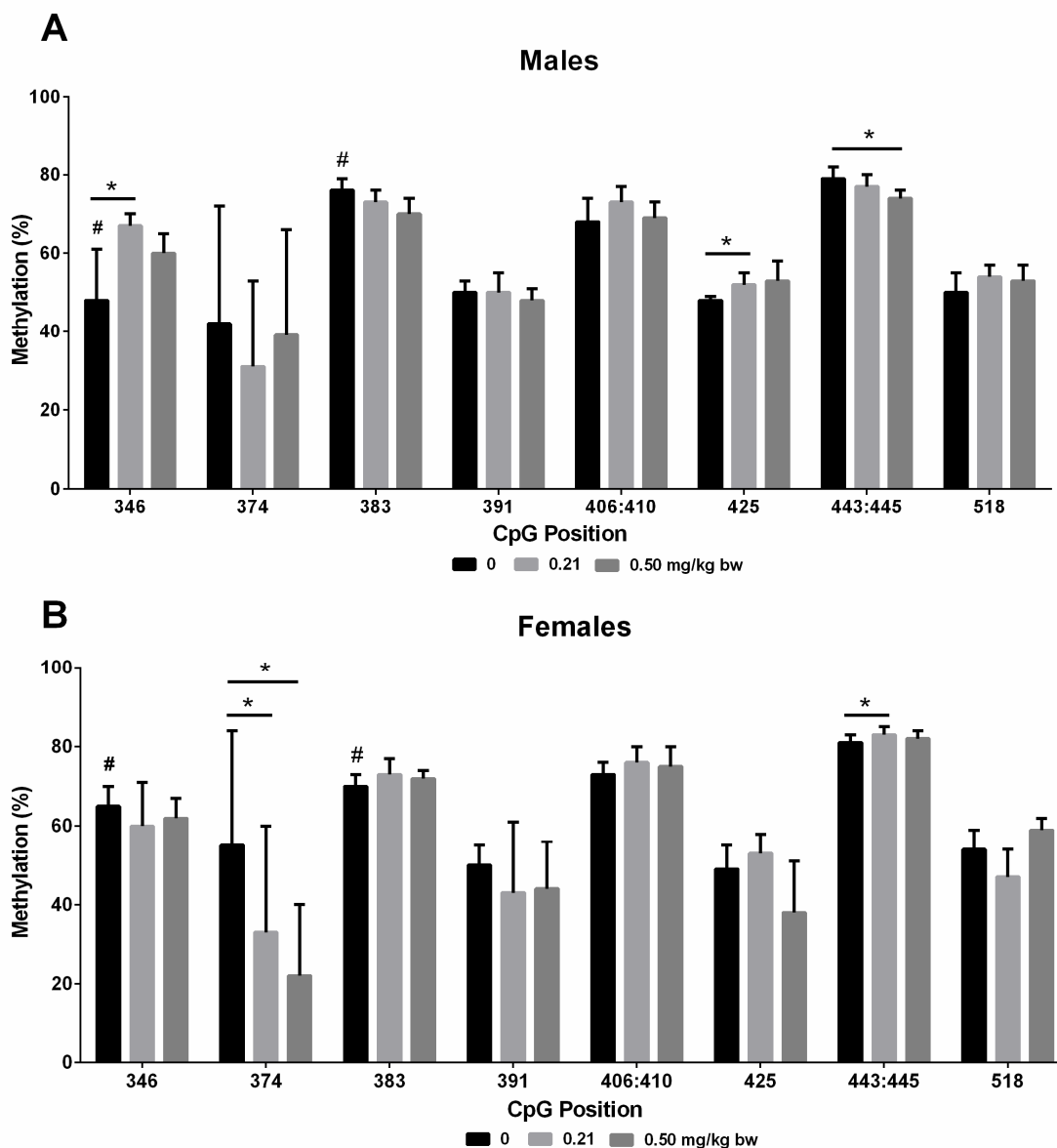


**Figure 1 Supplementary data. Hierarchical clustering** analysis of kidney samples from male (M) and female (F) rats treated for 21 days (n= 4) with the vehicle (NaHCO<sub>3</sub>; C: control) or OTA (0.21 or 0.50 mg/kg bw).

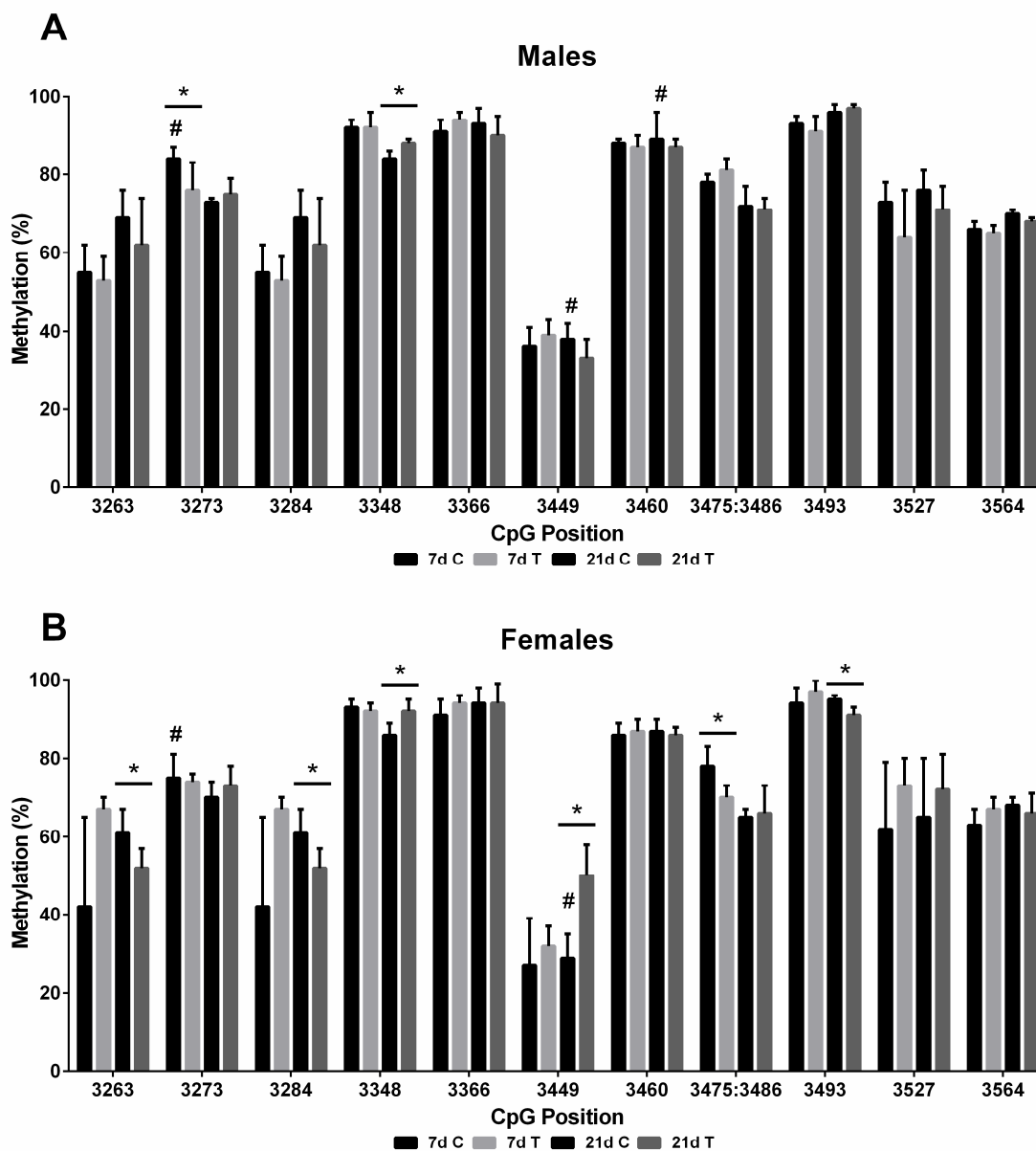


**Figure 2 Supplementary data.** DNA methylation profile of *Cyp2c11* promoter of kidney obtained from male (A) and female (B) F344 rats treated for 7 or 21 days with a daily oral dose of the vehicle ( $\text{NaHCO}_3$ ) or ochratoxin A (0.50 mg/kg bw). Percentage (%) of DNA methylation was shown as mean  $\pm$  standard deviation (SD). Statistical analyses were performed by Mann-Whitney *U* test and differences were considered significant when *p*-value was  $< 0.05$ : (#) male versus female control (C) animals or (\*) control versus treated animals in each sex and dose comparisons. Results were considered as biologically relevant when variation in DNA percentage was  $> 10\%$ . The selected region in our sequence (156 to 552) corresponds to 257672328 to 257672724 in the whole genome sequence.

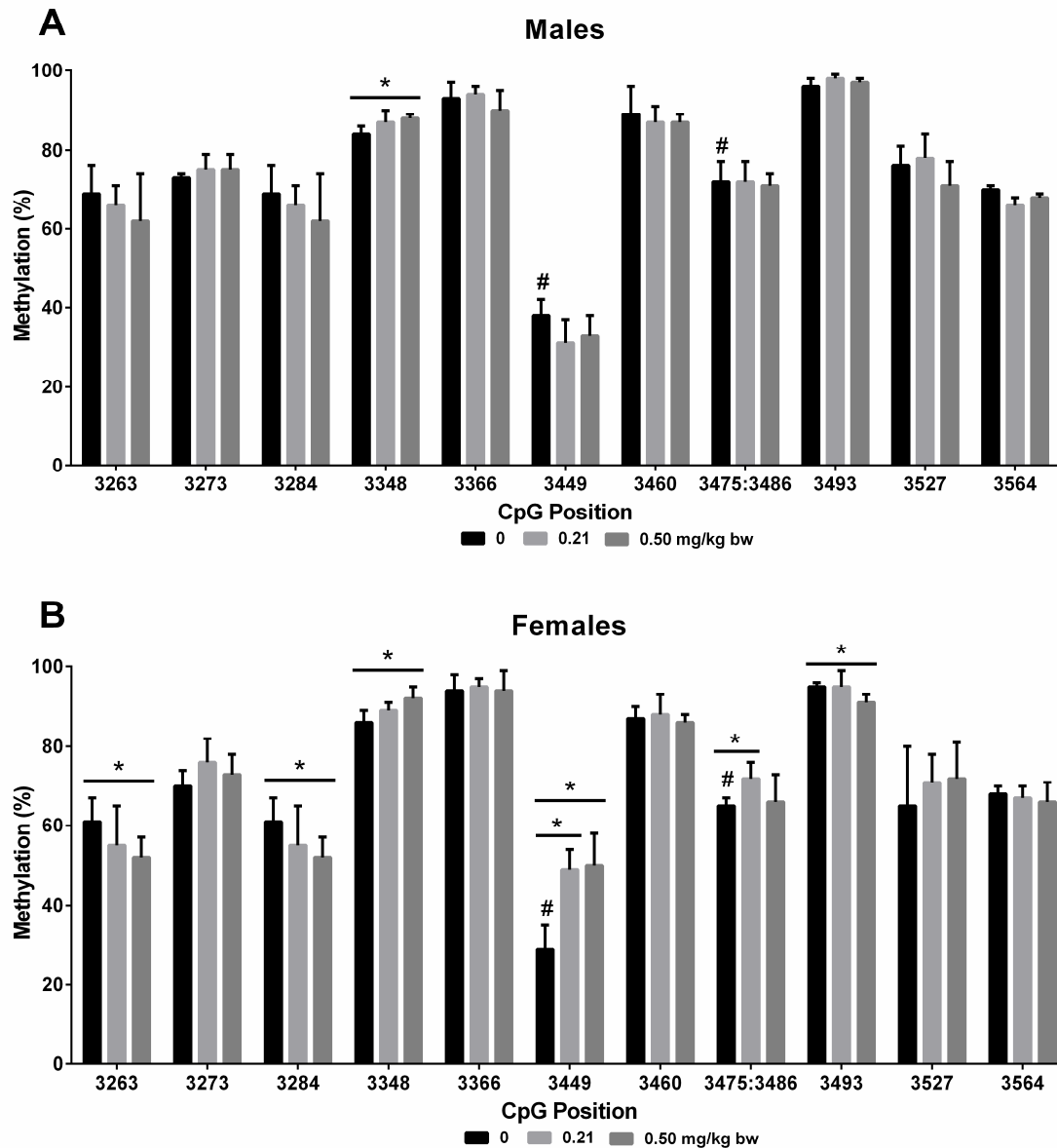




**Figure 3 Supplementary data. DNA methylation profile of Cyp2c11 promoter of kidney obtained from male (A) and female (B) F344 rats treated for 21 days with a daily oral dose of the vehicle ( $\text{NaHCO}_3$ ) or ochratoxin A (0.21 or 0.50 mg/kg bw). Percentage (%) of DNA methylation was shown as mean  $\pm$  standard deviation (SD). Statistical analyses were performed by Mann-Whitney *U* test and differences were considered significant when *p*-value was  $< 0.05$ : (#) male versus female control (C) animals or (\*) control versus treated animals in each sex and dose comparisons. Results were considered as biologically relevant when variation in DNA percentage was  $> 10\%$ . The selected region in our sequence (156 to 552) corresponds to 257672328 to 257672724 in the whole genome sequence.**



**Figure 4. Supplementary data. DNA methylation profile of Slc22a7 promoter of kidney obtained from male (A) and female (B) F344 rats treated for 7 or 21 days with a daily oral dose of the vehicle (NaHCO<sub>3</sub>) or ochratoxin A (0.50 mg/kg bw). Percentage (%) of DNA methylation was shown as mean  $\pm$  standard deviation (SD). Statistical analyses were performed by Mann-Whitney *U* test and differences were considered significant when *p*-value was < 0.05: (#) male versus female control (C) animals or (\*) control versus treated animals in each sex and dose comparisons. Results were considered as biologically relevant when variation in DNA percentage was > 10%. The selected region in our sequence (3219 to 3601) corresponds to 16930146 to 16930528 in the whole genome sequence.**



**Figure 5 Supplementary data.** DNA methylation profile of *Slc22a7* promoter of kidney obtained from male (A) and female (B) F344 rats treated for 21 days with a daily oral dose of the vehicle ( $\text{NaHCO}_3$ ) or ochratoxin A (0.21 or 0.50 mg/kg bw). Percentage (%) of DNA methylation was shown as mean  $\pm$  standard deviation (SD). Statistical analyses were performed by Mann-Whitney *U* test and differences were considered significant when *p*-value was  $< 0.05$ : (#) male versus female control (C) animals or (\*) control versus treated animals in each sex and dose comparisons. Results were considered as biologically relevant when variation in DNA percentage was  $> 10\%$ . The selected region in our sequence (3219 to 3601) corresponds to 16930146 to 16930528 in the whole genome sequence.