

**Chronic amiodarone administration causes liver damage via adipose tissue ER-stress dependent lipolysis, leading to hepatotoxic free fatty acid accumulation**

**Running title:** Amiodarone induces hepatic free fatty acid accumulation

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**Figures: 6**

**Supplementary material: 1 Table**

**Word count: 2571**

**Acknowledgements:** This work was supported by the German Israeli Fund grant # I-1188-74.9/2012 to O.S and H.B and the Israel Science Foundation grant # 970/12 to O.S. The authors thank Sanna P. Sihvo (HiLIPID) for excellent technical assistance.

**Authors contributions:** EH, OS, IH and RA performed the experiments, MH performed the fatty acid analysis, EH, MH, RK and IZ analyzed the data, SF, IZ and OS wrote the paper, which was edited and approved by all authors.

**Conflict of interest:** The authors declare no conflict of interest.

**Data availability statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

## Abstract

**Background and Purpose:** Drug induced liver injury is an emerging form of acute and chronic liver disease that may manifest as fatty liver. Amiodarone (AMD), a widely used anti-arrhythmic drug, can cause hepatic injury and steatosis by a variety of mechanisms, which are not completely understood.

**Experimental Approach:** Chronic damage was induced by daily gavage of 180mg/kg body weight AMD for four consecutive days to C57Bl/6OlaHsd male mice.

**Key Results:** Chronic AMD administration induced endoplasmic reticulum (ER) stress in both liver and adipose tissue. In adipose tissue, AMD reduced lipogenesis and increased lipolysis. Moreover, AMD treatment induced ER stress and ER stress-dependent lipolysis in 3T3L1 adipocytes *in vitro*. In the liver, AMD caused increased expression of genes encoding proteins involved in fatty acid (FA) uptake and transfer (*Cd36*, *Fabp1* and *Fabp4*) and resulted in increased hepatic accumulation of free FAs, but not of triacylglycerols. In line with this, there was increased expression of hepatic *de novo* FA synthesis genes (*Srebp1* and FA synthase encoded by *Fasn*). However, AMD significantly reduced the expression of the desaturase *Scd1* and elongase *Elovl6*, detected at mRNA and protein levels. Accordingly, the FA profile of hepatic total lipids revealed increased accumulation of palmitate, a SCD1 and ELOVL6 substrate, and reduced levels of palmitoleate and *cis*-vaccenate, products of the enzymes. In addition, AMD-treated mice displayed increased hepatic apoptosis, known to be induced by lipotoxic palmitate.

**Conclusions and Implications:** Chronic AMD induced ER stress and aggravated lipolysis in adipose tissue, while inducing a lipotoxic hepatic lipid environment.

**Key words:** drug-induced liver injury, amiodarone, lipogenesis, ER stress, lipolysis.

- **What is already known:** AMD chronic administration induces hepatic lipid accumulation by several mechanisms.
- These mechanisms include induction of hepatic ER stress, impairment of  $\beta$ -oxidation and inhibition of triacylglycerol secretion.
- **What this study adds:** AMD induces adipose tissue ER stress and lipolysis and hepatic accumulation of free fatty acids and enrichment of palmitate in the total lipids.
- **What is the clinical significance:** Hepatotoxicity is a common side effect of AMD treatment, and understanding the mechanisms of the toxicity may help in finding ways to limit the liver damage.

## Introduction

Amiodarone (AMD) is a class III anti-arrhythmic drug, used for the treatment and prevention of both ventricular and atrial arrhythmias. Its primary mechanism of action in the heart is the blocking of potassium channels, but it can also block sodium and calcium channels and the beta and alpha-adrenergic receptors. AMD use is associated with various adverse effects, among them hepatotoxicity (Greene et al, 1983). Approximately 25% of patients treated with the drug have elevated liver enzymes, and 3% develop “symptomatic” hepatitis (Lewis et al, 1989, Vassalo et al, 2007). There are two clinical forms of AMD hepatotoxicity. Severe acute hepatitis develops within 24 hours after intravenous (IV) infusion (Nasser et al, 2013). Chronic toxicity, following long-term oral therapy, usually involves mild asymptomatic elevation of aminotransferases (Adams et al, 1985). The pathogenesis of long-term AMD-induced steatosis and liver injury remains unclear. One suggested mechanism is the accumulation of triacylglycerols (TAGs) via inhibition of mitochondrial  $\beta$ -oxidation of fatty acids (FAs) (Begriche et al, 2011). There is evidence that AMD disrupts hepatic lipoprotein secretion via inhibition of microsomal TAG transfer protein (MTP) activity, thus decreasing ApoB-dependent VLDL secretion (Letteron et al, 2003). Another suggested mechanism of AMD-induced steatohepatitis is via induction of endoplasmic reticulum (ER) stress. The ER is the cellular site for protein folding and lipid transport. ER stress occurs when the load of unfolded proteins exceeds the ER folding capacity, and induces a reaction to re-establish the homeostasis (Ron & Walter 2007). This reaction is termed the unfolded protein response (UPR) and involves activation of several pathways including aberrations of lipid metabolism and trafficking by the ER (Almanza et al, 2019). ER stress signaling is regulated by the key molecular chaperone Grp 78 (glucose-regulated protein 78kDa, also known as Bip). When misfolded proteins accumulate in the ER, Grp78 is sequestered, initiating the UPR. Three major ER stress sensors, IRE1, PERK and ATF6, transduce the mammalian UPR. IRE1 and PERK are activated by autophosphorylation, while ATF6 is activated by intra-membrane cleavage. Activated IRE1 splices

the mRNA of XBP1 yielding the potent transcription factor XBP1s, inducing cellular stress responses and activating the immune system. Activated PERK phosphorylates eIF2 $\alpha$ , inducing selective transcription of *Atf4*, *Atf3* and *Ddit3* in a sequential manner. Activation of the UPR leads initially to attenuation of protein synthesis and protein translocation into the ER, preventing further misfolding, followed by an increase in ER folding capacity. If the stress is not relieved, cell death is triggered, mediated to prevent the damaging effect of misfolded proteins (Szegezdi et al, 2006). While acute ER stress leads to apoptosis, chronic sub-lethal ER stress causes cellular adaptation and resistance to apoptosis (Rutkowski et al, 2006). ER stress/UPR activation has been reported in most models of hepatic steatosis and contributes to lipid accumulation and lipotoxicity (Puri et al, 2008). Using a mouse model of acute AMD treatment, we detected liver damage and lipid accumulation, accompanied by activation of ER stress/UPR (Erez et al, 2017). Importantly, AMD-induced lipid accumulation was not mediated by *de novo* hepatic lipogenesis, increased adipose tissue lipolysis or increased hepatic uptake of TAG or free FA (FFA). Rather, AMD strongly increased hepatic mRNA expression of lipid droplet proteins, particularly Cidea and Cidec, suggesting a link between ER stress and increased TAG storage.

In the present study, we investigated the mechanism of chronic AMD-induced liver injury, which may have a different mechanism from the acute injury. Induction of ER stress in adipose tissue has been documented to induce lipolysis and increase levels of circulating FFAs (Deng et al, 2012, Bogdanovic et al, 2015). Several studies have demonstrated that pharmacological inhibition of adipose tissue lipolysis with synthetic long chain FAs or deletion of adipose tissue lipase (ATGL) in mice protects from development of hepatic ER stress (Fuchs et al, 2012, Kalderon et al, 2012). This suggests that drug-induced ER stress in the adipose tissue could affect hepatic ER stress and lipotoxicity. However, this avenue of research has not yet been explored in a drug-induced liver injury (DILI) model.

Based on the hypothesis that AMD induces ER stress not only in the liver, but also in adipose tissue, we have found a novel pathway of chronic AMD-induced liver lipotoxicity. In this pathway, increased lipolysis in adipose tissue results in enhanced circulating FFAs, which are then taken up by the liver and together with increased *de novo* lipogenesis lead to hepatotoxicity, liver injury and inflammation.

## **Methods**

### **Cell culture**

The 3T3L1 cell line was obtained from the American Type Culture Collection (ATCC, CL -173) and cultured in DMEM (high glucose) supplemented with 10% Bovine Calf Serum (BCS) (Biological Industries), 100 U/ml penicillin, 100 mg/ml streptomycin in a 5% CO<sub>2</sub> humidified atmosphere and allowed to reach 70% confluence. Cells were then seeded at a density of  $3 \times 10^3$  cells/cm<sup>2</sup> in DMEM + 10%BCS and allowed to reach 70% confluency. To initiate differentiation, cells were cultured in DMEM with 10% BCS, IBMX (0.5 mM), dexamethasone (1  $\mu$ M) and insulin (10 $\mu$ g/ml) for 3days. The medium was changed to DMEM with 10% FCS and insulin (10 $\mu$ g/ml). Medium was changed every 3 days. By day 10, fully differentiated adipocytes were obtained. Adipocytes were treated with 100 $\mu$ M AMD with or without 50 $\mu$ M of an ER stress inhibitor (BIX)(Sigma) for 8h. Medium was collected for testing lipolysis and cells were harvested for RNA extraction.

### **Animals and experimental protocol**

All animal care and experimental protocols use of animals was approved by the TASMC Animal Use and Care Committee (Ethical Approval Numbers 8-1-16, 12-6-17). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, Altman & Group, 2010) and with the recommendations made by the British Journal of Pharmacology. Wild type (WT) C57BL/6JOLA<sup>Hsd</sup> mice (Envigo) were maintained in the animal facility of the Tel Aviv Sourasky Medical Center on a standard rodent chow diet fed ad libitum, kept under controlled temperature of 22–24°C and 12h light-dark cycles. We used 10 weeks old male mice weighting 24–26g. Mice were randomly divided into three groups, mice receiving saline by gavage for 4 days, mice receiving acute AMD treatment and mice receiving chronic



AMD treatment. For the acute model of AMD injury, mice were injected intraperitoneally (i.p.) with 250mg.kg<sup>-1</sup> body weight AMD-HCl (AMD) (Sanofi Winthrop Industrie, France) (Erez et al, 2017). For the chronic model of AMD injury, mice received daily gavage with vehicle (saline) or 180mg.kg<sup>-1</sup> body weight of AMD for four consecutive days. The sample size in each group was determined based on our previous studies with similar experimental protocols. The number of animals for each analysis is described in the figure legends.

### **Analysis of serum and liver metabolic markers**

Mouse serum was collected and kept in -80°C until assessed. Serum and liver triglycerides (TG), liver enzymes ALT and AST were measured using the Advia 2000 Automatic Analyzer. Total free fatty acids (FFA) concentrations in serum and liver were measured by ELISA (Abcam, ab65341).

### **Oil Red O staining**

Oil Red O (ORO) (Sigma Aldrich USA #O1391) was dissolved in propylene glycol (1% weight/volume). Frozen sections were fixed in 4% formaldehyde for 10 min, followed by three washes for 30 sec in double distilled water (DDW). Slides were subjected to 100% polyethylene glycol (PEG) for 2 min followed by 15 min incubation with ORO. Then, slides were subjected to 60% PEG for 1 min, followed by three washes in tap water. Slides were stained with Gill III Hematoxylin for 1 min and washed three times with DDW. Cover slips were mounted with glycerol.

### **TUNEL assay**

Livers of control and AMD- treated mice were fixed in formalin and embedded in paraffin. Liver sections of 5µm were used to assess apoptosis with Dead End fluorimetric TUNEL system (Promega), as previously described (Hubel et al, 2017).

### **Lipolysis assay**

Differentiated 3T3-L1 adipocytes were treated with 100 $\mu$ M AMD with or without 50 $\mu$ M Bix and with 10 $\mu$ M isoproterenol as positive control for 8h in medium with no phenol. Media were collected and tested for free glycerol using an ELISA kit for glycerol (Abcam, ab65337).

### **Western blot analysis**

Western blot analysis was performed as previously described (Uzi et al, 2013) and complied with BJP guidelines. Total protein from liver was extracted by homogenization in ice-cold RIPA buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease and phosphatase inhibitors cocktails 1:100). After centrifuging at 12,000 *g* for 15 min at 4°C, the protein concentration was determined using a Protein Assay Dye Reagent concentrate (Bio-Rad Laboratories, USA). Proteins were separated by SDS-PAGE, blotted onto cellulose nitrate membranes (Whatman, Protran BA-83) and blots were blocked for 1 h in Tris-Tween buffered saline (TTBS) with 5% milk. Blots were incubated overnight at 4°C with primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies and subjected to chemi-luminescent detection using the Micro Chemiluminescent imaging system (DNR Bio Imaging Systems). Densitometry was performed using the Image J software and expression of proteins normalized to expression of housekeeping gene p97.

### **Quantitative RT-PCR**

Total RNA was extracted from isolated livers using TriReagent (Sigma) and from epididymal adipose tissue using Qiazol and Lipid RNA extraction kit (Qiagen). Total RNA was reverse-transcribed using the High capacity cDNA RT kit (Applied Biosystems). Real time qRT-PCR was performed with the Fast SYBR Green Master Mix (Applied Biosystems) using the Corbett rotor light cycler. Quantification of the PCR signals of each sample was performed by the  $\Delta$ Ct method normalized to *Rplp0* housekeeping genes. The primer sequences are shown in **Supplementary Table S1**.

### **Hepatic and serum fatty acid profile analysis**

The FA composition of liver and serum samples was determined with gas chromatography (GC). The liver samples were homogenized and serum samples evaporated near to dryness before the transmethylation according to the recommendations of Christie (Christie, 1993) and as described previously (Käkelä et al, 2005). In brief, samples were heated with 1% H<sub>2</sub>SO<sub>4</sub> in methanol under nitrogen, and the formed FA methyl esters were extracted in two steps with hexane. The samples were dried overnight in anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. The FA methyl esters were identified with GCMS-QP2010 Ultra (Shimadzu Scientific Instruments, Kyoto, Japan) with mass selective detector (MSD) and quantified using Shimadzu GC-2010 Plus with flame-ionization detector (FID), both equipped with a Zebron ZB-wax capillary column (30 m, 0.25 mm ID and film thickness 0.25 µm; Phenomenex, Torrence CA, USA). The FA proportions were expressed as mol%, and the FAs were marked using the following abbreviations: [carbon number]: [number of double bonds] n- [position of the first double bond calculated from the methyl end] (e.g., 16:1n-7 for palmitoleate).

### **Data and Statistical analysis**

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology. Randomization was used to assign mice to the experimental groups and treatment conditions for the *in vivo* studies. Data are expressed as the mean ± SEM. Statistical significance among groups was assessed by ANOVA, followed by a Bonferroni post hoc analysis using GraphPad Prism v5 for Windows (San Diego, CA; RRID:SCR\_002798). Post hoc tests were conducted only if F was significant, and there was no variance inhomogeneity. For analysis of statistical significance between two groups we used a two-tailed student t-test. Significance was set at  $p < .05$ .

### **Materials**

Amiodarone AMD-HCl (AMD) was purchased from Sanofi Winthrop Industrie, France. BIX was from Sigma. The primary antibodies used were: SCD1 (Abcam, ab39969), ELOVL6 (Abcam, ab69857), FAS (Santa Cruz Biotechnology, sc-20140), pEIF2 (Cell Signaling #119A11 ), pIRE1α

(Abcam USA, ab48187), Bip (Abcam, ab21685), C/EBP homologous protein (CHOP) (Cell Signaling, #2895), pJNK (Cell Signaling, #9251), pHSL (Cell Signaling, S660), CD36 (Abcam, ab133625), housekeeping P97 (rabbit polyclonal prepared in the lab of prof. Boaz Tirosh, Hebrew University, Jerusalem).

## Results

We have previously shown that acute AMD administration induces hepatic ER stress and hepatic steatosis (Erez et al, 2017). We now established a chronic model of AMD liver damage, which would be more similar to human liver damage, which is caused by long term-treatment with AMD. Based on previous publications, AMD administration by gavage for 4 days had the same effect on liver damage as a more prolonged administration for 11 days (Vitins et al, 2014). In the present study, the 4-day regimen of AMD administration by gavage led to significantly increased levels of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and also caused hepatic lipid accumulation (Figure 1a and 1b). Hence, the model of 4-day administration enables us to study chronic injury. We next determined the expression of ER stress markers induced by chronic administration of AMD. As in mice with acute treatment with AMD (Erez et al, 2017), chronic AMD administration strongly induced *Atf3*, *Atf4*, *Bip*, *Erdj4* and *Dnajc3*, but not *Ddit3* (Figure 1c). Analysis of ER stress sensors revealed increased phosphorylation of IRE1 after chronic AMD treatment, as well as increased levels of BIP protein (Figure 1d). These data show that chronic AMD induces strong hepatic ER stress and damage.

### Chronic AMD induces adipose tissue ER stress and lipolysis, but inhibits lipogenesis

Long-term treatment with AMD leads to its accumulation in adipose tissue (Brien et al, 1987, Plomp et al, 1990, Wyss et al, 1990). We therefore assessed whether AMD induces ER stress simultaneously in liver and epididymal adipose tissue (epiWAT), a part of the whole visceral fat. We first evaluated the effect of acute versus chronic AMD administration on expression of ER stress markers in adipose tissue. Acute AMD slightly increased expression of *Atf3* and *Atf4*, whereas chronic AMD strongly enhanced the expression of these transcription factors, in addition to increasing expression of *Bip*, *Ddit3* and *Erdj4* (Figure 2a). We further assessed markers of ER stress and UPR in adipose tissue following chronic AMD treatment. Western blot analysis revealed

increased levels of ER sensors, phosphorylated pIRE1 $\alpha$ , and phosphorylated EIF2 $\alpha$  in adipose tissue of AMD treated mice (Figure 2b). Protein levels of Bip and CHOP (encoded by *Ddit3* gene) were also induced following AMD treatment (Figure 2b). Adipose tissue also displayed increased phosphorylation of JNK (Figure 2b), as well as strongly increased expression of its downstream target *Tnfa* (Figure 2c).

Previous studies have documented that ER stress in adipocytes *in vitro* or in adipose tissue *in vivo* induces lipolysis (Bogdanovic et al, 2015, Deng et al, 2012, Wang et al, 2016). Accordingly, following AMD administration we observed increased levels of phosphorylated hormone sensitive lipase (pHSL), the rate-limiting enzyme regulating lipolysis (Figure 3a). In line, serum levels of FFAs were increased in the AMD-treated mice (Figure 3b). When addressing the saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in gas chromatographic FA profiles of serum, we found that the relative concentrations of oleate (18:1n-9) and *cis*-vaccenate (18:1n-7) were lower in the AMD group than in the controls (Figure 3c). This observation can be explained by reduced expression of the desaturase *Scd1*, which converts stearate (18:0) to 18:1n-9 and palmitate (16:0) to palmitoleate (16:1n-7), further elongated to 18:1n-7 (Figure 3d). Collectively, these results show that chronic AMD induces ER stress and lipolysis in adipose tissue and inhibits lipogenesis.

#### **AMD treatment induces ER stress and lipolysis in 3T3L1 differentiated adipocytes *in vitro***

We wanted to validate that AMD induces ER-stress dependent lipolysis in adipocytes. For this purpose, we differentiated 3T3L1 cells into adipocytes and treated them with different doses of AMD for 8 h, in order to determine the best dose for induction of ER stress. We chose the dose of 100  $\mu$ M AMD to produce increased expression of ER stress (Figure 4a) and investigated the effect of this dose on ER stress in adipocytes after 8, 24 and 48 h. We found that optimal ER stress was induced after 8 h of treatment (Figure 4b). In order to determine whether BIX, an ER stress

inhibitor, reduced AMD-induced ER stress in 3T3L1 adipocytes, we treated the cells for 8 h with 100  $\mu$ M AMD with or without 50  $\mu$ M BIX, and found that the AMD-induced expression of *Atf4*, *Bip*, *Erdj4* and *Dnajc3*, but not *Atf3*, was abolished in the presence of BIX (Figure 4c). Finally, we assessed whether AMD induced ER stress-dependent lipolysis in 3T3L1 differentiated adipocytes. AMD induced modest, albeit statistically significant, lipolysis, which was abolished in the presence of ER stress inhibitor BIX (Figure 4d).

### **Chronic AMD induces hepatic FFA, but not TAG, accumulation.**

In our chronic AMD mouse model, serum TAG level was significantly reduced, as expected, since AMD prevents TAG secretion by inhibiting the microsomal transfer protein that induces lipidation of ApoB and VLDL secretion (Letteron et al, 2003) (Figure 5a). Surprisingly, the hepatic TAG level was similar in the AMD and control groups. In contrast, significant accumulation of FFA was found in the AMD group (Figure 5b-c). The liver can internalize and traffic serum FFAs via several cell surface molecules, such as CD36 and FA binding protein 4 (FABP4) (Canbay et al, 2007). RT-PCR analysis showed significantly increased expression of both *Cd36* and *Fabp4* following chronic AMD treatment (Figure 5d). In addition, protein levels of CD36 were also increased in the livers of AMD-treated mice (Figure 5e). Altogether, the results suggest increased uptake and accumulation of FFA in the liver.

### **AMD induces genes involved in hepatic FFA synthesis, but inhibits expression of elongation and desaturation genes leading to hepatic palmitate accumulation and apoptosis**

Liver FFAs (like the FAs subsequently incorporated into hepatic lipids) may originate not only from circulating lipoproteins or adipose tissue but also from hepatic *de novo* lipogenesis. Therefore, we next examined hepatic expression of lipogenesis-related genes. This analysis revealed that AMD significantly induced the expression of master lipogenesis transcription factor *Srebp1* and its downstream target *Fasn* (Figure 6a). However, hepatic and adipose tissue expression of the

elongation and desaturation genes *Elovl6* and *Scd1* was significantly inhibited by AMD treatment (Figure 6a). Western blot analysis of FAS, SCD1 and ELOVL6 further demonstrated upregulation of FAS and downregulation of SCD1 and ELOVL6 in the AMD group (Figure 6b).

We assessed hepatic lipid profile, using 16:0 as the substrate, the product of ELOVL6 is 18:0 and that of SCD1 is 16:1n-7. The 18:1n-9 is produced from 16:0 by ELOVL6 and SCD1, whereas the 18:1n-7 is produced when these enzymes act in reverse order. Inhibition of these enzymes would lead to hepatic accumulation of the ELOVL6 substrate 16:0. Indeed, the hepatic FA profile showed relative increase of the FAS product 16:0 and reduced relative concentrations of the SCD1 product 16:1n-7 and the ELOVL6 and SCD1 product 18:1n-7 (Figure 6c). Moreover, livers of AMD-treated mice displayed increased apoptosis (Figure 6d).



## Discussion

AMD is a commonly used anti-arrhythmic drug known to induce both acute and chronic liver damage hallmarked by steatosis. The present study shows that while acute AMD treatment induces ER stress in the liver, chronic AMD administration induces stronger ER stress in both liver and adipose tissue. Moreover, chronic AMD administration increases adipose tissue lipolysis. We found that in the chronic model of AMD injury, there is increased hepatic accumulation of FFAs, but not of TAGs. This increase in FFAs may be due both to uptake of serum FFAs and to the increased FA synthesis observed in the liver. Interestingly, AMD treatment resulted in dramatically reduced expression of SCD1 in adipose tissue and liver, and ELOVL6 in liver, leading to increased relative concentrations of hepatic 16:0, the substrate for SCD1 and ELOVL6.

The phenomenon of ER stress-induced lipolysis in adipose tissue or adipocytes has been described by several groups (Bogdanovic et al, 2015, Deng et al, 2012, Wang et al, 2016). In the present work, we show that chronic AMD treatment induces adipose tissue ER stress and ER stress-dependent lipolysis. We show that AMD induces lipolysis in 3T3L1 adipocytes, and this effect is abolished in the presence of the ER stress inhibitor BIX.

Our previous study showed that acute AMD treatment stimulated hepatic lipid accumulation, despite strongly attenuated *de novo* lipogenesis (Erez et al, 2017). In the present study, a 4-day treatment with AMD stimulated hepatic expression of lipid uptake and transport proteins CD36 and FABP4. Interestingly, while the hepatic expressions of lipogenesis master transcription factor SREBP1c and its downstream target FASN are increased in AMD-treated mice, the expression of genes encoding elongation and desaturation enzymes ELOVL6 and SCD1 is strongly attenuated. In line with these changes of enzyme expression, the hepatic FA profiles revealed that AMD-treated mice had more of the hepatotoxic ELOVL6 and SCD1 substrate 16:0 and less of their products

16:1n-7 and 18:1n-7. Of note, *Scd1* expression was also strongly attenuated in adipose tissue of AMD-treated mice.

The liver protects itself from lipotoxicity by directing incoming FFA from the serum and from *de novo* lipogenesis to synthesis of TAG, which is then stored in lipid droplets (Canbay et al, 2007). The hallmark of the steatotic liver is accumulation of TAG. However, during the development of further liver injury and inflammation and progress to steatohepatitis, FFA, ceramides and free cholesterol also accumulate (Svegliati-Baroni et al, 2019). The elongation and desaturation of FAs, taken up from serum or *de novo* synthesized by liver, are essential for the generation of TAG (Aljohani et al, 2017, Gouillou et al, 2010). The ELOVL6 elongates 16:0 to 18:0, and SCD1 catalyzes desaturation of these SFAs to the respective MUFAs 16:1n-7 and 18:1n-9. These MUFAs are the preferential substrates for TAG synthesis. Genetic or pharmacological inhibition of SCD1 sensitizes cells to SFA-induced apoptosis (Li et al, 2009). Furthermore, mice with liver-specific SCD1 deficiency have been reported to be protected from high carbohydrate diet-induced obesity and insulin resistance (Miazaki et al, 2007). The 16:0 is a known inducer of both ER stress and hepatic apoptosis (Chen et al, 2018, Pfaffenbach et al, 2010), therefore the enrichment of 16:0 observed in the AMD-treated mice could be the cause of both the hepatic ER stress and apoptosis in these mice.

Several signals have been shown to down-regulate transcription of SCD1. First, activation of TLR-TRIF-IRF3 pathway inhibits SCD1 expression (Chen et al, 2017). Second, overexpression of transcription cofactor BTG1 (B cell translocation gene 1) suppresses activity of transcription factor ATF4, which leads to reduced SCD1 transcription (Xiao et al, 2016). Third, hepatic leptin signaling via Erk1/2 attenuates SCD1 mRNA (Mauvoisin et al, 2010). Interestingly, it appears that SCD1 deficiency induces ER stress/UPR via upregulation of PGC1 $\alpha$  and activation of mTORC1 (Aljohani et al, 2019). In contrast with increased hepatic FA synthesis, AMD-treated mice displayed reduced

expression of *de novo* lipogenesis enzymes (FASN and SCD1) and their serum FA profiles with reduced 18:1n-9 and 18:1n-7 reflected the reduced expression of SCD1.

In conclusion, we show that chronic AMD administration causes unique metabolic aberrations in liver and adipose tissue. Both contribute to hepatic lipotoxicity through accumulation of FFA and reduced FA desaturation.

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## Legends to figures

**Figure 1: Chronic AMD administration induces liver injury, steatosis and ER stress.** Chronic injury was induced by administration by gavage of AMD (180mg.kg<sup>-1</sup> body weight) for four consecutive days and the mice were sacrificed 24h later. (a) Serum ALT and AST. (b) Liver Oil Red O staining in mice with or without chronic AMD treatment. Bar=100µm. (c) Expression of hepatic ER stress genes was detected by qRT-PCR and gene expression was normalized to *Rplp0*. (d) Western blots showing hepatic expression for ER stress proteins with p97 showing equal loading. Results are expressed as averages ±SE. n=7-10, statistical significance between two groups was assessed by a two-tailed student t-test. Significance was set at \*p <0.05.

**Figure 2: Chronic AMD induces ER stress in adipose tissue.** C57Bl/6OlaHsd mice were used for the acute and chronic AMD injury. The acute AMD injury by administration of one intraperitoneal injection of AMD (250 mg.kg<sup>-1</sup> body weight). Chronic injury was induced by administration by gavage of AMD (180mg.kg<sup>-1</sup> body weight) for four consecutive days. (a) Epididymal adipose tissue expression of genes involved in ER stress/ UPR was assessed by qRT-PCR and normalized to *Rplp0*. (b) Western blots show epididymal adipose tissue expression for ER stress proteins. Below is the densitometry analysis of the proteins normalized to housekeeping gene p97. (c) Epididymal adipose tissue expression of *Tnfa* assessed by qRT-PCR and normalized to *Rplp0*. Results are expressed as averages ±SE. n= 7-15. Statistical significance between two groups was assessed using a two-tailed student t-test. Significance was set at \*p <0.05. Statistical significance between three groups was determined by one-way ANOVA, followed by Bonferroni's post hoc test, \*p <0.05 significantly different from control group.

**Figure 3: Chronic AMD administration leads to lipolysis in adipose tissue *in vivo*, but inhibits lipogenesis.** (a) Western blots showing epididymal adipose tissue expression of phosphorylated hormone sensitive lipase (pHSL). The right hand panel shows densitometry analysis of pHSL normalized to p97. (b) Serum FFA measured by ELISA. (c) Serum relative concentrations (mol%) of SFA and MUFA were determined with gas chromatography. Results are expressed as averages  $\pm$ SE.  $n=4$ ,  $*p < 0.05$ . (d) Expression of lipogenesis-related genes was measured in the epididymal adipose tissue by qRT-PCR and normalized to *Rplp0*. Results are expressed as averages  $\pm$ SE.  $n=10-12$ , statistical significance between two groups was assessed by a two-tailed student t-test. Significance was set at  $*p < 0.05$ .

**Figure 4: AMD treatment induces ER stress-dependent lipolysis in 3T3L1 differentiated into adipocytes.** (a) Effect of various doses of 8 h of AMD treatment on the expression of ER stress/UPR genes in 3T3L1 differentiated into adipocytes. Expression of the genes was assessed by qRT-PCR and normalized to *Rplp0*. (b) Effect of 100  $\mu$ M AMD treatment on 3T3L1 adipocytes on ER stress/UPR genes at different time points. Expression of the genes was assessed by qRT-PCR and normalized to *Rplp0*. (c) Effect of 100  $\mu$ M AMD, with or without 50  $\mu$ M BIX on 3T3L1 adipocytes ER stress/UPR genes detected by qRT-PCR and normalized to *Rplp0*. (d) Free glycerol release from adipocytes in response to 100  $\mu$ M AMD, with or without 50  $\mu$ M BIX. Isoproterenol 1  $\mu$ M was used as a positive control. Statistical significance between several groups was determined by one-way ANOVA, followed by Bonferroni's post hoc test,  $*p < 0.05$  significantly different from control,  $\#P < .05$ , significantly different from the BIX treatment,  $@p < 0.05$  significantly different from AMD treatment.

**Figure 5: Chronic AMD administration causes hepatic FFA accumulation and increased expression of lipid uptake molecules:** Concentrations of (a) Serum TAG, (b) Liver TAG. (c) Liver FFA measured by ELISA. (d) Expression of liver *Cd36* and *Fabp4* was determined by qRT-

PCR and normalized to *Rplp0* housekeeping gene. (e) Western blot analysis shows protein levels of CD36 in livers of control and AMD- treated mice. The right hand panel shows densitometry of CD36 normalized to p97. All results are expressed as averages  $\pm$ SE. Statistical significance between two groups was determined by a two-tailed student t-test. Significance was set at \* $p < 0.05$ .

**Figure 6: Chronic AMD reduces hepatic expression of ELOVL6 and SCD1, accompanied by a decrease in their products palmitoleate (16:1n-7) and *cis*-vaccenate (18:1n-7) and increased lipotoxic palmitate (16:0).** (a) Expression of lipogenesis-related genes was assessed in the liver by qRT-PCR and normalized to *Rplp0* housekeeping gene. (b) Western blots showing hepatic expression of SCD1 and ELOVL6 protein. The right hand panel shows densitometry of the proteins normalized to p97 expression. (c) Relative concentrations (mol%) of hepatic SFA and MUFA were determined with gas chromatography. (d) TUNEL assay showing apoptosis in livers of AMD-treated mice. All results are expressed as averages  $\pm$ SE. Statistical significance between two groups was determined by a two-tailed student t-test. Significance was set at \* $p < 0.05$ .