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Development of a fatty liver model using oleic acid in primary liver cells isolated from Atlantic salmon and the prevention of lipid accumulation using metformin

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Abstract

The following study aimed to develop a fatty liver model in primary hepatocytes isolated from Atlantic salmon. In order to induce the fatty liver, oleic acid (OA) at 0.2 or 0.4 mM was used. Metformin, known to prevent and cure fatty liver in mammalian cells, was used at 1 or 10 mM for 24 hr before addition of OA to test possible prevention effect of metformin on the OA-induced fatty liver phenotype. Cells grown in 0.2 mM OA did not increase the mean number of lipid droplets, while cells grown in 0.4 mM OA increased the number of lipid droplets within the liver cells ($p < 0.0001$). Metformin pretreatment prior to OA supplementation reduced the mean number of lipid droplets. Gene expression of *ApoB100*, *CD36* and *PPARa* increased in cells treated with metformin and most so at 10 mM. On the other hand, gene expression of *LXR*, *SREBP2* and *CPT-1* decreased at both concentrations of metformin, while OA treatment did not affect these genes. Gene expression of *IL-8* increased by 0.4 mM OA ($p = 0.002$). Metformin reduced the gene expression of *IL-8*. Thus, metformin efficiently enhanced the expression of genes related to transport and oxidation of lipids in hepatic cells of salmon, but required higher concentrations of OA and metformin than those required in rodent models to increase and prevent lipid accumulation, respectively.

KEYWORDS
AMPK activation, fatty liver, liver, metformin, TAG

1 | INTRODUCTION

Feeding Atlantic salmon diets with high inclusion of plant ingredients, low in nutrients naturally present in animal ingredients, results in a more obese salmon with increased visceral and plasma TAG (Torstensen, Espe, Stubhaug, & Lie, 2011). Likewise, undernutrition of methionine increased visceral mass (Espe, Hevrøy, Liaset, Lemme, & El-Mowafi, 2008) and liver TAG accumulation (Espe, Rathore, Du, Liaset, & El-Mowafi, 2010). These findings indicated that amino acid-imbalanced diets increase adiposity in fish. In humans, obesity is associated with non-alcoholic fatty liver disease (NAFLD) in combination with insulin resistance, high plasma glucose concentrations and increased plasma circulating lipids. Increased liver TAG is widely accepted as the onset of NAFLD, and this is strongly associated with central obesity and insulin resistance in humans (Cohen, Horton, & Hobbs, 2011). In addition, methionine choline deficiency (Chawla et al., 1998; Slow & Garrow, 2006) or high-cysteine diets concomitantly containing low dietary methionine and choline result in liver

TAG accumulation in rodents (Toohey, 2014). Treatment with oleic acid (OA) is a well-recognized method for experimentally promoting the development of fatty liver in rodent models (Lian et al., 2011; Vidyashankar, Varma, & Patki, 2013). Whether TAG accumulation present in salmon liver is similar to NAFLD described in both rodent and human models is not known. Although obesity seems closely related to the presence and balance of amino acids, including sulphur amino acids, within tissue compartments in salmon (Espe et al., 2010; Torstensen et al., 2011), details in its signalling molecules and energy regulation promoting TAG accumulation in salmon are still largely unknown. Recently, Zhou et al. (2019) reported that high-fat-fed liver cells had increased TAG and cholesterol as compared to low-fat-fed cells. Further, cells treated with metformin upregulated AMP-activated protein kinase (AMPK) and had a higher expression of genes associated with lipid metabolism.

Metformin has been used to treat diabetes 2 during the past 50 years (He et al., 2009) as it increases plasma glucose clearance (Mazza et al., 2012) and reduces glucose production (Cao et al., 2014). Metformin reduced TAG accumulation in diet-induced fatty liver model in rodent (Liu et al., 2014). The reduction in lipid accumulation probably occurs through sensing of energy status as metformin phosphorylates and activates AMPK, the main regulator of energy sensing inside cells (Viollet et al., 2012). Further, metformin administration reduced body weight and obesity in humans. However, the mechanisms behind this weight reduction are not fully understood (Malin & Kashyap, 2014). Additionally metformin administration is reported to reduce endoplasmatic reticulum stress (Simon-Szabó, Kokas, Mandi, Keri, & Csala, 2014) which is often associated with increased inflammation and lack of appropriate antioxidative compounds (Esser, Legrand-Poels, Piette, Scheen, & Paquot, 2014; Matsunada & Shimomura, 2013; Pitocco, Tesauro, Allesandro, Ghirlanda, & Cardillo, 2013; Vonghia, Michielsen, & Francque, 2013). Panserat et al. (2009) reported that rainbow trout fed high-carbohydrate diets reduced hyperglycaemia following metformin treatment and had higher activities of lipogenic enzymes. Nutrients' ability to prevent TAG accumulation is of high interest in fish nutrition, while treatment of TAG accumulation is of lower interest. The following experiments aimed at developing a model for TAG accumulation using primary liver cells isolated from Atlantic salmon using OA, furthermore, to assess whether metformin might have the ability to prevent the OA-induced TAG accumulation in these cells, thus making the model suitable to be used in studies evaluating effect of nutrient supply on changes leading to liver TAG accumulation.

2 | MATERIALS AND METHODS

2.1 | Isolation of primary liver cells

The aquarium in Bergen housed the Atlantic salmon used in the current study. The fish were fed the same commercial feed. Primary liver cells were isolated from Atlantic salmon as described elsewhere (Espe & Holen, 2013). In brief, primary liver cells were isolated from Atlantic salmon with an average BW of 512 ± 75 g (two males and three females). The liver was perfused with 0.09 M HEPES buffer containing 1.4 M NaCl, 0.067 M KCl and 0.03 M EDTA (pH 7.4) until free from blood; thereafter, livers were digested with collagenase (0.1% dissolved in the HEPES buffer). Cells were squeezed through a Falcon cell strainer, and the isolated cells were dissolved in Leibovitz-15 (L-15) media and washed three times in L-15 media. Cell viability and counting were done using Trypan blue (Lonzo, MedProbe). Before handling, fish were anaesthetized with metacaine (MS222, 5–8 g/L) and killed with a blow to the head before liver cells were isolated. The Norwegian Board of Experiments with Living Animals approved the protocol applied.

2.2 | Cell studies

The isolated primary liver cells were plated in laminin-coated 6-well plates at a concentration of 0.8 million/cm^2 as described elsewhere (Espe & Holen, 2013; Holen et al., 2014). Isolated cells were plated and grown in complete Leibovitz-15 media (cL-15), that is L-15, added 1% glutamax, 1% antibiotics and 10% calf serum (Espe & Holen, 2013). In the pilot study, the media was supplemented with 1.0 mM or 10 mM metformin for 1 or 24 hr prior to OA treatment (0.2 and 0.4 mM OA). The OA used was a water-soluble OA from Sigma. All cells were harvested 24 hr post-OA addition. In the main trial, the cells were pretreated with metformin at 1 mM and 10 mM for 24 hr before the OA was added and harvested 24 hr post-OA supplementation. In both trials, cells added only OA or metformin were included. In addition, a negative control grown in the cL-15 media without addition of OA or metformin was included. Plates were incubated in a dark normal atmosphere incubator at 9°C (Sanyo Incubator). Mean accumulation of lipid droplets and the expression of genes associated with lipid metabolism and lipid transport were used to evaluate the concentrations and time necessary for the model to work. The outlines of the used protocols are shown in Table 1 and Supporting Information Figure S1.

TABLE 1 The trial based on the pilot results used 0.2 or 0.4 mM oleic acid (OA) and 10 or 1 mM metformin following the design below

Control 1	1 mM metformin 2	10 mM metformin 3
0.2 mM OA 4	1 mM metformin + 0.2 mM OA 5	10 mM metformin + 0.2 mM OA 6
0.4 mM OA 7	1 mM metformin + 0.4 mM OA 8	10 mM metformin + 0.4 mM OA 9

Cells were treated with metformin for 24 hr or not added any metformin before wells were added OA at either 0.2 or 0.4 mM. All cells were harvested 24 hr post addition of OA. A total of 9 wells (i.e. treatments) and cells isolated from five individual fish were sampled (n = 5).

2.3 | Harvesting of the cells

Monolayer liver cells without media were added 300 µl cold lysis buffer (CelLytic™ M cell lysis reagent, Sigma-Aldrich) of which were added protease/phosphatase inhibitor cocktail 100× from Cell Signaling (BioNordica#5872) as described by the supplier. The supernatant from lysed cells was harvested after centrifugation (15 min at 10,000 *g*). Supernatants were stored at −80°C until analysed by Western blots. Likewise, monolayer liver cells without media were added 600 µl RLT Plus buffer (RNeasy® Plus Kit, Qiagen) as described by the supplier, homogenized using a syringe and frozen at −80°C until RNA extraction.

2.4 | Gene expression analysis

RNA was extracted using RNeasy® Plus Kit (Qiagen) according to the manufacturer's instructions and frozen at −80°C for further processing. The quantity and quality of RNA were tested using the NanoDrop Spectrophotometer and the RNA 6000 Nano LabChip® Kit (Agilent Technologies, Palo Alto, CA, USA) as described earlier (Espe et al., 2015; Holen et al., 2014). A two-step real-time quantitative polymerase chain reaction (RT-qPCR) protocol was used to measure the mRNA levels of the target genes in liver cells as described elsewhere (Espe et al., 2015; Holen et al., 2014). The C_t values obtained were normalized using the geNorm tool as described (Olsvik, Lie, Jordal, Nilsen, & Hordvik, 2005) and presented as mean normalized gene expression (NGE). EGF1a and beta-actin were used as reference genes. Genes involved in lipid synthesis, degradation and transport including some markers for inflammation were addressed (Holen et al., 2014). The primers used are shown in Supporting Information Table S1.

2.5 | Cells for Transition Emission Chromatography

Electron microscopy was used to evaluate structural changes in mitochondria and counting mean lipid droplets within liver cells following each treatment. Lipid droplets were counted in TEM pictures of 20 different cells per treatment. Results are presented as mean number of lipid droplets/cell. Transmission electron microscope (TEM, Jeol JEM-1230, Molecular Imaging Center, University of Bergen) was used to image the cells.

2.6 | Western blot

Western blots were run as described previously (Espe & Holen, 2013) with modifications published elsewhere (Espe et al., 2015). In short, samples were mixed with Laemmli buffer (1:1) and run on precast 10% SDS gels using a Bio-Rad Mini-PROTEAN® Cell. Thereafter, the proteins were blotted on polyvinylidene difluoride (PVDF) membranes, blocked and incubated overnight with the respective antibodies tested (1:1,000). The following primary rabbit antibodies from Cell Signaling Technology (BioNordika, Oslo, Norway) were used: P-AMPKα (#2531), AMPKα (#2532), P-ACC (#11818) and ACC (#3662). Then, HRP-linked anti-rabbit IgG (Cell Signaling, #7074, 1:500) was used as the secondary antibody. Amersham ECL Advance™ Western Blotting Detection Kit (GE Healthcare and Chemi Chemiluminescence) and Image Capture (Syngene, Cambridge, UK) were used to detect the proteins. Results are reported as abundance of proteins normalized against actin (Cell Signaling # 4970) and shown relative to the negative control.

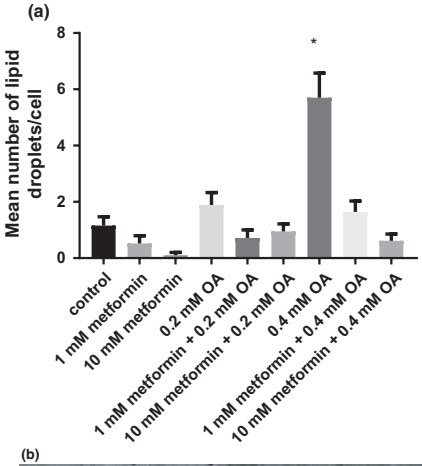
2.7 | Statistical analysis

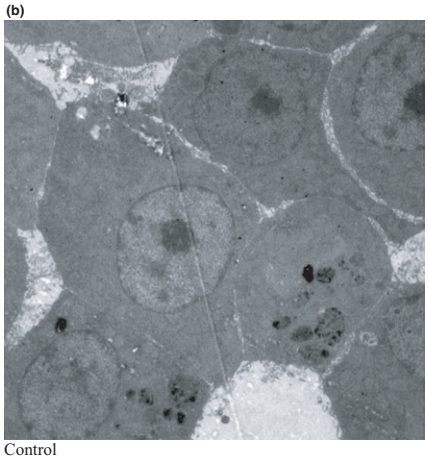
Differences in normalized gene expression (NGE) between treatment means were analysed by ANOVA followed by Tukey's post hoc test. *p*-values < 0.05 were accepted statistically different. When the data did not fulfil the assumptions for normality and homogeneity in variance, the non-parametric Kruskal–Wallis test was used. Western blot data and mean number of lipid droplets per cell were analysed using Dunett test ($p < 0.05$). Values are reported as the means ± standard error (*SE*, 6 or 5 individual fish, the pilot and trial, respectively) with exception of Western blot analyses that were analysed in three fish per treatment.

3 | RESULTS

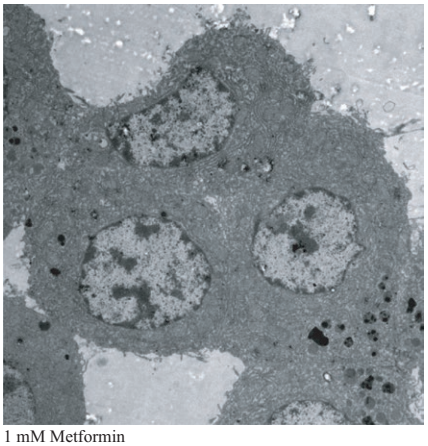
3.1 | Determination of time and concentration necessary to develop the model

First, a regression using metformin concentration ranging from 0.1 mM to 40 mM was run with the aim of testing what concentration of metformin the primary liver cells tolerated. Exceeding 10 mM metformin did not give any change in gene expression of the tested gene (Supporting Information Figure S2 showing *manganese superoxide dismutase*, *MnSOD*). A pilot study using OA and metformin at different concentrations and times of metformin pretreatment was run to address the time of pretreatment necessary to affect gene expression in the liver cells of salmon. The concentrations and times tested are outlined in Supporting Information Figure S2. The pilot model with 1 hr pretreatment with metformin (1 mM) followed by 24-hr OA treatment (0.2 mM) did not result in any change in gene expression of genes associated with lipid metabolism as compared to the control. Here, it is shown *peroxisome proliferator-activated receptor alpha* (*PPARa*, Supporting Information Figure S3a) and *acetyl CoA carboxylase* (*ACC*, Supporting Information Figure S3b). Increasing the pretreatment time of metformin from 1 to 24 hr prior to the OA supplementation did not result in any difference in the gene expression either. However, at 24 hr and 10 mM metformin affects the gene expression of (*PPARa*), while OA concentration at the double of the rodent model (0.4 mM) did not affect *PPARa*, but affect the gene expression of *ACC*. This was also the case of several other tested genes (not shown). It was evident that pretreatment with metformin for a longer period than 1 hr was necessary to affect gene expression. The experiment therefore used 24 hr pretreatment with 1.0 or 10 mM metformin, followed by 0.2 or 0.4 mM OA treatment. Cells were harvested 24 hr post-OA addition. Controls not added anything as well as controls added only OA or only metformin were included. Table 1 shows the arrangement of the wells used in the trial.





Control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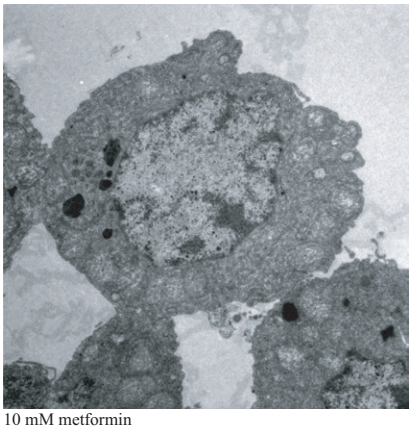
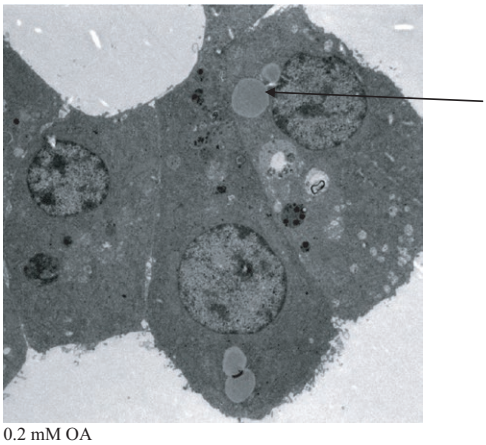
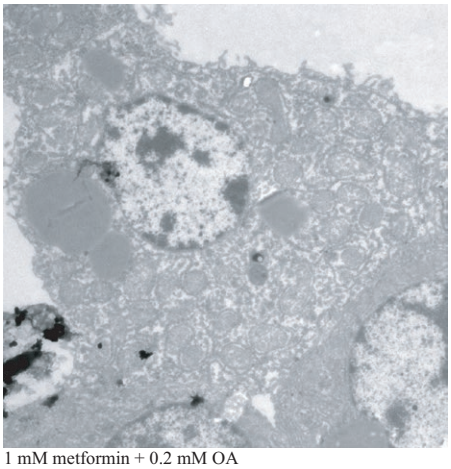
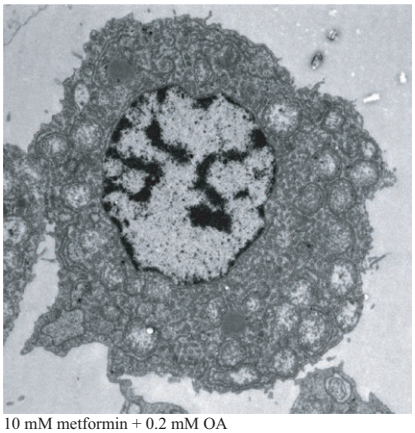

10 mM metformin


0.2 mM OA


1 mM metformin + 0.2 mM OA


10 mM metformin + 0.2 mM OA

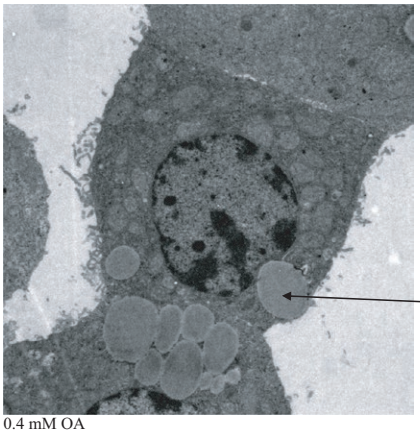

0.4 mM OA

FIGURE 1

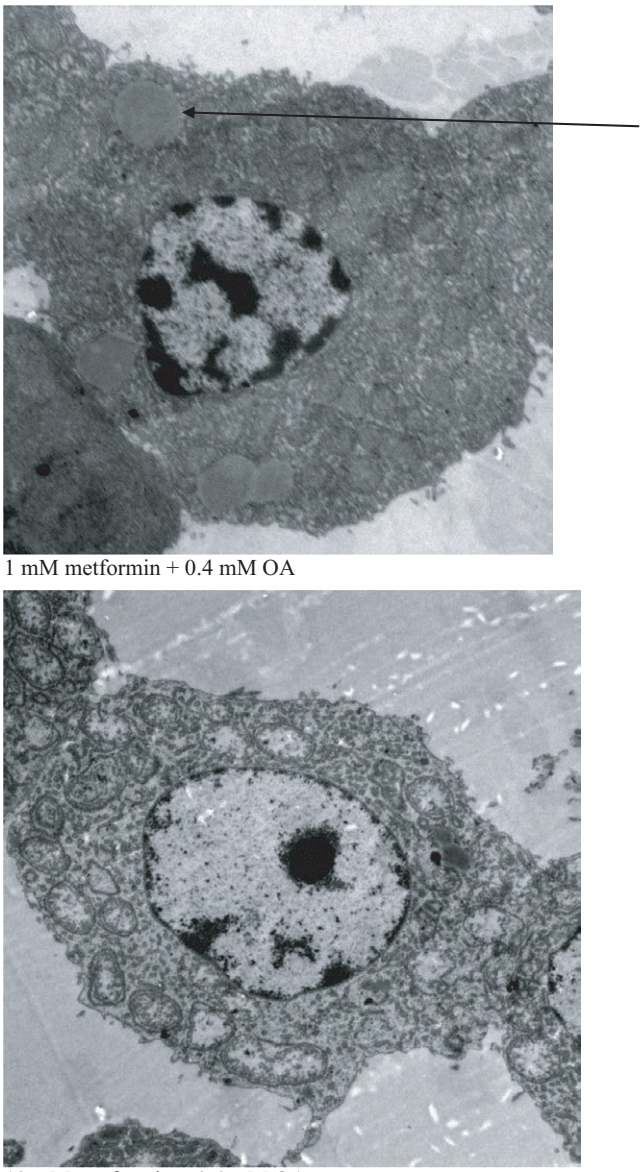


FIGURE 1 The mean number of lipid droplets following the different treatments. Droplets were counted in 20 cells per treatment from TEM pictures (a). Cells grown in 0.4 mM oleic acid (OA) contained significantly more lipid droplets than the other treatments ($p < 0.0001$, Dunett). Values are mean lipid droplets per cell ± *SE*. The respective TEM from each treatment is shown (b). Arrows show lipid droplets. Cells treated with 10 mM metformin started to degrade the mitochondria as compared to cells treated with 1 mM metformin

3.2 | The development the fatty liver model and possibly preventive effect of TAG accumulation by metformin

Cells grown in 0.4 mM OA had significantly more ($p < 0.0001$) lipid droplets in the liver cells as compared to the negative control (Figure 1a). Even though the number of lipid droplets also increased in cells grown in 0.2 mM OA, they were not significantly different from the control cells. Pretreatment with metformin prior to OA supplementation prevented the accumulation of lipid droplets at both concentrations tested. However, the TEM pictures showed that cells grown in 10 mM metformin seem to have degraded mitochondria (Figure 1b). Metformin increased phosphorylation and thus activation of adenosine monophosphate-activated protein kinase (AMPK) in a dose-dependent manner ($p = 0.041$, Figure 2). Treatment with the higher metformin concentration (10 mM) prior to OA treatment at 0.4 mM tended to increase the amount of P-AMPK, but not significantly. Phosphorylation and thus inactivation of ACC were not affected by treatments ($p = 0.32$, not shown). Neither were the respective unphosphorylated proteins affected by treatments ($p = 0.51$ and $p = 0.54$ for AMPK and ACC, respectively, not shown).

Cells pretreated with 10 mM metformin had higher gene expression of *PPARa* when treated with OA at both 0.2 and 0.4 mM (Figure 3a, $p < 0.0001$ OA versus metformin pretreatment +OA), while cells grown in 1 mM metformin did not affect the *PPARa* gene expression. Cells grown without pretreatment with metformin, but added OA did not differ from cells grown in the cL-15 media (the control group). Likewise, the gene expression of the *cluster of differentiation 36* (*CD36*) increased in cells grown in 10 mM metformin (Figure 3b, $p < 0.0001$). Cells pretreated with 10 mM metformin before OA supplementation increased *CD36* expression. The gene expression of *Apolipoprotein B100* (*ApoB100*) was similar to *CD36* as 10 mM metformin pretreatment increased its expression independent of adding OA at 0.2 or 0.4 mM (Figure 3c, $p = 0.0006$). Gene expression of *ACC* was similar to *ApoB100* and *CD36* as 10 mM metformin pretreatment prior to OA supplementation increased its gene expression (Figure 3d, $p = 0.02$).

On the other hand, both 1 and 10 mM metformin reduced the gene expression of *liver x receptor* (*LXR*) and this reduced gene expression also persisted after OA treatments at both 0.2 and 0.4 mM OA (Figure 3e, $p < 0.0001$). Gene expression of *sterol regulatory element-binding protein* 2 (*SREBP2*) showed the same trend as *LXR* expression in that metformin decreased its gene expression (Figure 3f, $p < 0.0001$) at both 1 and 10 mM. The gene expression of *carnitine palmitoyltransferase 1 (CPT-1)* followed the same pattern as *LXR* (Figure 3g, $p < 0.0001$). Treatment with either 0.2 or 0.4 mM OA had no effect on gene expression as compared to the control cells grown in the cL-15 media. The gene expression of *caspase-3*, *acetyl CoA oxidase* (*ACO*), *PPARg* and *fatty acid synthase* (*FAS*) was unaffected by treatments (not shown).

Liver cells treated with OA tended to have higher expression of the pro-inflammatory *interleukin-8* (*IL-8*) at both 0.2 and 0.4 mM OA, but IL-8 expression only differed significantly from control cells treated with 10 mM metformin (Figure 4, $p = 0.0005$). Treating cells with metformin prior to the OA treatment generally decreased the gene expression of *IL-8*, but did not reach a statistical difference. The gene expression of the pro-inflammatory *interleukin-1b* and *interleukin-6* (*IL-1b and IL-6*) was too low to be calculated.

4 | DISCUSSION

Metformin reduces accumulated liver lipids (Kim et al., 2016; Liu et al., 2014; Malin & Kashyap, 2014), but is mainly used to treat TAG

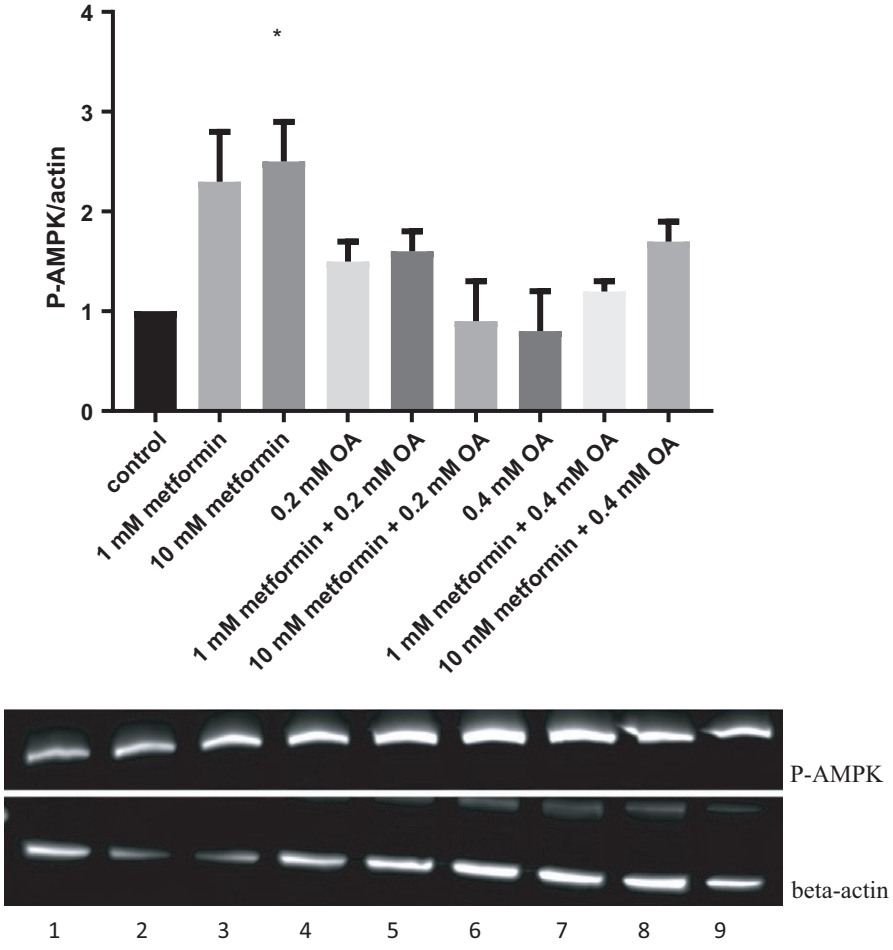


FIGURE 2 As in mammalian models, metformin phosphorylated and activated AMPK (i.e. treatments 1–3; $p = 0.041$). Even though P-AMPK also increased following 0.4 mM OA and metformin, the differences were not statistically different from the control. Activation of AMPK was normalized towards actin and calculated relative to the control group (Dunett, $p < 0.05$). Values are mean ± *SE*, $n = 3$

accumulation associated with diabetes and not to prevent lipid accumulation in humans (He et al., 2009). The current trial did not use metformin to reduce and cure the fatty liver. Metformin was used to test whether metformin had the possibility to prevent TAG accumulation, which is more relevant for fish nutrition as deficiency of nutrients increases TAG accumulation and liver size (Espe et al., 2010; Skjærven et al., 2018). The establishment of a fatty liver model using OA in primary liver cells isolated from Atlantic salmon succeeded. Addition of 0.4 mM OA increased the mean number of lipid droplets in the liver cells, while 0.2 mM OA did not differ from the control cells (Figure 1a). Our findings are in line with Liu et al. (2014) who reported that primary hepatocytes isolated from mice treated with OA (0.2 mM and 18 hr) accumulated TAG, while cells grown in media pretreated with metformin (0.5 mM and 12 hr) prior to OA treatment reduced their TAG content within the cells. Opposite to the rodent models (Liu et al., 2014), fish cells needed longer pretreatment time with metformin prior to OA treatment to prevent lipid accumulation within the liver cells. Likewise, Moracova et al. (2015) increased OA from 0.1 to 2.0 mM in primary hepatocytes isolated from rats, which resulted in a dose-dependent increase in TAG accumulations. These results also are in line with the current study, but HepG2 cells needed 0.25 mM OA to induce lipid accumulation (Lian et al., 2011), which is less than the 0.4 mM needed by the salmon cells. Lately, also Zhou et al. (2019) used 0.4 mM OA and 0.2 mM metformin in liver cells isolated from blunt snout bream and found that metformin upregulated AMPK and attenuated lipid accumulation in high-fat-fed cells. Thus, salmon liver cells responded as the rodent model towards OA treatment, but needed higher concentration of OA as compared to the mammalian models as well as longer pretreatment time with metformin to attenuate the OA-induced TAG accumulation. These differences are probably due to the fact that salmon is a cold-water species and cells were incubated at lower temperatures than those commonly used for mammalian cells.

As in the mammalian models, metformin administration phosphorylated and activated AMPK, while phosphorylation and inactivation of ACC did not occur. This is opposite to the results reported by Tokubuchi et al., (2017) who reported an increase in both P-ACC and P-AMPK following OA treatment. Activation of AMPK by phosphorylation promotes ATP-producing pathways as glycolysis, fatty acid oxidation and mitochondrial biogenesis, concomitantly inhibiting ATP-consuming pathways as glucogenesis, glycogen synthesis, fatty acid synthesis and protein synthesis (Wong, Quinn, & Brown, 2006). In addition, AMPK activation inactivates ACC and activates malonyl-CoA carboxylase, thereby decreasing fatty acid and cholesterol synthesis (Choi et al., 2007; Mazza et al., 2012; Wong et al., 2006). Furthermore, activation of AMPK is known to activate PGC1a, the main mitochondrial biogenesis sensor mirroring mitochondrial DNA quality and maintenance (Barbieri et al., 2016).

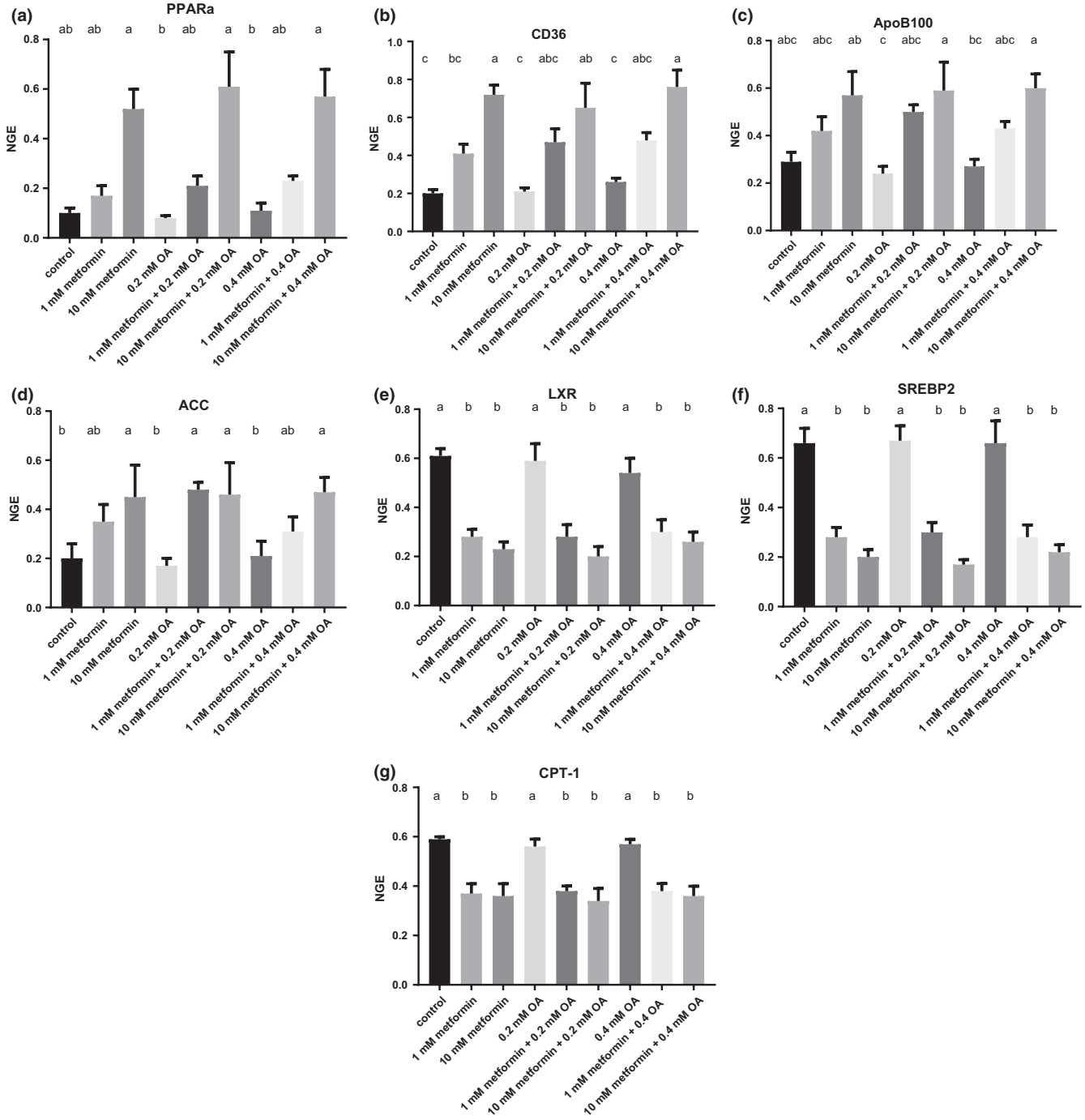


FIGURE 3 Metformin increased gene expression of *PPARa* (a), *CD36* (b), *ApoB100* (c) and *ACC* (d) and attenuated gene expression of *LXR* (e), *SREBP2* (f) and *CPT-1* (g). All values are means ± *SEM*, $n = 5$ (ANOVA, Tukey $p < 0.05$). 10 mM metformin generally resulted in higher or lower response than 1 mM metformin. The supplementation of oleic acid (OA) had low impact on the gene expression of the analysed genes

Acetyl CoA carboxylase is a key regulator of mitochondrial fatty acid oxidation and is involved in regulating both oxidation and synthesis of fatty acids depending on being phosphorylated or not. P-ACC is inactivated, and less malonyl-CoA is produced. Low malonyl-CoA thus reduces the fatty acid synthesis and increases the fatty acid oxidation (Song et al., 2015). The present trial could not report inactivation of ACC in salmon, probably due to relative high variations between the analysed fish. However, unphosphorylated ACC might act opposite to P-ACC reducing oxidation of fat in line with the reduced gene expression of CPT-1 indicating a reduced fat oxidation in the current trial. Inactivation of fatty acid oxidation in the proposed model following metformin pretreatment was not expected. Therefore, more studies testing which protein is activated or not are needed to unravel which proteins are present.

The effects observed in expression of genes affecting lipid metabolism were due to metformin, and minor effects of OA treatments were present in the current trial. Woo et al. (2014) reported that gene expression in cells isolated from obese mice showed reduced gene

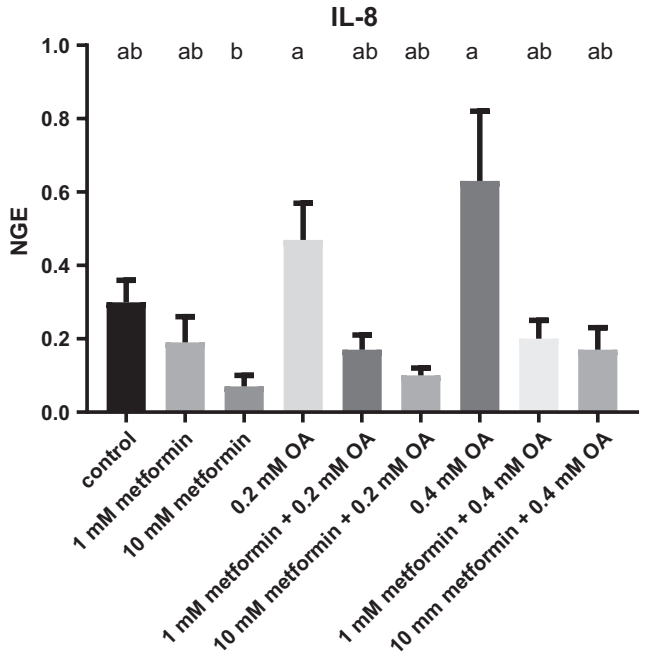


FIGURE 4 Addition of oleic acid (OA) increased the gene expression of *IL-8* at both 0.2 and 0.4 mM compared to cells grown in the respective controls ($p = 0.002$, ANOVA followed by Tukey, $p < 0.05$), indicating that OA promotes inflammation in primary liver cells and metformin has a protective role in reducing inflammation

expression of *ACC*, *FAS* and *CPT-1* in cells pretreated with 0.5 mM metformin prior to treatment with 0.25 mM palm oil. Metformin also increased the gene expression of *PGC1a* as well as the diameter of the adipocytes (Aatsinki et al., 2014). *PGC1a* is a master regulator of oxidative phosphorylation as well as mitochondrial biogenesis. Increased gene expression of *PGC1a* also increases the expression of *PPARa* also improving the mitochondrial fatty acid oxidation, while any increased bile acids will suppress *PGC1a* through *LXR*. The present study did not find any differences in *PGC1a* expression, but *PPARa* and *ACC* both increased following metformin administration, which indicates a possible higher oxidation of lipid in these cells. One should expect increased expression of CPT-1 following metformin pretreatment, but in the current trial expression of *CPT-1* was reduced in metformin-pretreated cells, while *FAS* and *ACO* were unaffected by treatment. The reduced expression of *CPT-1* decreased following metformin pretreatment, and no effects on *ACO* and *FAS* were unexpected. Zhou et al. (2019) reported that the gene expression of PPARa, CPT-1 and PGC1a all was upregulated following metformin treatment in liver cells isolated from blunt snout bream. The reason we did not find increased CPT-1 expression in salmon liver following metformin needs to be investigated in more detail. In the rodent model, *CPT-1* expression generally increased following treatment with metformin (Tokubuchi et al., 2017). It should be noted that *CPT-1* expression was not significantly affected by treatment in the pilot study. Metformin pretreatment at 10 mM significantly increased the transcription of *PPARa*, which is well known for regulating and increasing several genes in lipid metabolism and increasing several genes related to lipid oxidation. The increase in *PPARa* by 10 mM metformin was independent of the added concentration of OA, while cells grown in either 1 mM metformin or in OA did not differ from the controls. This is in line with a previous study involving rainbow trout hepatocytes where an increase in *PPARa* following OA (1 mM) treatment was reported (Woo et al., 2014).

ApoB100 is necessary for assembly of VLDL and exports of lipids from the liver (Vance, Walkey, & Cui, 1997; Watkins, Zhu, & Zeisel, 2003). In the current trial, gene expression of *ApoB100* was significantly higher following pretreatment with metformin especially at the 10 mM concentration. Depletion of *CD36* reduced VLDL secretion from liver in mice (Nassir, Adewole, Brunt, & Abumrad, 2013). In the current study, pretreatment with metformin increased the *CD36* gene expression indicating a possible increased secretion of VLDL contributing to the reduced amount of lipid droplet observed in these groups. Probably, both ApoB100 and CD36 participated in increased export of lipids from the liver cells leading to the low lipid phenotype observed in the current trial. The metformin concentration needed to prevent lipid accumulation in Atlantic salmon cells should be followed up using lower metformin concentrations than 10 mM. Especially as the TEM pictures indicated that 10 mM might have been too high as the mitochondria seem degraded.

SREBP-2 is known to upregulate genes involved in biosynthesis of cholesterol and its uptake, while LXR is a cholesterol sensor known to upregulate cholesterol efflux genes (Wong et al., 2006). Both *SREBP2* and *LXR* gene expression were significantly downregulated following the addition of metformin in hepatocytes from salmon, indicating that metformin administration reduced cholesterol synthesis and its efflux from the hepatocytes. This is in line with previous studies on metformin treatment of obese children with metabolic syndrome, where metformin treatment reduced circulating cholesterol (Luong, Oster, & Ashraf, 2015). AMPK activation, as is present following metformin administration in the current trial, was reported to suppress *LXR* expression and reducing lipid accumulation in HepG2 cells (Lee et al., 2015). As P-AMPK was higher following metformin treatment in the current trial (i.e. cells incubated in 10 mM), this might have influenced the expression of LXR expression.

The increased transcription of the cytokine *IL-8* after OA addition indicates a pro-inflammatory response to the free fatty acid supplementation, while the addition of metformin at the higher concentration (10 mM) prevented this response. High concentration of free fatty acids is toxic to the liver cells, and some of this toxicity is expressed through increased inflammatory response (Feldstein et al., 2004; Yao et al., 2011). The reduced transcription of cytokines following metformin pretreatment is in line with results reported by Aatsinki et al. (2014); Arai et al. (2010), who found reduced expression of *IL-1b* and *IL-6* following metformin treatment. The present study analysed both *IL-1b* and *IL-6* together with IL-8. However, only *IL-8* is reported due to the very low gene expression of *IL-1b* and *IL-6*. The lower gene expression of *IL-8* following metformin treatment at both 1 and 10 mM indicated that metformin might have an anti-inflammatory function also in the salmon liver cells. This is in line with a report (Simon-Szabó et al.,

2014) where it was observed that metformin influences pro-inflammatory cytokines and reduced active cleaved caspase-3 thus improving viability of the cells. The active cleaved caspase-3 was not analysed in the present study, only its gene expression of which did not change upon treatments; thus, it is not possible to conclude whether cell viability was affected in the current study.

5 | CONCLUSION

The present study succeeded in building a model of the fatty liver in primary liver cells isolated from Atlantic salmon. The model in question seems to be pretty much like the mammalian, though requiring higher concentration of OA. Metformin treatment probably reduced liver lipid accumulation by increasing *PPARa*, *ACC* and transport of lipids out of the hepatocytes (*ApoB100* and *CD36*). In addition, OA supplementation without any metformin pretreatment increased the gene expression of *IL-8* compared to cells treated with 10 mM metformin, probably affecting inflammation. To prevent accumulation of lipid droplets in liver cells of Atlantic salmon, higher concentration of metformin and longer treatment time are required than the proposed mammalian model.

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CONFLICT OF INTEREST

There is no conflict of interest to report.

AUTHOR CONTRIBUTION

ME planned and ME and EH ran the cell study with the help of SX and SC. All authors analysed the samples collected. ME was responsible for drafting the manuscript. All authors approved the final manuscript.

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