



# Involvement of hepatic lipid droplets and their associated proteins in the detoxification of aflatoxin B<sub>1</sub> in aflatoxin-resistance BALB/C mouse

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## ABSTRACT

The highly potent carcinogen, Aflatoxin B<sub>1</sub>, induces liver cancer in many animals including humans but some mice strains are highly resistant. This murine resistance is due to a rapid detoxification of AFB<sub>1</sub>. Hepatic lipid droplets (LDs) ultimately impact the liver functions but their potential role in AFB<sub>1</sub> detoxification has not been addressed. This study describes the structural and functional impacts on hepatic LDs in BALB/C mice after exposure to 44 (low dose) or 663 (high dose) µg AFB<sub>1</sub>/kg of body weight. After 7 days, the liver of AFB<sub>1</sub>-dosed mice did not accumulate any detectable AFB<sub>1</sub> or its metabolites and this was associated with a net increase in gene transcripts of the *AhR*-mediating pathway. Of particular interest, the livers of high-dose mice accumulated many more LDs than those of low-dose mice. This was accompanied with a net increase in transcript levels of LD-associated protein-encoding genes including *Plin2*, *Plin3* and *Cideb* and an alteration in the LDs lipid profiles that could be likely due to the induction of *lipoxygenase* and *cyclooxygenase* genes. Interestingly, our data suggest that hepatic LDs catalyze the *in vitro* activation of AFB<sub>1</sub> into AFB<sub>1</sub>-*exo*-8,9-epoxide and subsequent hydrolysis of this epoxide into its corresponding dihydrodiol. Finally, transcript levels of *CYP1A2*, *CYP1B1*, *GSTA3* and *EH1* genes were elevated in livers of high-dose mice. These data suggest new roles for hepatic LDs in the trapping and detoxifying of aflatoxins.

## 1. Introduction

Aflatoxins (AF) are a group of lipid-derived toxins secreted by certain common fungi including *Aspergillus flavus* and *A. parasiticus* [1–3]. AFs cause both chronic and acute toxicity in humans and animals following consumption of fresh and/or stored AF-contaminated food and feed. In terms of chronic exposure, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), [(6aR,9aS)-2,3,6a,9a-Tetrahydro-4-methoxy-1H,11H-cyclopenta[c]furo[3,2':4,5]furo[2,3-h][1]benzopyran-1,11-dione], is the most toxic form of AFs and is regarded as the most potent environmental carcinogen identified to date, where the exposure to AF is considered as a major causal factor of hepatocellular carcinoma (HCC) [1,3,4]. Acute exposure to AFB<sub>1</sub> also provokes many immunotoxicological effects and alterations in cytokine expression in various animal species [5–7]. Of particular interest, it was shown that AFB<sub>1</sub> affects the expression of lipid metabolizing genes in rat liver, suggesting a potential connection between the AFB<sub>1</sub>-induced lipid metabolism and in the long term a possible elevated risk of coronary heart disease [8].

It is well known that the livers of AF-exposed animals are the most

active accumulating organs of such toxins, and that the liver plays important roles in the sequestration and biotransformation of AF [9,10]. The hepatic detoxifying capability of AFs, which are highly lipophilic molecules, is ensured by the high intracellular lipid content plus a battery of AF-metabolizing enzymes, most notably microsomal cytochrome P450 s. The biological connection between AF and hepatic lipids has been demonstrated in AFB<sub>1</sub>-producing fungal cells where biosynthesis, trafficking and exporting of AFB<sub>1</sub> are strictly modulated by fungal LDs and their associated proteins, especially caleosin/peroxygenase AfPXG [11,12]. Addition of exogenous AFB<sub>1</sub> also causes alterations in plasma and liver lipid levels in exposed animals [8,13,14]. Moreover, the integrated analysis of transcriptomic and metabolomic profiles of AFB<sub>1</sub>-induced hepatotoxicity in AFB<sub>1</sub>-dosed rats revealed that dysfunction of lipid metabolism was a major metabolic effect, suggesting its potential use as a biomarker for detecting AFB<sub>1</sub>-induced acute hepatotoxicity [15].

The efficient and rapid detoxification of AFB<sub>1</sub> requires a set of enzymes that actively metabolize such xenobiotics into more hydrophilic metabolites that are more readily excreted in the urine (via the kidney)

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or in the bile (via the liver). This process varies between different animal species and even between strains of the same species. In the case of common laboratory animals, mice are less sensitive than similar mammalian species such as rats, guinea pigs, and rabbits [16]. Moreover, different inbred mouse strains have differential cellular susceptibilities towards AFB<sub>1</sub>, with the BALB/C strain being highly resistant compared with strains such as C57B1/6, B10A and CBA/J [17]. In this context, murine resistance to AFB<sub>1</sub> is related to, but not limited to, the activation of AFB<sub>1</sub> by hepatic  $\alpha$ -glutathione-S-transferases (GSTAs), mainly GSTA1 and GSTA3, that rapidly conjugate the AFB<sub>1</sub>-exo-8,9-epoxide (AFBO) with glutathione forming the AFB<sub>1</sub>-exo-glutathione [18], suggesting GSTA as a primary pathway responsible for the detoxification of the AFB<sub>1</sub> in mice [19,20]. Although the AFBO conjugation reaction by GSTA is well characterized in mice, the upstream catalytic activating of AFB<sub>1</sub> is still uncertain. This is because mice do not harbor an ortholog of human CYP3A4 that catalyzes the epoxidation of AFB<sub>1</sub>, but do express an ortholog of CYP1A2 that putatively epoxidizes AFB<sub>1</sub> in other mammals, an activity that has never been reported in mice [21], suggesting that the AFB<sub>1</sub>-resistance is probably due to the involvement of other pathways.

Mammalian liver cells are well known as active sites of lipid metabolism and this is often reflected in the accumulation, or even hyperaccumulation, of hepatic LDs that typically contain a mainly triacylglycerol core that is surrounded by a specific population of lipid-associated proteins [22–24]. In some cases, hepatic LDs can also serve as storage organelles for lipophilic molecules such as retinoids [25]. Recent studies have demonstrated that the murine hepatic LD proteome is highly dynamic and can undergo rapid compositional changes in response to fasting and refeeding [26]. Hepatic LDs are also implicated in a variety of pathologies, most notably alcohol-related and non-alcoholic fatty liver disease [27,28] and hepatitis C infection [29,30]. However, although LDs have been shown to sequester lipophilic toxins, such as AFB<sub>1</sub>, in fungi an analogous role for hepatic LDs has yet to be elucidated in animals.

In this study, we report the involvement of hepatic LDs in the sequestration/trafficking, and possibly, the biotransformation of AFB<sub>1</sub> in BALB/C mice, an AFB<sub>1</sub>-resistant strain. To investigate this, hepatic LDs from female mice, exposed to low or high doses of AFB<sub>1</sub> for 7 days, were isolated, purified and characterized with respect to their abundance, size, lipid and protein content. Furthermore, the *in vitro* biotransformation activity of hepatic LDs towards AFB<sub>1</sub> was measured together with transcript abundances of key genes involved in the binding and activation of AFB<sub>1</sub>.

## 2. Materials and methods

### 2.1. Chemicals, animals, conditions and treatments

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) standards from *Aspergillus flavus*, aniline, cumene hydroperoxide and all organic solvents were purchased from Sigma-Aldrich, USA. Oligonucleotides (Table S1) were supplied by the Unit of primers synthesizer at AEC. Twelve-week old female mice, with average weight of 20 g, of BALB/C were obtained from the Breeding Unit for Inbred Mice at the Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria (AECS). Mice were housed in clean cages, received feed and clean tap water *ad libitum* and kept under standard 12-h light/dark cycles at 25 ± 2 °C and 40–60 % humidity. Fifteen mice were used in the experiments which were randomly divided into three groups of five animals. Dose-related toxicological effects have been reported in AFB<sub>1</sub>-exposed laboratory animals notably in mice [17,31–33]. Apparently, these different effects are produced by different pathways. For this reason, in the current work we studied the effects of a low dose (L-dose) and a high dose (H-dos) of AFB<sub>1</sub> on the hepatic LDs to determine the responsive and functional capacities of these organelles as a function of AFB<sub>1</sub> dose. To do this, groups I and II mice orally received a single 50  $\mu$ L-dose of corn oil

containing 44 (low dose) or 663 (high dose)  $\mu$ g AFB<sub>1</sub>/kg of body weight while group III was received a similar dose of corn oil alone [31]. Seven days later, mice were weighed and euthanized. The livers were rapidly removed, rinsed with 0.9 % NaCl, weighed, immediately frozen in liquid nitrogen and stored at -20 °C until further use. All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. This study was approved by the AECS Committee of Animal Ethics.

### 2.2. Extraction and thin layer chromatography (TLC) analysis of aflatoxins

Liver tissue (about one gram) of each experimental mouse was used for analysis of aflatoxin B<sub>1</sub> and its potential biotransformation products. Extraction of AFs was done according to Hanano et al., [12] using 2 mL of chloroform for one hour on a rotary-shaker. Recovery of the extracted AFB<sub>1</sub> was evaluated using a spiked sample with 50 ng AFB<sub>1</sub> for one gram of liver tissue. Thus, the recovered concentration of AFB<sub>1</sub> was considered in the subsequent calculations. The extracts were analyzed by thin layer chromatography (TLC) according to [11]. Samples were spotted onto a C<sub>18</sub> reverse-phase TLC plate (aluminum sheets 20 × 20 cm, 200  $\mu$ m layer, Merck, Germany) and the chromatogram developed using a solvent system of chloroform/acetone (90:10, v/v). The detection of AFB<sub>1</sub> was compared with a AFB<sub>1</sub>-standard point containing 5 ng by spotting 1  $\mu$ L of AFB<sub>1</sub> standard (5  $\mu$ g/mL). After development, the spot having a *R<sub>f</sub>* value similar to AFB<sub>1</sub> standard (20  $\mu$ g/mL) was scraped then re-extracted with chloroform and evaporated to dryness under nitrogen. The extract was resuspended with 100  $\mu$ L chloroform and the concentration of AFB<sub>1</sub> was measured by spectrophotometer at 360 nm.

### 2.3. Isolation of LDs and microsomal fractions from animal livers

The isolation of LDs was based on their buoyant density, which is less than 1 g/cm<sup>3</sup>, by differential centrifugation and using a gradient floating buffer in cooling conditions [34]. The pooled livers of each animal group (about 3 g) were vigorously ground in a mortar and pestle and in the presence of liquid nitrogen. The liver powder was immediately homogenized with 6 mL of homogenization buffer (HB) (50 mM Tris–HCl, 1 mM EDTA, 1 M sorbitol, pH 7.5). The total homogenate was divided into four fractions of 1.5 mL each, transferred into 2-mL microcentrifuge tube and centrifuged at 5000 × g for 15 min at 4 °C. The supernatant (about 1 mL) containing LDs was taken into a clean 2-mL tube, overlaid with an equal volume of floating buffer (FB) (50 mM Tris–HCl, 1 mM EDTA, 1 M sorbitol, pH 7.5) and centrifuged at 21,130 × g for 1 h at 4 °C. After centrifugation, three fractions were obtained, an upper creamy floating layer corresponding to the LDs, an infranatant phase corresponding to cytosolic proteins fraction, and a pellet corresponding the microsomal fraction (M). LD fractions were separately and carefully taken and subjected to a one-step washing with 1 mL of FB, then centrifuged at 21,130 × g for 1 h at 4 °C. Finally, the respective fractions, LDs or M, were re-suspended in 100  $\mu$ L of suspension buffer (50 mM Tris–HCl pH-7.5, 1 mM EDTA pH-8.0 and 10 % glycerol) and stored at 4 °C.

### 2.4. Characterization of LDs

Isolated LDs were examined under light microscope (Olympus with a mercury light source) at a magnification of × 40. LDs were stained by Nile Blue dye and examined by a fluorescent microscope. For that, the lipophilic Nile Blue dye was dissolved in dimethyl sulfoxide (DMSO) at concentration of 1 mg mL<sup>-1</sup> then diluted 10 × and freshly used to stain 1  $\mu$ L of LDs for 15 min at room temperature. Stained LDs were immediately examined under a fluorescent microscope (Nikon Ti-U microscope supplied with an Olympus FE-4000 camera) using red and green fluorescence filters (excitation, 545 and 480 nm; emission, 620

and 535 nm, respectively) at  $\times 40$  magnification. The concentration of proteins associated with LDs was determined by the Bradford method using bovine serum albumin (BSA) as a standard [35]. The absorbance at 595 nm was measured using a Jenway 6840 spectrophotometer.

### 2.5. Lipid profile using TLC

The organic mixture (chloroform/acetone) described above for protein precipitation was also used for extraction of lipids from LDs. After centrifugation, the organic phases containing the lipid fraction were transferred into new tubes and evaporated to dryness under nitrogen. Extracted lipids were re-dissolved in 50  $\mu$ L of the same solvent mixture and analyzed by TLC. Samples were spotted onto a  $C_{18}$  reverse-phase TLC plate (aluminum sheets 20  $\times$  20 cm, 200  $\mu$ m layer, Merck, Germany) and the chromatogram developed using a solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Developed TLC plates were dried at room temperature and lipids visualized by iodine vapor.

### 2.6. Hydroxylation enzymatic activity

The potential fatty acid-oxygenation activities of LD-associated proteins were assayed by a rapid test based on measurement of aniline hydroxylation [11,36]. This was performed by incubating an increasing amount of LDs containing about 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6  $\mu$ g of total proteins with 1 mM of aniline in a final volume of 1 mL of 10 mM sodium acetate buffer, pH 5.5 and 20 % (v/v) glycerol. The enzymatic activity was initiated, at room temperature, by adding 4 mM of cumene hydroperoxide as an oxygen donor. The accumulation of hydroxylaniline was spectrophotometrically measured at 310 nm.

### 2.7. Genes, primers and transcripts analysis

Three sets of genes were selected to study hepatic transcriptional changes in response to the AFB<sub>1</sub>. Set I refers to key genes involved in the reception and detoxification pathway of AF, e.g. aryl hydrocarbon receptor (*AhR*), aryl hydrocarbon receptor nuclear translocator (*ARNT*), nuclear factor kappa (*NFκB*), cytochrome P450 1A1 (*CYP1A1*), cytochrome P450 1A2 (*CYP1A2*), cytochrome P450 1B1 (*CYP1B1*), glutathione S-transferase  $\alpha$ -1 (*GSTA1*), glutathione S-transferase  $\alpha$ -3 (*GSTA3*) and epoxide hydrolase 1 (*EH1*). Set II comprises the lipoxigenases (*LOX5*, *LOX12* and *LOX15*) and cyclooxygenases (*COX1* and *COX2*) catalyzing oxygenation of polyunsaturated fatty acids. Set III contains a collection of genes encoding LD-associated proteins, e.g. Perilipin 2 (*Plin2*), Perilipin 3 (*Plin3*), Perilipin 4 (*Plin4*), Perilipin 5 (*Plin5*), Cell death-inducing DFFA-like effector b (*Cideb*), Cell death-inducing DFFA-like effector c (*Cidec*), peroxisome proliferator-activated receptor  $\alpha$  (*Ppara*), peroxisome proliferator-activated receptor gamma (*Pparg*) and peroxisome proliferator-activated receptor delta (*Ppard*). Gene expression was normalized to that of a set of reference genes, i.e. actin-beta (*Actb*), ribosomal protein L13a (*RPL13a*), hypoxanthine phosphoribosyl transferase 1 (*Hprt1*) and succinate dehydrogenase complex flavoprotein subunit A (*SdhA*). Table 1 summarizes gene name, NCBI-accession number, forward and reverse oligonucleotides and expected amplicon size.

Changes in relative transcriptional abundance of three sets of genes in response to AFB<sub>1</sub> exposure were analysed by reverse-transcription quantitative PCR (RT-qPCR) as described [37]. For RNA extraction, 30 mg of liver from each animal group were finely ground in the presence of liquid nitrogen and the total RNA was extracted using an RNeasy kit according to the manufacturer's instructions (Qiagen, Germany). DNA traces were removed by treating the samples for 1 h at 37 °C with 2 U of RNase-free RQI DNase (Promega, USA). RNAs were diluted to 50 ng/ $\mu$ L using RNase-free water and stored at - 80 °C. Aliquots of 1  $\mu$ g total RNA were used for first-strand cDNA synthesis using M-MLV RT (Invitrogen), for more details please refer to Hanano et al.,

[38]. Real-time PCR was performed in 48-well plates using an AriaMx Real-time PCR System from Agilent technologies, USA. In brief, 25  $\mu$ L reaction mixtures contained 0.5  $\mu$ M of each specific oligonucleotide primer for the target and reference genes, 12.5  $\mu$ L of SYBR Green PCR mix (Bio-Rad, USA) and 100 ng cDNA. qPCR conditions were as described before [36]. Each point was replicated in triplicate and the average of  $C_T$  was taken for calculation of the relative quantification  $RQ = 2^{(-\Delta\Delta CT)}$ .

### 2.8. In vitro biotransformation of AFB<sub>1</sub>

The activity of LD and microsomes fractions isolated from control and AFB<sub>1</sub>-treated mice were assayed for biotransformation of AFB<sub>1</sub>. An aliquot of 100  $\mu$ L containing about 15  $\mu$ g protein from each fraction was separately incubated with 15 ng of standard AFB<sub>1</sub> pre-dissolved in DMSO at 37 °C for 1 h with gentle shaking ( $\sim 200$  rpm). After incubation, the AFB<sub>1</sub> and its metabolites were immediately extracted with 1 mL of chloroform by vigorous vortexing for 15 min and a brief centrifugation at 14,500 rpm for 5 min. The organic phase was evaporated under nitrogen and resolved in 50  $\mu$ L chloroform for further analysis. AFB<sub>1</sub> metabolites were analyzed by TLC onto a  $C_{18}$  reversed-phase plate (Aluminum sheets 20  $\times$  20 cm, 200  $\mu$ m layer, Merck, Germany) using a solvent system of chloroform/acetone (9:1 v/v). Plates were examined under UV light at 365 nm. UV-fluorescent metabolites of AFB<sub>1</sub> was scraped and re-extracted from the silica gel by 0.5 mL of chloroform as described above.

### 2.9. Detection of AFB<sub>1</sub> metabolites by HPLC-FD

Purified AFB<sub>1</sub> and its metabolites were analyzed on a Waters Alliance e2695 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 2475 fluorescence detector. Samples were run at 25 °C on a ZORBAX SB-C<sub>18</sub> column. The mobile phase, the gradient schedule, and detection conditions were as described previously [39]. The flow rate was 1 mL/min and the injection volume was 50  $\mu$ L. Fractions corresponding to each metabolites were collected and the spectral characterization of both metabolites, AFB<sub>1</sub> 8,9-epoxide and AFB<sub>1</sub>-exo-8,9-dihydrodiol, was performed by UV- and Fluorescent-spectroscopy as described by Johnson et al., [40]. First, absorbance ( $Abs_{300-400}$ ) spectra were recorded using a Jenway 6840 spectrophotometer with a wavelength range from 300 to 400 nm. Fluorescence spectra were recorded using a Varian SF-330 spectrofluorometer, with a wavelength range from 350 to 550 nm.

### 2.10. Statistics

All data presented were expressed as means  $\pm$  standard deviation (SD). Statistical analysis was performed using IBM SPSS statistics 23p4. Statistical significance between control and treatments was evaluated by ANOVA.

## 3. Results

### 3.1. Detection of AFB<sub>1</sub> and its metabolites in mouse liver

AFB<sub>1</sub> and its metabolites were extracted from livers of healthy and AFB<sub>1</sub>-dosed animals and analyzed by TLC. No blue-fluorescent spots were detected in the extracts of livers from L- (low AF doses) and H-dosed (high AF doses) animals corresponding to the AFB<sub>1</sub> standard  $R_f$  of 0.316 (Fig. 1 A). The rapid detoxification of AFB<sub>1</sub> was accompanied with an increase in transcripts levels of selected genes involved in cytotoxicological responses of animal cells towards aflatoxins, i.e., aryl hydrocarbon receptor (*AhR*), aryl hydrocarbon receptor nuclear translocator (*ARNT*), nuclear factor kappa (*NFκB*). These gene products potentially regulate expression of genes involved in the initial steps of AFB<sub>1</sub>-activation. The relative quantification of genes transcripts, shown

**Table 1**  
Names and nucleotide sequences of primers used in this study.

Name	Accession #	Forward Primer	Reverse Primer	Amplicon
Actb	NM_007393.5	TCCAGGCTGTGCTGTCCCTGT	ACGCAGGATGGCGTGAGGGA	125
RplL13a	NM_009438.5	CGAGCCCCAGCCGCATTTT	AGCAGGGACCACCATCCGCT	147
Hprt1	NM_013556.2	GCAGCGTTTCTGAGCCATTG	TCATCGCTAATCACGACGCT	172
SdhA	NM_023281.1	ACCTGACAGCTACAGGACCA	GACAAAGTCTGGCGCAACTC	173
AhR	NM_001314027.1	TCCACCGTGTGGTGAGGT	CTGTGTGGCAAGCCGAGT	117
ARNT	NM_001037737.2	GCTCATTCCCTCCTAACCCC	GTCTTGGCTGTAGCCTGGG	217
NFkB	NM_001177369.1	CGCCACCTGTGTATGGCACA	GCAGAGCGTCTGGTGCAGG	163
CYP1A1	NM_009992.4	CCTGTGGTGGTGTGAGCGG	CAGGGCATTCTGGGCCAGGC	183
CYP1A2	NM_009993.3	TCACTAACGGCAAGAGCATGA	TGGCTGACTGGTTCGAAGTG	223
CYP1B1	NM_009994.1	GCGCAGTATCCCTCCGGGCTG	CTCATGCAGGGCAGGCGGTC	193
GSTA1	NM_008181	CCCCGACACCAAGAGAAGCCA	ACCCTGGTTCAGCCTGTTGCC	131
GSTA3	NM_001077353	TACCCCCACATGCCCCCTGA	AGCCCTGTCTAGCCTGTTGC	144
EH1	NM_001312918	ATTCCTTGACCCCTCTCTGGG	CCCACAGTGTCCGCTTGGT	160
LOX5	NM_009662.2	CCCCTGGAGAGATAACCCA	TGAAAAGGGGATGCACAGTA	192
LOX12	NM_007440.5	TTTGACTTCGACGTTCCCGA	GGAGGCTCAGGATTCCTCT	181
LOX15	XM_006532036.3	GAAGATGTAAACCCACCGTTC	CCAAGACAGAGGAACACAGGG	174
COX2	NM_011198.4	AACCGCATTGCCTCTGAAT	CATGTTCCAGGAGGATGGAG	130
COX1	XM_017316496.1	TTTCTCTAGCCTCTTCGGG	GGTTCAATCCCTCCAGCTC	244
Plin2	NM_007408.3	ACCGTGACCTCTGCGGCCAT	TCGCCCAAGTACGGCACCT	181
Plin3	NM_025836.3	CAGCAGCAGCGACAGGAGCA	AGCCTCTGGTCCACACCTGT	191
Plin4	NM_020568.3	AAGGCACAGCGCAGATGGGT	ACAGCCCTCTGAGCCCTGT	187
Plin5	NM_025874.3	GCGCAGCGTGGATGCTCTACA	GGCCCGCAGGACCAATCCA	145
Cideb	NM_009894.3	AGCCTCAACCCCAATGGCCTG	ACACGGAAGGTCGCTGAGGT	105
Cidec	NM_178373.4	TGCTCCGCTGGACCCTCTTCA	GCTTGGCCTTGGCAGGCTGT	117
Ppara	NM_011144.6	TCCGCTGAAGCTGGTGTACGA	CCCGACAGCAGGCACTTGTG	106
Pparg	NM_001127330.2	CAGGTTTGGGCGGATGCCACA	TCGCCCTCGCCTTGGCTTTG	167
Ppard	NM_011145.3	AAAGACGGGCTGTGGTGGC	CGCGATGAAGAGCGCCAGGT	162

in Fig. 1 B, reveals a brief raise in *AhR* gene transcript levels while those of *ARNT* and *NFkB* increased by 27.9 and 5.5-fold respectively. These results show that, after seven days, the livers of AFB<sub>1</sub>-dosed mice do not accumulate detectable traces of AFB<sub>1</sub> or its metabolites, possibly due to a rapid and efficient hepatic AFB<sub>1</sub>-detoxifying system mediated by the *AhR* pathway.

### 3.2. The exposure to AFB<sub>1</sub> modulates the number and composition of hepatic LDs in a dose-dependent manner

LDs were firstly fractionated from liver of healthy mice. The quality and purity of LD fractions were examined by light and fluorescence microscopy. As shown in Fig. 2, isolated LDs appeared as intact spherical structures under the light microscopy and confirmed by Nile Blue staining under fluorescence microscopes, where the stained LDs were highly fluorescent in green and in red under according to the filter used. The LDs were subsequently fractionated from livers of control (Fig. 3 A I and II) and AFB<sub>1</sub>-exposed animals of L-dose (Fig. 3 B I and II) and H-dose (Fig. 3 C I and II), respectively. The LD count was about of  $60 \times 10^4$  per mL in the fraction isolated from the liver of control mice (Fig. 3 A III), decreased to about of  $45 \times 10^4$  per mL L-dose mice (Fig. 3 B III), but considerably increased to about of  $148 \times 10^4$  per mL in H-dose mice (Fig. 3 C III). The concentration of LD-associated proteins was much lower in L-dose fractions, not exceeding 0.22 mg/mL (Fig. 3 A IV), but was considerably higher in the H-dose fraction, reaching about  $2.5 \text{ mg mL}^{-1}$  (Fig. 3 B IV). This compared to an LD-associated protein concentration in the control fraction of about 1.8 mg/mL (Fig. 3 C IV).

Unexpectedly, although the concentration of LD-associated proteins decreased in L-dose fraction, we did not detect any significant change in the transcript levels of their respective genes (Fig. 3 D). In contrast, the increase in the concentration of LD-associated proteins in the H-dose fraction was accompanied by increases in the transcript levels of key LD-associated proteins encoding genes, notably *Plin2*, *Plin3* and *Cideb*. The transcript levels of these genes increased about of 25-, 9- and 6-fold, respectively (Fig. 3 D). These data indicate that exposure of mice to AFB<sub>1</sub> affects the accumulation of hepatic LDs and possibly their proteomic signature in a dose-dependent manner. Also, while, a chronic dose of AFB<sub>1</sub> leads to a net decrease the hepatic LDs, an acute dose

stimulates their accumulation.

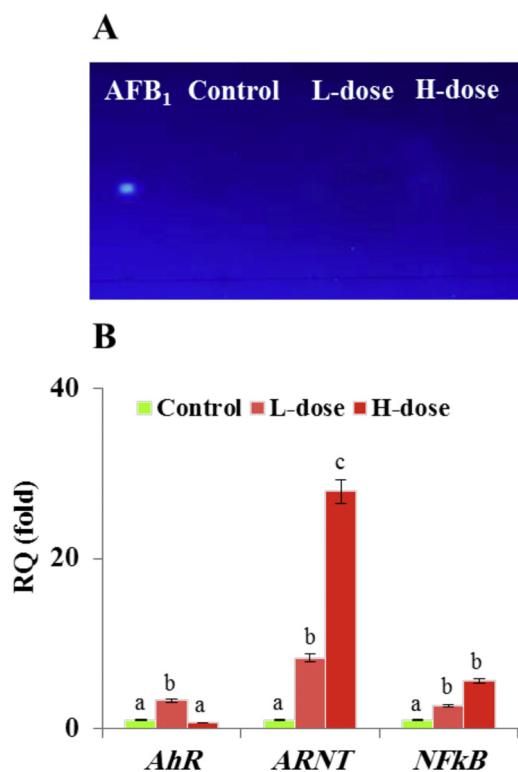
### 3.3. Exposure to AFB<sub>1</sub> affects the lipid profile of hepatic LDs

Both the composition of LD-associated proteins and the nature of their lipidic core can play important roles in the diverse biological functions of LD organelles. The lipid composition was evaluated for each fraction of LDs using TLC. Compared to controls, the lipidic profile of LDs extracted from L-dose set contained only small amounts of TAG and a surprising amount of MAG (Fig. 4 A). However, the LD lipids of H-dose mice contained the expected main five classes, namely as MAG, DAG, TAG, free fatty acids (FFA) and sterol esters (SE) albeit with reduced relative amounts of MAG and DAG. Polyunsaturated fatty acids and their oxygenated metabolites, collectively termed oxylipins, that are present in the lipidic core of LDs are crucial for their biological functions. Therefore, the expression of selected genes involved in the oxygenation of polyunsaturated fatty acids pathways was examined, i.e., Lipoxygenases (*LOX5*, *LOX12* and *LOX15*) and Cyclooxygenases (*COX1* and *COX2*) respectively catalyzing the formation of leukotrienes, prostaglandins and thromboxanes. As shown in Fig. 4 B transcripts levels of these genes varied as a function of AFB<sub>1</sub> dose. In particular, transcripts levels of *LOX5*, *LOX12*, *LOX15* and *COX2* were significantly more induced in the livers of L-dose animals while *COX1* transcripts were only increased in the livers of H-dose animals. Furthermore, the induction of *LOX* genes in the livers of L-dose animals was synchronized with a similar induction of the hydroperoxide reductase activity of the LDs fraction prepared from the livers of L-dose animals. Compared to controls, this activity was about of 0.2 Abs<sub>310</sub> greater in the LDs of L-dose animals and about of 0.1 Abs<sub>310</sub> less in the LDs of H-does animals (Fig. 4 C). These results indicate that exposure of mice to AFB<sub>1</sub> modifies, in a dose-dependent manner, the lipid profile of hepatic LDs and the expression of key oxylipin biosynthesis pathway genes.

### 3.4. Purified hepatic LDs can catalyse the biotransformation of AFB<sub>1</sub> in vitro

The sequestration of AFB<sub>1</sub> into LDs of fungal cell was recently demonstrated [11]. However, the biochemical connection between the





**Fig. 1.** Hepatic metabolism of AFB<sub>1</sub> and activation of genes expressed from the AhR-mediated pathway. **A**, TLC-analysis of AFB<sub>1</sub> extracted from livers of control and AFB<sub>1</sub>-treated mice at low (L) or high doses (H). The *R<sub>f</sub>* values of samples were compared to the *R<sub>f</sub>* of an AFB<sub>1</sub> standard. **B**, Relative quantification (RQ) data of AhR, ARNT and NfκB gene transcripts as described in Methods. Two independent measurements were taken of cDNAs prepared from three individual animals for each treatment. For each dose, the expression level for a given gene in the control was defined as 1 and the corresponding abundance changes in L- and H-dosed animals were calculated. Uppercase letters indicate significant differences in the genes expression between control and AFB<sub>1</sub>-dosed animals, where columns with different upper case letters (a,b) were statistically significant (<sup>b</sup>  $P < 0.05$ ) and (a,c) were statistically very significant (<sup>c</sup>  $P < 0.01$ ), as determined by the ANOVA test.

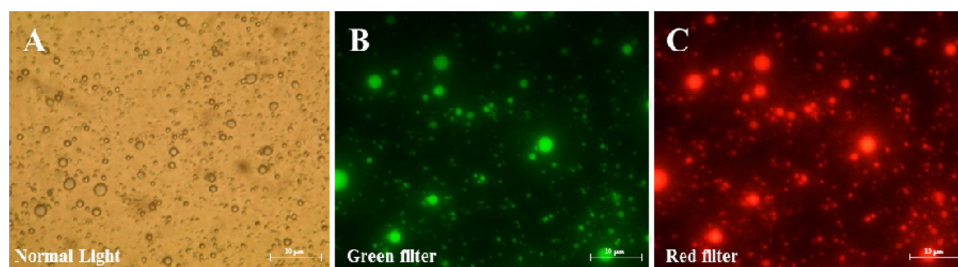
AFB<sub>1</sub> and LDs in animal cells has yet to be elucidated. To determine whether a such connection exists, AFB<sub>1</sub> was incubated with LD fractions from livers of control, L-dose and H-dose mice. The TLC data in Fig. 5 show that LD fractions from L-dose and H-dose mice metabolized AFB<sub>1</sub> similarly to each other but differently from controls. Most of the AFB<sub>1</sub> was still intact after incubation with control LDs but it was metabolized in the treated mice into two derivatives (1 and 2) with *R<sub>f</sub>* values of 0.57 and 0.44, respectively (Fig. 5 A). To characterize the AFB<sub>1</sub> metabolites, the two spots were re-extracted and separately analyzed by TLC (Fig. 5 B). In parallel, the purified metabolites were subsequently analyzed by HPLC-FD, where the less retained metabolite was eluted at retention time (Rt) of 8.75, while the other metabolite was eluted at Rt of 20.58, compared with the intact AFB<sub>1</sub> that has a Rt of 25.62 (Fig. 5 C). In

respect to their *R<sub>t</sub>* values, compared with those that have been earlier reported, the both metabolites could probably correspond to AFB<sub>1</sub>-exo-8,9-dihydrodiol and AFB<sub>1</sub>-exo-8,9-epoxide, respectively. To gain more information about the biochemical identity for these metabolites, both was characterized in the respect to their respective spectral features and this was summarized in Table 2. First, in term of absorbance changes (*Abs*<sub>300–400</sub>), the absorbance spectra indicate a *λ*<sub>max</sub> of 348 nm for the AFB<sub>1</sub>-exo-8,9-epoxide and a *λ*<sub>max</sub> of 364 nm for the hydrolysis product, AFB<sub>1</sub>-exo-8,9-dihydrodiol. A comparison of spectra shows that the widest change in *Abs* upon hydrolysis of the epoxide was between 370 and 390 nm. Moreover, the difference in the fluorescence spectra between the AFB<sub>1</sub>-exo-8,9-epoxide and the AFB<sub>1</sub>-exo-8,9-dihydrodiol was significant. The *λ*<sub>max</sub> for AFB<sub>1</sub>-exo-8,9-epoxide was 382 nm, however, the *λ*<sub>max</sub> for AFB<sub>1</sub>-exo-8,9-dihydrodiol was 454 nm. In addition, compared to their levels in controls, transcripts levels of *CYP1A2*, *CYP1B1*, *GSTA3* and *EHI* were much higher in the H-dose animals (2.1, 5.02, 3.75 and 4.94-fold, respectively) (Fig. 5 D). These data suggest that hepatic LDs of AFB<sub>1</sub>-dosed can effectively catalyze the biotransformation of AFB<sub>1</sub> and this activity conducts the transformation of AFB<sub>1</sub>-exo-8,9-epoxide into its corresponding dihydrodiol.

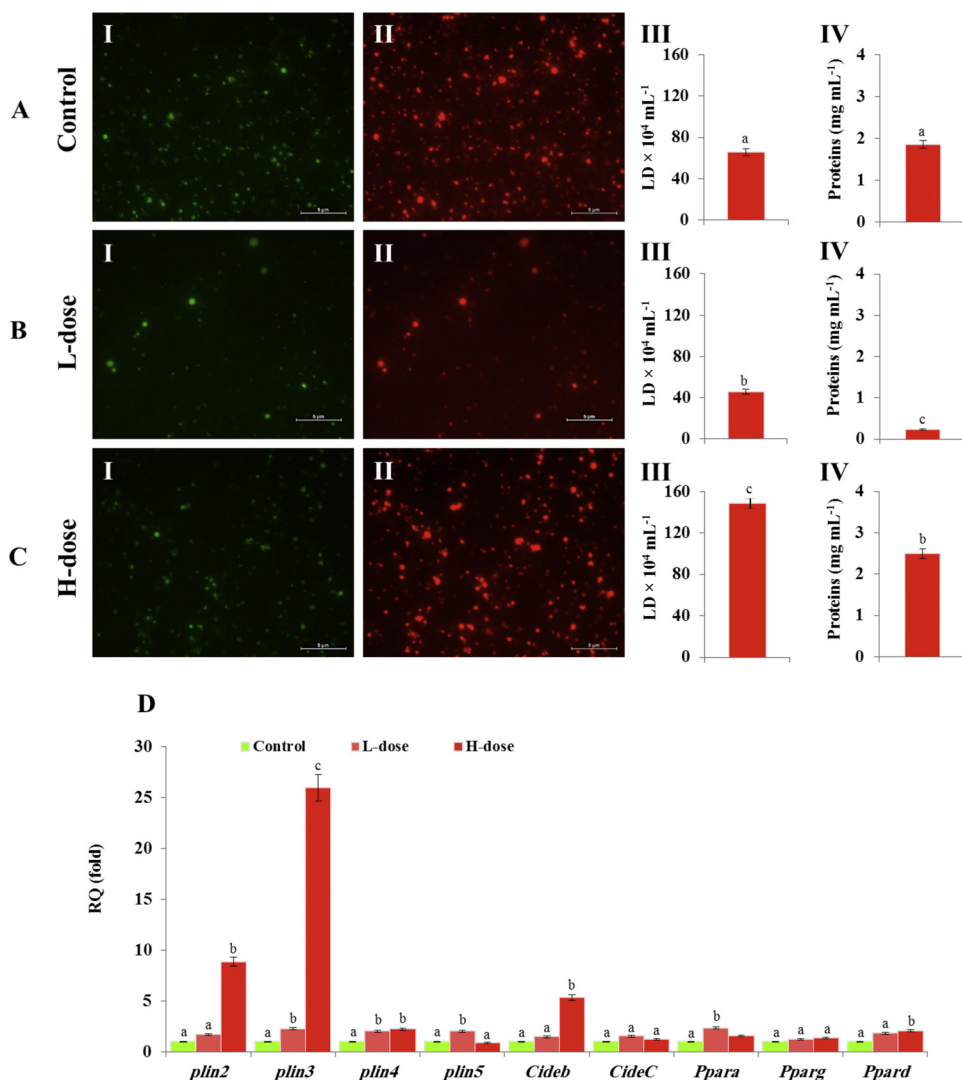
#### 4. Discussion

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a highly potent poison that contaminates both human foods and animal feedstuffs and particularly targets the liver of exposed individuals. The acute-hepatotoxicity of AFB<sub>1</sub> is typified by the induction of apoptosis and genotoxicity in humans, with a potential additional risk of hepatocellular carcinoma [41–44]. Minimizing the cytotoxicity of AFB<sub>1</sub> is dependent on the presence of an efficient hepatic detoxifying system that ensures a rapid elimination of AFB<sub>1</sub>. In this report, we describe new evidence for the involvement of hepatic LDs in the detoxification of an acute dose of AFB<sub>1</sub> in the AF-resistant strain of BALB/C mouse. The results show that, after seven days, the livers of AFB<sub>1</sub>-dosed mice did not accumulate detectable traces of AFB<sub>1</sub> or its potential metabolites and that this could be due to a rapid and efficient hepatic AFB<sub>1</sub>-detoxifying system mediated by the AhR pathway. These data support earlier reports of the rapid elimination of AFB<sub>1</sub> from liver tissue of BALB/C mouse and its correlation with a specific transcriptional pattern of the AhR-mediated pathway [17,45]. Such a rapid elimination of AFB<sub>1</sub> is in accordance with a recent report of increased transcripts levels of aryl hydrocarbon receptor (*AhR*), aryl hydrocarbon receptor nuclear translocator (*ARNT*) and nuclear factor kappa (*NfκB*), which are key genes implicated in the initial steps of AFB<sub>1</sub>-activation [46].

Our data also indicate that the exposure of mice to AFB<sub>1</sub> affects the accumulation of hepatic LDs and possibly their proteomic signature in a dose-dependent manner. While, a chronic dose of AFB<sub>1</sub> caused a net decrease in hepatic LD numbers, an acute dose stimulated their accumulation, suggesting involvement of two different pathways relating to AFB<sub>1</sub> detoxification by hepatic LDs. Interestingly, dose-differential responses to AFB<sub>1</sub> have been demonstrated by several groups [19,32]. Although acute aflatoxicosis is less common than the chronic condition, it can occur occasionally as shown in episodes correlated with acute-dose-specific gluconeogenesis and lipid metabolism disorders [15]. The



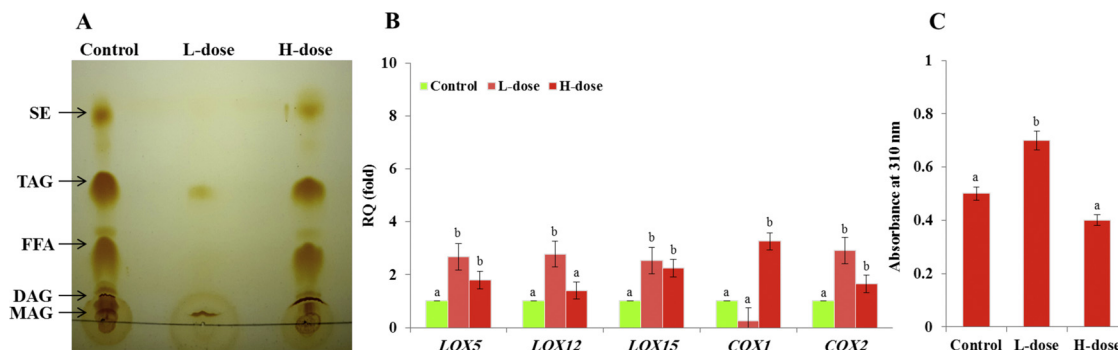
**Fig. 2.** Detection of hepatic LDs fraction under light and fluorescent microscopy. **A**, micrograph of LDs fraction from liver of control animal under normal light at ×40 magnification. **B** and **C**, micrographs of LDs after staining by Nile Blue. Stained LDs were examined under a fluorescent microscope (Nikon Ti-U microscope supplied with an Olympus FE-4000 camera) using green and red fluorescence filters (excitation, 515–560 nm; emission, > 590 nm) at magnification of ×100. Bars represent 10 μm.



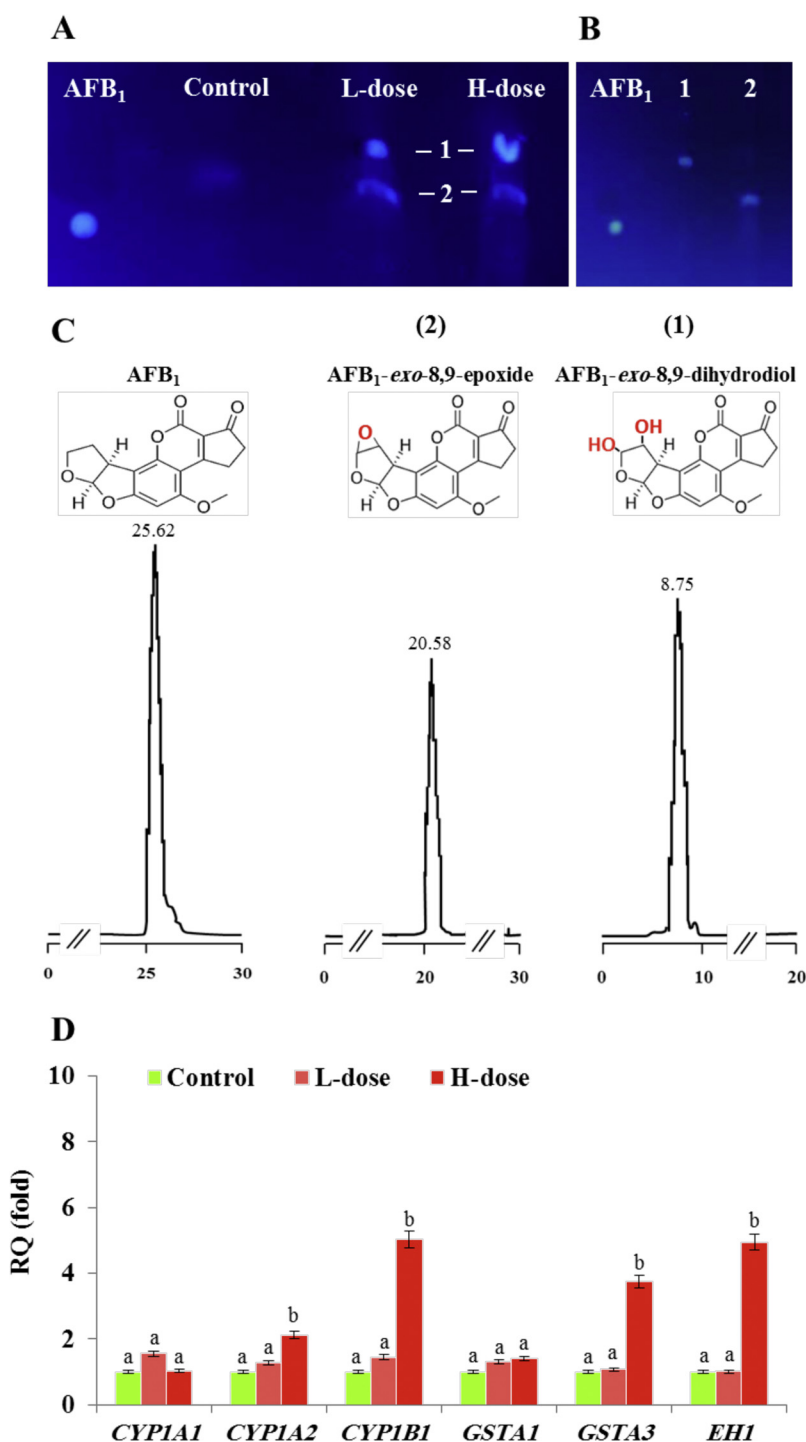
**Fig. 3.** AFB<sub>1</sub> affects accumulation of hepatic LDs and expression of their associated proteins in a dose-dependent manner. **A, B** and **C**, micrographs of LD fractions from control, L and H dosed animals under a fluorescent microscope using green (panel I) and red (panel II) filters at magnification of ×40. Bars represent 5 μm. LD counts and the concentration of their associated proteins are presented for each fraction in panel III and IV, respectively. **D**, transcript levels of LD-associated protein-encoding genes analysed by qRT-PCR. Two independent measurements were taken of cDNAs prepared from three individual animals for each treatment. For each dose, the expression level for a given gene in the control was defined as 1 and corresponding abundance changes in L- and H-dosed animals calculated. Uppercase letters indicate significant differences in the genes expression between control and AFB<sub>1</sub>-dosed animals, where columns with different uppercase letters (a,b) were statistically significant (<sup>b</sup> *P* < 0.05) and (a,c) were statistically very significant (<sup>c</sup> *P* < 0.01), as determined by the ANOVA test.

determination of the proteomic signature of LDs requires isolation of highly pure LDs fractions. The purity of LDs in this study was confirmed by two sequential washings to eliminate endoplasmic reticulum-associated proteins contaminants as previously discussed [26]. Several previous investigations have highlighted the dynamic character of LDs and the often rapid changes in LD proteomes in response to a wide range of developmental, environmental, physiological and

pathophysiological factors [24,26,47–50]. Perilipins are major components of the mammalian LD proteome and are involved in their formation and subsequent functions [51]. In this context, our data showed that expression of certain perilipins, notably *Plin2* and *Plin3* was induced by TCDD which is in line with earlier reports indicating the essential role of these perilipins in the structural stability of LDs [52]. It is suggested that *Plin2* promotes LD formation and thereby protects the



**Fig. 4.** AFB<sub>1</sub> modifies the lipid profile of hepatic LDs and the expression of key genes in the oxylipin biosynthesis pathway. **A**, TLC-analysis of neutral lipids of hepatic LD isolated from control, low- (L-) and high- (H-) dosed animals. Abbreviations: MAG, monoacylglycerol; DAG, diacylglycerol; FFA, free fatty acids; TAG, triacylglycerol; SE, sterol ester. **B**, relative quantification of transcripts for key genes of the oxylipin biosynthesis pathway. **C**, enzymatic activity of the LD-associated oxygenases measured by differential absorption at 310 nm. For each set, two independent measurements were taken for three individual animals. Uppercase letters indicate significant differences in the genes expression or enzymatic activity between control and AFB<sub>1</sub>-dosed animals (<sup>b</sup> *P* < 0.05).



**Fig. 5.** Trapping and biotransformation of AFB<sub>1</sub> by hepatic LDs. **A**, TLC-separation of AFB<sub>1</sub> metabolites (spots 1 and 2) resulting from *in vitro* incubation of standard AFB<sub>1</sub> with a pure fraction of liver LDs from control, L and H dosed mice. **B**, sequential TLC-analysis for the purified metabolites 1 and 2 compared to AFB<sub>1</sub> standard. **C**, HPLC-FD-analysis of AFB<sub>1</sub> and its metabolites 1 and 2 compared with their respective standards with retention times of 25.62, 20.58 and 8.75, respectively. **D**, relative quantification of transcripts for key genes involved in the activation and biotransformation of AFB<sub>1</sub>. Two independent measurements were taken of cDNAs prepared from three individual mice for each treatment. For each dose, the expression level for a given gene in the control was defined as 1 and the corresponding abundance changes in L- and H-dosed animals were calculated. Uppercase letters indicate significant differences in the genes expression between control and AFB<sub>1</sub>-dosed animals (<sup>b</sup> *P* < 0.05).

lipidic core from lipolysis [53,54]. More interestingly, the activation of Plin2 is normally modulated by peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) and  $\gamma$  (PPAR $\gamma$ ) signaling in various tissues, including the liver and kidney [55–57] which is in agreement with our data showing a brief but significant increase in PPAR $\alpha$  transcripts. Moreover, our data demonstrate that TCDD induced the expression of Cideb, a member of cell death activator proteins. This observation is of special importance regarding the biological roles of this protein in mediating of LD growth as well as LD-LD interactions, especially in adipocytes, and in promoting exchange of lipids and other components between LDs [58].

In addition to their protein profiles, our data indicate that exposure to AFB<sub>1</sub> also modifies LD lipid profiles and expression of key oxylipin

biosynthesis pathway genes. In this context, lipids have been used as biomarkers to assess cell status under various conditions and even as clinical diagnostic tools. For example, specific lipids associated with diabetes and obesity are routinely used in diagnosis [59,60]. The accumulation and lipid patterns of adipocyte LDs was found to be conditioned by several factors and this was mediated via fatty acid uptake or lipogenesis [61]. We also found that the acute-AFB<sub>1</sub>-dosed mice had high level of hepatic TAGs. This is in line with reports showing that acute exposure to AFB<sub>1</sub> increased levels of plasma and liver lipids notably TAGs [8,14]. Our results show that AFB<sub>1</sub>-related changes in LDs lipid profiles were accompanied by increasing transcript levels of some fatty acid metabolizing genes, notably LOXs and COXs, which were differentially induced as a function of AFB<sub>1</sub> dose. In agreement with

**Table 2**  
Values of  $R_f$ , Rt and  $\lambda_{\max}$  of AFB<sub>1</sub> metabolites.

Metabolite	Absorbance ( $\lambda_{\max}$ )	Fluorescence ( $\lambda_{\max}$ )	TLC ( $R_f$ ) <sup>a</sup>	HPLC-FD (Rt) <sup>b</sup>	Reference
Metabolite 1	364	454	0.57	8.75	This study
AFB <sub>1</sub> -exo-9,8-dihydrodiol	365	450		12.2	1,2
Metabolite 2	348	382	0.44	20.58	This study
AFB <sub>1</sub> -exo-9,8-epoxide	350	380		21.20	1,2,3,4

1 [64].

2 [40].

3 [39].

4 [74].

<sup>a</sup> Retention factor in TLC.

<sup>b</sup> Retention time in HPLC-FD.

this, several lines of evidence have demonstrated induction of LOX and COX genes in response to inflammatory stimuli and to high risk of carcinogenesis [62,63].

Interestingly, our results show that the purified LDs can catalyze the biotransformation of AFB<sub>1</sub> into the corresponding dihydrodiol and this activity is enhanced in the LD fraction isolated from AFB<sub>1</sub>-dosed animals. Indeed, the biochemical identities of the resulting metabolites, as suggested as AFB<sub>1</sub>-exo-8,9-dihydrodiol and AFB<sub>1</sub>-exo-8,9-epoxide, can be supported by several lines of analytical evidence. First, each metabolite was eluted from HPLC-FD system at retention time that is relatively similar to the values reported before [39,64]. More specifically, the purified metabolites exhibited absorbance and fluorescence spectral features that are identical to those of AFB<sub>1</sub>-exo-8,9-dihydrodiol and AFB<sub>1</sub>-exo-8,9-epoxide [40]. The formation of these metabolites under the action of a purified fraction of hepatic LD necessarily the presence of the enzymes that are typically involved in a such process, i.e., an AFB<sub>1</sub>-epoxidase and possibly a hepatic AFB<sub>1</sub>-epoxide hydrolase. The affinity of AFB<sub>1</sub> for LDs has been experimentally proven in several previous studies [11]. However, the integration of AFB<sub>1</sub>-metabolizing enzymes into the LDs was unexpected. Indeed, the enrichment of LDs with such enzymes could be explained by two possibilities; the first is that AFB<sub>1</sub>-metabolizing enzymes, or a subset of them, are LD-associated proteins, and the second is that these enzymes, or a subset of them, are stored/trapped in the LDs. Whatever the method of association, there are multiple reports of the presence of CYP450 proteins in hepatic LDs and their increasing abundance increased during diet-induced hepatic steatosis [65–67].

Although our observations suggested that the biotransformation of AFB<sub>1</sub> by LDs is likely mediated by an AFB<sub>1</sub>-epoxidase and a microsomal AFB<sub>1</sub>-epoxide hydrolase, the involvement of hepatic  $\alpha$ -glutathione S-transferases, especially GSTA3, in this process is also possible. This can be concluded by the net increase in the level of gene transcripts that we detected in the liver of highly AFB<sub>1</sub>-dosed mice. Accordingly, the most likely mechanism for the extreme sensitivity of some animal species and humans is due to the absence of functional hepatic GSTA3 and its analogs [68–70]. As a result, the AFB<sub>1</sub>-epoxide remains freely active to form DNA and RNA adducts inducing mutations, block transcription and/or alter translation [71,72]. The functional implication of GSTA3 in the detoxification of AFB<sub>1</sub> was also clearly demonstrated in the GSTA3-knockout mice which, when exposed to AFB<sub>1</sub>, exhibited a strong induction of hepatocellular carcinomas or cholangiocarcinomas [73]. Inversely, AFB<sub>1</sub>-resistant wild-type mice strains harbored a high activity of GSTA3 which is consistent with our observations in this study [19].

## 5. Conclusions

This study highlights the potential role of hepatic LDs in the rapid detoxification of AFB<sub>1</sub> when BALB/C mice, known for high resistance to AFB<sub>1</sub>, were exposed to an acute dose of AFB<sub>1</sub> for 7 days. An acute dose of AFB<sub>1</sub> induced accumulation of LDs in the livers of exposed animals.

Of particular interest, purified fraction of LD was likely able to detoxify AFB<sub>1</sub> *in vitro* into the corresponding dihydrodiol. Although the current work presents some of interesting indication on a possible involvement of hepatic LDs in the biotransformation of AFB<sub>1</sub>, future research is required for better characterization of this new mechanism. So, we suggest to pay a particular attention to the hepatic LDs and their potential roles in the detoxification of AFB<sub>1</sub>. It also opens up new horizons for additional roles of LDs in the sequestration, biotransformation and excretion of lipid-soluble toxins in general.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxrep.2020.06.005>.

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