doi: 10.4149/gpb_2019016

Effect of glucagon-like peptide-1 analogue liraglutide on primary cultures of rat hepatocytes isolated from lean and steatotic livers

Josef Fontana[1], Otto Kučera[2], Michal Anděl[1] and Zuzana Červinková[2]

[1] *Centre for Research on Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Prague, Czech Republic*
[2] *Department of Physiology, Faculty of Medicine in Hradec Králové, Charles University, Hradec Králové, Czech Republic*

Abstract. Non-alcoholic fatty liver disease and its complications are frequent causes of liver-related morbidity and mortality. Incretin glucagon-like peptide-1 (GLP-1) affects liver functions and metabolism. Although GLP-1 analogues are widely used in clinical practice, information regarding their potential toxic effect on hepatocytes *in vitro* is missing. Therefore, we evaluated the effect of GLP-1 analogue liraglutide on activity of caspases 3/7, cell viability and oxidative stress in primary cultures of hepatocytes. Primary cultures isolated from male Wistar rats fed a standard (ST1-group, 10% energy from fat) or a high-fat diet (HF-group, 71% fat) for 10 weeks were incubated with liraglutide (0.1–1000 nmol/l) for 24 h. Activities of caspases 3/7 and cellular dehydrogenases (WST-1), lactate dehydrogenase (LDH) leakage and oxidative stress (malondialdehyde concentration and DCFDA assay) were evaluated. HF-groups *vs.* ST1-groups showed higher caspases activity, LDH leakage and MDA production ($p < 0.001$) and lower cellular dehydrogenases activity ($p < 0.01$). Liraglutide induced a dose-dependent decrease of caspases activity in both groups, reduction of oxidative stress in HF-animals and exerted no negative effects on other parameters. In conclusion, GLP-1 analogue liraglutide decreased activity of caspases 3/7, reduced ROS production and didn't exhibit negative effects on cell viability and oxidative stress in primary cultures of hepatocytes isolated from lean and steatotic livers.

Key words: GLP-1 — Liraglutide — Hepatocytes — Cell viability — ROS

Abbreviations: DCFDA, dichlorodihydrofluorescein diacetate; DPP4, dipeptidyl peptidase 4; ER, endoplasmic reticulum; Ex4, exenatide; FA, fatty acids; GLP-1, glucagon-like peptide-1; HFGD, high-fat gelled diet; LAP, laparotomy; LEAN, liraglutide efficacy and action in non-alcoholic steatohepatitis clinical trial; LIRA, liraglutide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; SIRT1, silent mating type information regulation 2 homolog 1; TAG, triacylglycerol; TBARS, thiobarbituric acid-reactive substances; TGF-β1, transforming growth factor β1.

Introduction

Glucagon-like peptide-1 (GLP-1), along with gastric inhibitory polypeptide (GIP, also known as the glucose-dependent insulinotropic peptide), belong to a group of peptide hormones referred as incretins. These hormones are produced in response to the presence of chymus in gastrointestinal tract, GLP-1 predominantly in endocrine L-cells of human ileum and colon. GLP-1 occurs as two active molecules: GLP-1(7-36) amide and GLP-1(7-37) in blood plasma (Eissele et al. 1992; Baggio and Drucker 2007; Holst 2007). Their biological half-life in the bloodstream is very short (1–2 min) due to a cleavage catalyzed by a degrading enzymes dipeptidyl peptidase 4 (DPP4) and neutral endopeptidases (Vilsboll et al. 2003). Molecules of active GLP-1 have several important functions in the human body: e.g. stimulation of postprandial insulin release from pancreatic beta cells, suppression of gastrointestinal tract motility and appetite or modulation of metabolic processes in tissues (Nauck et al. 1986; Baggio

Correspondence to: Josef Fontana, Centre for Research on Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Ruska 87, 100 00 Prague 10, Czech Republic
E-mail: josef.fontana@lf3.cuni.cz

and Drucker 2007). Actions of GLP-1 can be mimicked by its analogues that have a longer half-life compared to original hormone molecule. There are several GLP-1 analogues available, e.g. exenatide (Ex4) and liraglutide. Liraglutide is a GLP-1 analogue with 97% amino acid sequence homology to native human GLP-1. It contains a fatty acid side-chain that enables its bound to plasma protein albumin resulting in prolongation of its half-life to 13 h. This analogue is routinely used in treatment of patients with type 2 diabetes mellitus (Agersø et al. 2002; Russel-Jones and Gough 2012).

Excessive accumulation of triacylglycerols (TAG) in the liver is termed as fatty liver disease. Non-alcoholic fatty liver disease (NAFLD) is one of the most frequent liver diseases in western countries, affecting 20–30% of adult population. In its initial stages, NAFLD is characterized by an accumulation of ectopic fat in the liver. NAFLD is histologically defined by the presence of liver steatosis exceeding 5% of hepatocytes, regardless of whether it is macrovesicular, mixed or microvesicular (Kleiner et al. 2005; Kučera and Červinková 2014). 10–25% of patients with NAFLD can develop more severe form termed as non-alcoholic steatohepatitis (NASH). NASH development is characterized by several processes: immune cells recruitment, endoplasmic reticulum stress, mitochondrial dysfunction, oxidative stress and defective autophagy, resulting in hepatocyte ballooning, apoptosis and activation of hepatic stellate cells (Tsuchida and Friedman 2017; Gonzalez-Rodriguez et al. 2018). Their subsequent differentiation to myofibroblasts producing collagen I promotes development of liver fibrosis (Mehal et al. 2011; Valdecantos et al. 2018). 10–15% of patients with NASH can develop hepatocellular carcinoma. Etiopathogenesis of NAFLD and NASH is closely linked to insulin resistance (liver manifestation of the metabolic syndrome), lipotoxicity, oxidative stress and apoptosis (Ruhl and Everhart 2004; Kim and Younossi 2008; Vanni et al. 2010). Scheme of the possible progression of NAFLD is depicted in Fig. 1. Nowadays, no widely established and highly effective treatment option for this complex pathological condition exists which leads to an intensive search for new therapeutic approach (Ratziu et al. 2015).

GLP-1 and its analogues led in *in vitro* and *in vivo* studies of several experimental animal models to modulation of liver lipid metabolism and to decrease in hepatic lipid content (Ding et al. 2006; Sharma et al. 2011; Svegliati-Baroni et al. 2011; Lee et al. 2012a; Trevaskis et al. 2012) showing its beneficial effect on liver metabolism under NAFLD conditions. Results of LEAN clinical trial (Liraglutide Efficacy and Action in Non-alcoholic steatohepatitis; Clinicaltrials.gov#NCT01237119) showed that 48 weeks of treatment with liraglutide (once-daily 1.8 mg s.c.) led to an improvement of histological results of NASH (Armstrong et al. 2013, 2016a). This result was accompanied by decreased liver steatosis and ballooning of hepatocytes. No statistically significant difference in Kleiner fibrosis stage was observed, however, patients treated with liraglutide had lower worsening of histological results of fibrosis when compared to placebo group (8 of 22 patients in placebo group showed its worsening *vs.* 2 of 23 patients in liraglutide group (Armstrong et al. 2016a). In a sub-study involving 14 patients of the LEAN study, Armstrong et al. described improvement of liver lipid and glucose metabolism reducing metabolic dysfunction in NASH after 12 weeks of liraglutide treatment (Armstrong et al. 2016b). These results make liraglutide more attractive for therapy of NAFLD and NASH.

Although GLP-1 analogues are widely used in clinical practice for treatment of type 2 diabetes mellitus, data about their potential toxic effect on hepatocytes in *in vitro* conditions are missing in literature, especially for liraglutide.

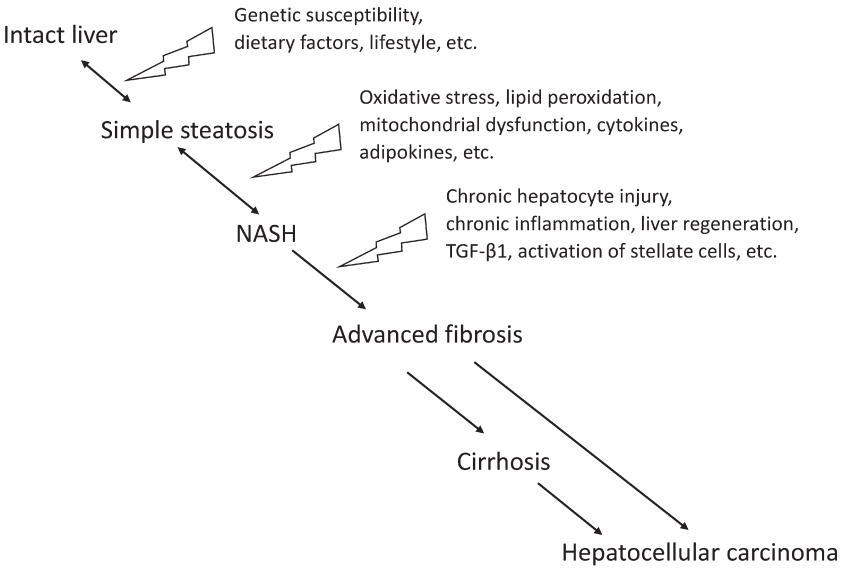

Figure 1. Scheme of the possible progression of non-alcoholic fatty liver disease. TGF-β1, transforming growth factor β1; NASH, non-alcoholic steatohepatitis. (Adopted from Kučera and Červinková 2014).

Therefore, in our present work, we evaluated an effect of liraglutide on activity of caspases 3/7, cell viability and oxidative stress parameters in primary cultures of hepatocytes isolated from rats fed a standard or a high-fat diet.

Materials and Methods

Chemicals

Liraglutide was purchased from Bachem AG (Bubendorf, Switzerland). William's E medium without phenol red, penicillin, streptomycin, fetal bovine serum and glutamine were supplied by BioChrom GmbH (Germany). Collagenase (from Clostridium histolyticum, Serva, Germany), insulin (Actrapid, Hoechst, Germany), glucagon (Novo Nordisk, Denmark), prednisolone (Merck, Germany), kit for lactate dehydrogenase (DiaSys, Germany), Cell Proliferation Reagent WST-1 (Roche, Germany), DCFDA (Life Technologies, Carlsbad, California, USA) and Caspase Glo 3/7 kit (Promega, Madison, Wisconsin, USA) were obtained from suppliers mentioned in the brackets. All other chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Animals and experimental design

Male Wistar rats (Biotest, Konarovice, Czech Republic) with initial body weight 235 ± 15 g, age 8 weeks were used throughout the study. The animals were housed at 23 ± 1°C, 55 ± 10% relative humidity, and air exchange 12–14 times/h, 12-h light/dark cycles (6:00 h to 18:00 h) and has had a free access to tap water and different diets as described below. All animals received care according to the guidelines set out by the Animal-Welfare Body of the Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic and the committee approved our experiment. All planned experiments were in agreement with law No. 246/1992 (for protection of animals against cruelty), notice No. 419/2012 (the protection of animals for experimental purposes) and Council Directive 86/609/ECC (on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes).

The animals were fed *ad libitum* a standard laboratory pelleted diet (ST-1, Velaz, Prague, Czech Republic; 10% energy fat, 30% energy proteins, 60% energy saccharides – ST1-group) or high-fat gelled diet (HFGD; 71% energy fat, 18% energy proteins, 11% energy saccharides – HF-group; described by Lieber et al. 2004 and Kučera et al. 2011) for 10 weeks. Supplements for HFGD diet were purchased from MP Biomedicals (Solon, OH, USA). Food and water intakes and mean body weight gain were monitored 3 times a week.

Hepatocyte preparation, cultivation and treatment

Hepatocytes were isolated from animals under ether anaesthesia by two-step collagenase perfusion as described previously (Berry et al. 1991). The body weight at time of isolation was 390 ± 12 g for ST-1 and 405 ± 14 g for HFGD. The cell viability of freshly isolated hepatocytes was determined with a trypan blue exclusion test – for all isolations, it was higher than 90%. Cell density was counted using a Cellometer (Nexcelom Bioscience, Lawrence, Massachusetts). Subsequently, isolated hepatocytes were suspended in William's E medium enriched with 6% fetal bovine serum, glutamine (2 mmol/l), penicillin (100 IU/ml), streptomycin (10 mg/ml), insulin (0.08 IU/ml), prednisolone (0.5 μg/ml), glucagon (0.008 μg/ml) and allowed to attach on collagen-coated cultivating 6-well (at a density of 1×10^6 cells/well), 24-well (density of 3×10^5 cells/well) and 96-well (density of 3×10^4 cells/well) NUNC plates (Thermo Scientific, Waltham, Mass.) in a gassed atmosphere (5% CO_2) at 37°C for 2 h. After the establishment of monolayers, the medium was removed and replaced with a fresh medium containing liraglutide with concentrations ranging from 0.1 to 1000 nmol/l. Control hepatocytes were incubated in William's E medium, with no liraglutide. The treatment period lasted 24 h (5% CO_2, 37°C). After this period, the medium was collected for the biochemical assays and cells were harvested.

Microscopic evaluation – phase contrast imaging

The morphological changes of hepatocytes in culture were assessed using Olympus IX51 microscope equipped with the digital camera Olympus E-600 (Olympus Imaging Corp., Tokyo, Japan) and Quick Photo Camera 3.0 software (Promicra, Prague, Czech Republic). The objective magnification was set 20× for phase contrast imaging. No image editing was performed.

Markers of hepatocyte damage and viability

Lactate dehydrogenase (LDH) leakage test consisted of measurement of LDH activity in the culture medium and in the cell lysate (hepatocytes were frozen for 10 min at –80°C and lysed in distilled water) using a commercial kit from DiaSys (Holzheim, Germany). The ratio of extracellular and total LDH was calculated.

Cell viability was assessed using the WST-1 assay (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, Roche, Germany) evaluating the activity of intracellular dehydrogenases (described by Lotková et al. 2005). In the assay, the maternal tetrazolium salt WST-1 is cleaved to formazan by intracellular dehydrogenases. The higher the number of viable cell is, the higher the activity of the enzymes leading

to the higher production of the formazan dye. The change directly correlates to the number of metabolically active cells in the culture. The medium was collected and the WST-1 reagent (diluted 1:10 in PBS; obtained from Roche, Penzberg, Germany) was added to the well-plates. Changes in absorbance of the dye solution were measured at time 0, 1 h and at 2 h using a TECAN Infinite M200 spectrophotometer (Tecan Group AG, Männedorf, Switzerland) at a wavelength of 440 nm. The difference between these values was calculated and used for statistical analysis.

Activity of caspases 3/7

Activity of executive caspases 3/7 was evaluated using a Caspase-Glo 3/7 kit delivered from Promega (Madison, Wisconsin, USA). Incubated cells were loaded with Caspase Glo 3/7 reagent that induced cell lysis and provided a substrate for caspases 3/7. The total luminescence at time 0, 35 and 70 min was measured using TECAN Infinite M200 spectrophotometer (Tecan Group AG, Männedorf, Switzerland). For statistical analysis was used delta of total luminescences at time 0 and 70 min.

Markers of oxidative stress

Intensity of lipid peroxidation in hepatocytes was determined by evaluating malondialdehyde (MDA) production by measuring the level of thiobarbituric acid-reactive substances (TBARS) in the medium (Ohkawa et al. 1979). In the method thiobarbituric acid (TBA) is reacted with MDA resulting in the formation of the chromogen that can be determined spectrophotometrically.

The production of reactive oxygen species (ROS) was evaluated using the fluorescence probe dichlorodihydrofluorescein diacetate (DCFDA) obtained from Life Technologies (Carlsbad, California, USA). DCFDA is a fluorogenic dye that detects activity of ROS (e.g. hydroxyl and peroxyl) in cells. After diffusion through membrane, intracellular esterases cleave off the acetate groups forming a non-fluorescent molecule that is subsequently oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected. After the incubation, well-plates were rinsed with free culture medium and consequently loaded by DCFDA diluted in medium (1 μmol/l) for 30 min incubation at 37°C. Then the substrate was removed and the well-plates were washed with the medium. After an additional 45 min, the increase of fluorescence intensity was assessed in a TECAN Infinite M200 spectrophotometer (Tecan Group AG, Männedorf, Switzerland) using an excitation and emission wavelength of 485 and 535 nm, respectively. The results were related to the amount of total protein extracted from the cells determined by the Bradford assay (Bradford 1976).

Statistical analysis

Results are expressed as the mean ± SD. GraphPad Prism 6.01 software (La Jolla, California, USA) was used for statistical analysis. Kolmogorov-Smirnov test was used for proving the normality. Data with Gaussian distribution were analyzed using ANOVA followed by Tukey-Kramer's *post hoc* test for group comparison. In non-Gaussian distribution, non-parametric Kruskal-Wallis tests and Dunn's post hoc test were used. For the assessment of caspases´ activity over concentrations of liraglutide we used simple linear regression, which was done by using SPSS Statistics software. Values of $p < 0.05$ were considered statistically significant.

Results

Phase contrast microscopy

Morphological changes of hepatocytes are shown on representative microphotographs of primary cultures (Fig. 2). Hepatocytes isolated from HF-group animals exerted mi-

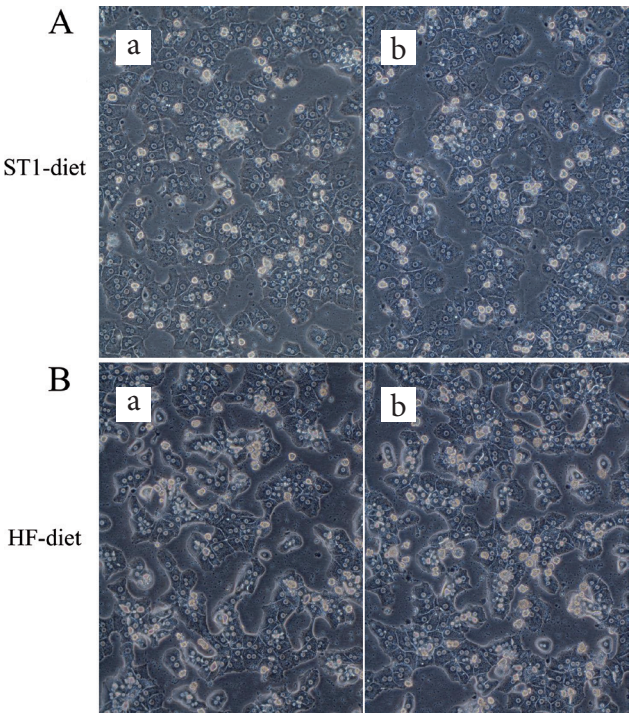

Figure 2. Morphology of primary cultures of hepatocytes after 24 h of incubation. Representative microphotographs of hepatocytes from rats on ST1-diet (**A**) and HF-diet (**B**). **a.** Control hepatocytes. **b.** Hepatocytes treated with LIRA 100 nmol/l. Phase contrast, objective magnification 20× for all microphotographs. ST-1 diet, the animals were fed a standard laboratory diet, HF-diet, the animals were fed a high-fat diet; LIRA, liraglutide.

crovesicular steatosis (Fig. 2Ba) in comparison with hepatocytes isolated from lean ST1-group animals (Fig. 2Aa). Incubation with liraglutide for 24 h was not associated with any significant changes on morphology of cultivated hepatocytes (Figs. 2Ab and Bb – concentration of liraglutide 100 nmol/l).

Hepatocyte damage and viability

In both assays, LDH leakage and WST-1 test, hepatocytes from HF-group showed a lower cell viability as documented by higher LDH leakage and lower activity of intracellular dehydrogenases ($p < 0.001$ for all comparisons in LDH leakage; $p < 0.001$ for LIRA0.1 and LIRA1, $p < 0.001$ for LIRA10, LIRA100 and LIRA1000 in WST-1 assay). Liraglutide treatment did not exert any negative effect on cell viability in both assays (Fig. 3 and Fig. 4).

Activity of caspases 3/7

Activities of caspases 3/7, as a marker of apoptosis, were higher in all HF-groups when compared to respective ST1-groups ($p < 0.001$ for all comparisons). Simple linear regression revealed linear relationship between the activity and concentration of liraglutide for ST1-groups (–1521.48, SE = 241.1, R^2 = 0.464) which is statistically significant ($p < 0.001$). We obtained similar association for HF-groups (–1306.05, SE = 189.9, R^2 = 0.507) which is also statistically significant ($p < 0.001$; Fig. 5).

ROS production

Significantly higher concentrations of MDA were observed in hepatocytes isolated from animals fed a high-fat diet in comparison to respective ST1-group ($p < 0.001$ for all comparisons). No effect of liraglutide on this parameter was found in any group. On the other hand, DCFDA assay revealed a reduction of ROS production in HF-group after liraglutide exposure (HF-C *vs.* HF-LIRA100: $p < 0.05$; HF-C *vs.* HF-LIRA1000: $p < 0.001$; Fig. 6 and Fig. 7).

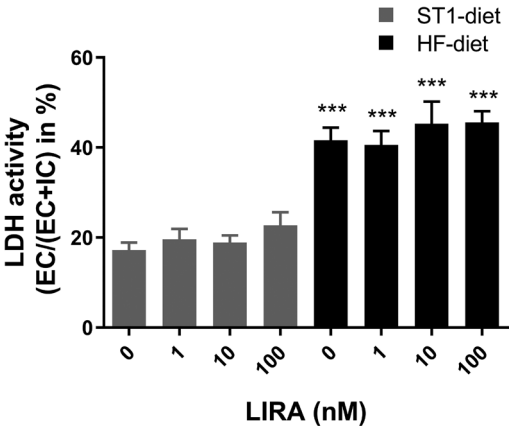

Figure 3. LDH leakage after 24 h of incubation with William's E medium (0 – controls) and with medium containing LIRA at concentration of 1, 10 and 100 nmol/l, respectively. Data are expressed as ratio between EC and total LDH activity (IC + EC) ($n = 6$; *** $p < 0.001$ *vs.* corresponding ST1-groups). EC, extracellular; IC, intracellular; LDH, lactate dehydrogenase. (For more abbreviations, see Fig. 2).

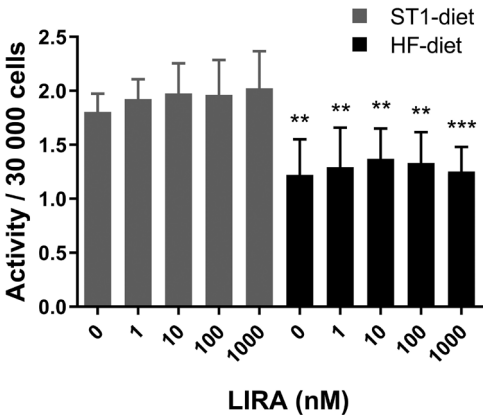

Figure 4. WST-1 assay. Activity of cellular dehydrogenases after 24 h of incubation with William's E medium (0 – controls) and with medium containing LIRA at concentration of 1, 10, 100 and 1000 nmol/l, respectively ($n = 8$; ** $p < 0.01$, *** $p < 0.001$ *vs.* corresponding ST1-groups). For abbreviations, see Fig. 2).

Discussion

The potential of incretin based therapies in diabetology has been theoretically described for more than 20 years ago (Gutniak et al. 1992). Subsequently the basic experimental and clinical-physiological pillars of this therapy have been developed mainly by the groups around Holst (Holst 2007, 2008), Nauck (Nauck et al. 1997, 1998; Nauck 2008) and Ahren (Ahren 1998). Nowadays, there are two groups of drugs with known therapeutic potential based on modulation of the incretin system, GLP-1 analogues and DPP4 inhibitors. Both groups have proven to be effective option and have been a part of treatment of type 2 diabetes for many years now (Tuch 2016).

In the literature, there are many works studying the effect of GLP-1 analogues on metabolism of hepatocytes and liver function. Intensively studied field is especially hepatic metabolism of lipids, since several studies postulated possible beneficiary effect of incretins and related incretin-based therapies in the treatment of NAFLD/NASH condition (Lee 2012b; Samson and Bajaj 2013; Armstrong et al. 2016a). Several *in vitro* and *in vivo* animal studies assessed the effect of GLP-1 and its analogues on parameters of liver lipid metabolism – e.g. hepatic lipid content, gene expression of key enzymes and transport proteins of fatty acids (FA) and

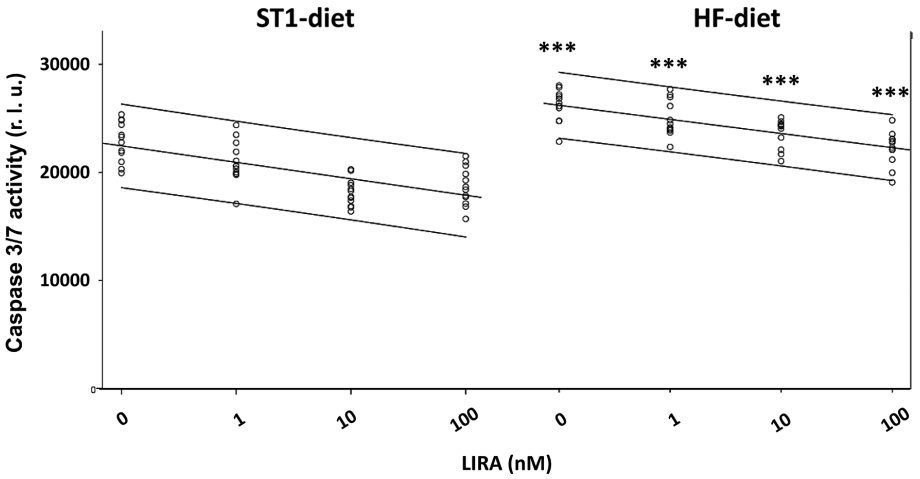

Figure 5. Activity of caspases 3/7 after 24 h of incubation (n = 12; *** $p < 0.001$ *vs.* corresponding ST1-groups). Simple linear regression of caspases´ activity over concentrations of LIRA for ST1-groups and HF-groups revealed linear relationship ($p < 0.001$ for both groups). r.l.u., relative light units. (For more abbreviations, see Fig. 2).

TAG metabolism etc. – concluding with promising results (Ding et al. 2006; Sharma et al. 2011; Svegliati-Baroni et al. 2011; Lee et al. 2012a; Trevaskis et al. 2012). These animal experiments were supported by works describing positive findings of incretin-based therapies on liver parameters of patients with diabetes in clinical practice (Tushuizen et al. 2006; Buse et al. 2007; Klonoff et al. 2008; Kenny et al. 2010; D'Amico 2011; Sathyanarayana et al. 2011; Cuthbertson et al. 2012; Ohki et al. 2012; Vilsbøll et al. 2012). Subsequently, LEAN clinical trial (Armstrong et al. 2013), which focused on liraglutide action in overweight patients with a histological evidence of NASH, described an improvement of histological results of NASH, decreased liver steatosis and ballooning of hepatocytes and improvement of liver lipid metabolism reducing metabolic dysfunction in NASH (Armstrong et al. 2016a, 2016b). However, in the literature there is a gap in knowledge of effects of liraglutide on the hepatocyte viability and oxidative stress. This missing information we found as crucial since both parameters are modified in the NAFLD/NASH condition. In the current study we try to elucidate the effect of liraglutide on these parameters in both, normal and steatotic liver.

Excess of fatty acids (esp. saturated FA) may induce an endoplasmic reticulum (ER) stress response that can lead to an activation of signalling pathways (including caspase cascade) causing hepatocyte cell death (Zhang et al. 2011; Gentile et al. 2011). We described, that activity of caspases 3/7 was higher in hepatocytes isolated from HF-animals when compared to respective ST1-groups, which is a finding

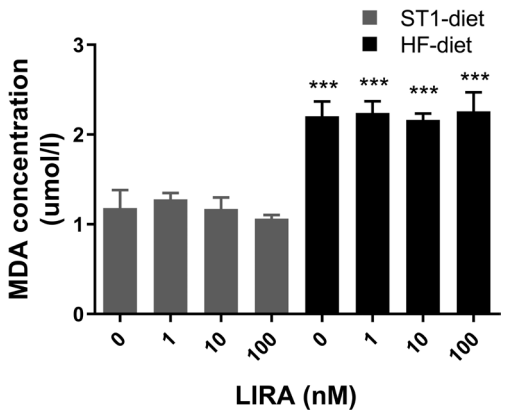

Figure 6. Concentration of MDA after 24 h of incubation with William's E medium (0 – controls) and with medium containing LIRA at concentration of 1, 10 and 100 nmol/l, respectively (n = 6; *** $p < 0.001$ *vs.* corresponding ST1-groups). MDA, malondialdehyde. (For more abbreviations, see Fig. 2).

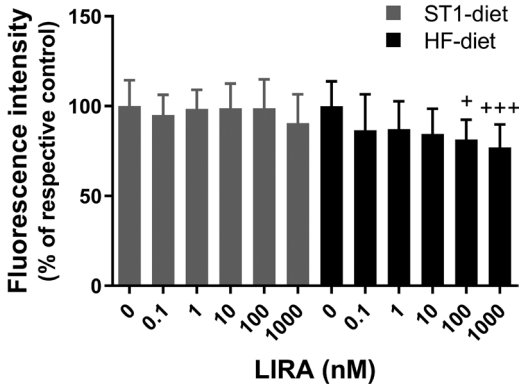

Figure 7. DCFDA assay. Production of ROS after 24 h of incubation with William's E medium (0 – controls) and with medium containing LIRA at concentration of 0.1, 1, 10, 100 and 1000 nmol/l, respectively. Data are expressed as percentage of fluorescence intensity of respective control (n = 16; + $p < 0.05$, +++ $p < 0.001$ *vs.* HF-control group). DCFDA, dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species. (For more abbreviations, see Fig. 2).

described previously in *in vitro* models of NAFLD/NASH induced by cultivation of primary human hepatocytes (Sharma et al. 2011), HepG2 cells (Lee et al. 2014) and primary cultures of rat hepatocytes (Moravcová et al. 2015) with different FA. Cultivation of hepatocytes from both groups in the medium with liraglutide led to a significant decrease of activity of caspases 3/7 (thus suppression of apoptosis). Based on the statistical analysis, the described effect was dose-dependent. To our best knowledge, there are no previous studies describing a positive effect of liraglutide on activity of caspases 3/7 in primary cultures of rat hepatocytes. In the literature, there are several studies concerning the effect of other GLP-1 analogue exenatide on caspases activity (Sharma 2011; Gezginci-Oktayoglu et al. 2011; Lee et al. 2014). Lee et al. described that Ex4 improves steatohepatitis in mice fed with a high-fat diet by increasing SIRT1 signalling (Lee et al. 2012a). Similar decline in activity of caspases as in our present work was described for primary human hepatocytes (Sharma et al. 2011) and HepG2 cells (Lee et al. 2014). A possible underlying mechanism is attenuation of ER stress (enhanced capacity to handle the unfolded protein response) *via* SIRT-1 dependent mechanisms leading to prevention of hepatocyte cell death *via* apoptosis (Sharma et al. 2011; Lee at al. 2014).

Hepatocytes from HF-animals when compared to ST1-hepatocytes showed, based on the results of WST-1 assay and LDH leakage, significantly increased cell damage and lower viability. This finding for fat-laden hepatocytes has been already described in our previous experiment with the same experimental animal model (Kučera et al. 2012), or in experimental models of *in vitro* induction of steatosis by incubation of primary cultures of hepatocytes with different FA (Sharma et al. 2011; Moravcová et al. 2015). Liraglutide treatment did not exert any negative effect on cell viability in both assays. Sharma et al. and Aviv et al. described in *in vitro* conditions a positive effect of Ex4 on hepatocyte viability assessed by MTT and XTT assays that are similar to WST-1 assay used in our study (Aviv et al. 2009; Sharma et al. 2011). Our results thus support and correspond with the previous literature data that GLP-1 analogues do not cause negative effects on hepatocyte viability.

Concerning the oxidative stress, we assessed markers evaluating two distinct segments of ROS metabolism: concentration of MDA, marker of lipid peroxidation, and DCFDA assay. MDA level was elevated for hepatocytes from HF-group when compared to respective ST1-hepatocytes. This is in good accordance with our previous results: for the same experimental model as in the present study (Kučera et al. 2012), or for primary cultures of rat hepatocytes exposed *in vitro* to FA (Moravcová et al. 2015). Liraglutide treatment led in our present study to a decrease in ROS production evaluated by DCFDA assay in HF-group. Reduced oxidative stress has already been described: for Ex4 in liver homogenates obtained for ob/ob mice (assessed by TBARS/MDA concentration; Ding et al. 2006); and for GLP-1-derived nonapeptide GLP-1(28-36)amide in a study on primary cultures of hepatocytes isolated from diet-induced obesity (DIO) mice and normal (C57bl/6J) mice or on H4IIe hepatocyte cell line (evaluated by DCFDA assay; Tomas et al. 2011).

Conclusion

In conclusion, our results suggest that GLP-1 analogue liraglutide expresses a beneficial action in primary cultures of hepatocytes isolated from male Wistar rats fed a standard laboratory or a high-fat diet by decreasing the activity of caspases 3/7 and reducing oxidative stress. No negative effects on cell viability were described. These findings can support a further research and usage of liraglutide in treatment of human liver diseases, especially NAFLD/NASH.

Brief summary

In our article, we described that glucagon-like peptide-1 analogue Liraglutide decreased activity of caspases 3/7, reduced oxidative stress and did not exhibit negative effects on cell viability in primary cultures of hepatocytes isolated from male Wistar rats fed a standard laboratory or a high-fat diet. These findings can support a further research and usage of Liraglutide in treatment of human liver diseases, especially NAFLD/NASH.

Acknowledgments. This work was supported by grant from Charles University in Prague PROGRES Q40/02.

Conflict of interest. The authors state that there are no conflicts of interest regarding the publication of this article.

References

Agersø H, Jensen LB, Elbrønd B, Rolan P, Zdravkovic M (2002): The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. Diabetologia **45,** 195-202 https://doi.org/10.1007/s00125-001-0719-z

Ahrén B (1998): Glucagon-like peptide-1 (GLP-1): a gut hormone of potential interest in the treatment of diabetes. Bioassays **20,** 642-651 https://doi.org/10.1002/(SICI)1521-1878(199808)20:8<642::AID-BIES7>3.0.CO;2-K

Armstrong MJ, Barton D, Gaunt P, Hull D, Guo K, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hübscher SG, Newsome PN; LEAN trial team (2013): Liraglutide efficacy and action in non-alcoholic steatohepatitis (LEAN): study protocol for

a phase II multicentre, double-blinded, randomised, controlled trial. BMJ Open **3,** e003995
https://doi.org/10.1136/bmjopen-2013-003995

Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K; LEAN trial team, Abouda G, et al. (2016a): Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet **387,** 679-690
https://doi.org/10.1016/S0140-6736(15)00803-X

Armstrong MJ, Hull D, Guo K, Barton D, Hazlehurst JM, Gathercole LL, Nasiri M, Yu J, Gough SC, Newsome PN, Tomlinson JW (2016b): Glucagon-like peptide 1 decreases lipotoxicity in non-alcoholic steatohepatitis. J. Hepatol. **64,** 399-408
https://doi.org/10.1016/j.jhep.2015.08.038

Aviv V, Meivar-Levy I, Rachmut IH, Rubinek T, Mor E, Ferber S (2009): Exendin-4 promotes liver cell proliferation and enhances the PDX-1-induced liver to pancreas transdifferentiation process. J. Biol. Chem. **284,** 33509-33520
https://doi.org/10.1074/jbc.M109.017608

Baggio LL, Drucker DJ (2007): Biology of incretins: GLP-1 and GIP. Gastroenterology **132,** 2131-2157
https://doi.org/10.1053/j.gastro.2007.03.054

Berry MN, Edwards AM, Barritt GJ (1991): High-yield preparation of isolated hepatocytes from rat liver. In: Isolated Hepatocytes: Preparation, Properties and Application. (Eds. Burdon RH, van Knippenberg PH), pp. 15-58, N.Y., Elsevier, New York
https://doi.org/10.1016/S0075-7535(08)70023-8

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248-254
https://doi.org/10.1016/0003-2697(76)90527-3

Buse JB, Klonoff DC, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, Maggs DG, Wintle ME (2007): Metabolic effects of two years of exenatide treatment on diabetes, obesity, and hepatic biomarkers in patients with type 2 diabetes: an interim analysis of data from the open-label, uncontrolled extension of three double-blind, placebo-controlled trials. Clin. Ther. **29,** 139-153
https://doi.org/10.1016/j.clinthera.2007.01.015

Cuthbertson DJ, Irwin A, Gardner CJ, Daousi C, Purewal T, Furlong N, Goenka N, Thomas EL, Adams VL, Pushpakom SP, et al. (2012): Improved glycaemia correlates with liver fat reduction in obese, type 2 diabetes, patients given glucagon-like peptide-1 (GLP-1) receptor agonists. PLoS One **7,** e50117
https://doi.org/10.1371/journal.pone.0050117

D'Amico E (2011): Efficacy of liraglutide in a patient with type 2 diabetes and cryptogenic cirrhosis. Acta Biomed. **82,** 160-161

Ding X, Saxena NK, Lin S, Gupta NA, Anania FA (2006): Exendin-4, a glucagon-like protein-1 receptor agonist, reverses hepatic steatosis in ob/ob mice. Hepatology **43,** 173-181
https://doi.org/10.1002/hep.21006

Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, Goke B (1992): Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. Eur. J. Clin. Invest. **22,** 283-291
https://doi.org/10.1111/j.1365-2362.1992.tb01464.x

Gentile CL, Frye MA, Pagliassotti MJ (2011): Fatty acids and the endoplasmic reticulum in nonalcoholic fatty liver disease. Biofactors **37,** 8-16
https://doi.org/10.1002/biof.135

Gezginci-Oktayoglu S, Sacan O, Yanardag R, Karatug A, Bolkent S (2011): Exendin-4 improves hepatocyte injury by decreasing proliferation through blocking NGF/TrkA in diabetic mice. Peptides **32,** 223-231
https://doi.org/10.1016/j.peptides.2010.10.025

Gonzalez-Rodriguez A, Valdecantos MP, Rada P, Addante A, Barahona I, Rey E, Pardo V, Ruiz L, Laiglesia LM, Moreno-Aliaga MJ, et al. (2018): Dual role of protein tyrosine phosphatase 1B in the progression and reversion of non-alcoholic steatohepatitis. Mol. Metab. **7,** 132-146
https://doi.org/10.1016/j.molmet.2017.10.008

Gutniak M, Orskov C, Holst JJ, Ahrén B, Efendic S (1992): Antidiabetogenic effect of glucagon-like peptide-1 (7-36)amide in normal subjects and patients with diabetes mellitus. N. Engl. J. Med. **326,** 1316-1322
https://doi.org/10.1056/NEJM199205143262003

Holst JJ (2007): The physiology of glucagon-like peptide 1. Physiol. Rev. **87,** 1409-1439
https://doi.org/10.1152/physrev.00034.2006

Holst JJ (2008): Pharmacology of GLP-1 based therapies. Brit J. Diab. Vasc. Dis. **8** (Suppl. 2), S10-S18
https://doi.org/10.1177/1474651408100523

Kim CH, Younossi ZM (2008): Nonalcoholic fatty liver disease: a manifestation of the metabolic syndrome. Cleve. Clin. J. Med. **75,** 721-728
https://doi.org/10.3949/ccjm.75.10.721

Kenny PR, Brady DE, Torres DM, Ragozzino L, Chalasani N, Harrison SA (2010): Exenatide in the treatment of diabetic patients with non-alcoholic steatohepatitis: a case series. Am. J. Gastroenterol. **105,** 2707-2709
https://doi.org/10.1038/ajg.2010.363

Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology **41,** 1313-1321
https://doi.org/10.1002/hep.20701

Klonoff DC, Buse JB, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, Wintle ME, Maggs DG (2008): Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic bio markers in patients with type 2 diabetes treated for at least 3 years. Curr. Med. Res. Opin. **24,** 275-286
https://doi.org/10.1185/030079907X253870

Kučera O, Garnol T, Lotková H, Staňková P, Mazurová Y, Hroch M, Bolehovská R, Roušar T, Červinková Z (2011): The effect of rat strain, diet composition and feeding period on the development of a nutritional model of non-alcoholic fatty liver disease in rats. Physiol. Res. **60,** 317-328

Kučera O, Al-Dury S, Lotková H, Roušar T, Rychtrmoc D, Červinková Z (2012): Steatotic rat hepatocytes in primary culture are more susceptible to the acute toxic effect of acetaminophen. Physiol. Res. **61** (Suppl. 2), S93-101

Kučera O, Červinková Z (2014): Experimental models of non-alcoholic fatty liver disease in rats. World J. Gastroenterol. **20,** 8364-8376

https://doi.org/10.3748/wjg.v20.i26.8364
Lee J, Hong SW, Chae SW, Kim DH, Choi JH, Bae JC, Park SE, Rhee EJ, Park CY, Oh KW, et al. (2012a): Exendin-4 improves steatohepatitis by increasing Sirt1 expression in high-fat diet-induced obese C57BL/6J mice. PLoS One **7,** e31394
https://doi.org/10.1371/journal.pone.0031394
Lee J, Hong SW, Rhee EJ, Lee WY (2012b): GLP-1 receptor agonist and non-alcoholic fatty liver disease. Diabetes Metab. J. **36,** 262-267
https://doi.org/10.4093/dmj.2012.36.4.262
Lee J, Hong SW, Park SE, Rhee EJ, Park CY, Oh KW, Park SW, Lee WY (2014): Exendin-4 attenuates endoplasmic reticulum stress through a SIRT1-dependent mechanism. Cell Stress Chaperones **19,** 649-656
https://doi.org/10.1007/s12192-013-0490-3
Lieber CS, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, Ponomarenko A, DeCarli LM (2004): Model of nonalcoholic steatohepatitis. Am. J. Clin. Nutr. **79,** 502-509
https://doi.org/10.1093/ajcn/79.3.502
Lotková H, Červinková Z, Kučera O, Kriváková P, Kanďár R (2005): Protective effect of S-adenosylmethionine on cellular and mitochondria l membranes of rat hepatocytes against tert-butylhydroperoxide-induced injury in primary culture. Chem. Biol. Interact. **156,** 13-23
https://doi.org/10.1016/j.cbi.2005.06.008
Mehal WZ, Iredale J, Friedman SL (2011): Scraping fibrosis: expressway to the core of fibrosis. Nat. Med. **17,** 552-523
https://doi.org/10.1038/nm0511-552
Moravcová A, Červinková Z, Kučera O, Mezera V, Rychtrmoc D, Lotková H (2015): The effect of oleic and palmitic acid on induction of steatosis and cytotoxicity on rat hepatocytes in primary culture. Physiol. Res. **64** (Suppl. 5), S627-636
Nauck MA, Homberger E, Siegel EG, Allen RC, Eaton RP, Ebert R, Creutzfeldt W (1986): Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. J. Clin. Endocrinol. Metab. **63,** 492-498
https://doi.org/10.1210/jcem-63-2-492
Nauck MA, Niederreichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, Schmiegel WH (1997): Glucagon-like peptide-1 inhibition of gastric emptying outweights its insulinotropic effects in healthy humans. Am. J. Physiol. **273,** E981-988
https://doi.org/10.1152/ajpendo.1997.273.5.E981
Nauck MA, Weber I, Bach I, Richter S, Orskov C, Holst JJ, Schmiegel W (1998): Normalization of fasting glycaemia by intravenous GLP-1 ([7-36 amide] or [7-37]) in type 2 diabetic patients. Diabet. Med. **15,** 937-945
https://doi.org/10.1002/(SICI)1096-9136(1998110)15:11<937::AID-DIA701>3.3.CO;2-S
Nauck MA (2008): The modulating effects of GLP-1 in type 2 diabetes. Br. J. Diabetes. Vasc. Dis. **8** (Suppl. 2), S1-S2
https://doi.org/10.1177/1474651408100521
Ohkawa H, Ohishi N, Yagi K (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. **95,** 351-358
https://doi.org/10.1016/0003-2697(79)90738-3
Ohki T, Isogawa A, Iwamoto M, Ohsugi M, Yoshida H, Toda N, Tagawa K, Omata M, Koike K (2012): The effectiveness of liraglutide in nonalcoholic fatty liver disease patients with type 2 diabetes mellitus compared to sitagliptin and pioglitazone. Scientific World Journal **2012,** 496453
https://doi.org/10.1100/2012/496453
Ratziu V, Goodman Z, Sanyal A (2015): Current efforts and trends in the treatment of NASH. J. Hepatol. **62** (Suppl. 1), S65-75
https://doi.org/10.1016/j.jhep.2015.02.041
Ruhl CE, Everhart JE (2004): Epidemiology of nonalcoholic fatty liver. Clin. Liver Dis. **8,** 501-519
https://doi.org/10.1016/j.cld.2004.04.008
Russel-Jones D, Gough S (2012): Recent advances in incretin-based therapies. Clin. Endocrinol. (Oxf) **77,** 489-499
https://doi.org/10.1111/j.1365-2265.2012.04483.x
Samson SL, Bajaj M (2013): Potential of incretin-based therapies for non-alcoholic fatty liver disease. J. Diabetes Complications **27,** 401-406
https://doi.org/10.1016/j.jdiacomp.2012.12.005
Sathyanarayana P, Jogi M, Muthupillai R, Krishnamurthy R, Samson SL, Bajaj M (2011): Effects of combined exenatide and pioglitazone therapy on hepatic fat content in type 2 diabetes. Obesity **19,** 2310-2315
https://doi.org/10.1038/oby.2011.152
Sharma S, Mells JE, Fu PP, Saxena NK, Anania FA (2011): GLP-1 analogs reduce hepatocyte steatosis and improve survival by enhancing the unfolded protein response and promoting macroautophagy. PLoS One **6,** e25269
https://doi.org/10.1371/journal.pone.0025269
Svegliati-Baroni G, Saccomanno S, Rychlicki C, Agostinelli L, De Minicis S, Candelaresi C, Faraci G, Pacetti D, Vivarelli M, Nicolini D, et al. (2011): Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis. Liver Int. **31,** 1285-1297
https://doi.org/10.1111/j.1478-3231.2011.02462.x
Tomas E, Stanojevic V, Habener JF (2011): GLP-1-derived nonapeptide GLP-1(28-36)amide targets to mitochondria and suppresses glucose production and oxidative stress in isolated mouse hepatocytes. Regul. Pept. **167,** 177-184
https://doi.org/10.1016/j.regpep.2011.01.003
Trevaskis JL, Griffin PS, Wittmer C, Neuschwander-Tetri BA, Brunt EM, Dolman CS, Erickson MR, Napora J, Parkes DG, Roth JD (2012): Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of non-alcoholic steatohepatitis in mice. Am. J. Physiol. Gastrointest. Liver Physiol. **302,** G762-772
https://doi.org/10.1152/ajpgi.00476.2011
Tsuchida T, Friedman SL (2017): Mechanisms of hepatic stellate cell activation. Nat. Rev. Gastroenterol. Hepatol. **14,** 397-411
https://doi.org/10.1038/nrgastro.2017.38
Tuch BE (2016): Clinical use of GLP-1 agonists and DPP4 inhibitors. Pancreatology **16,** 8-9
https://doi.org/10.1016/j.pan.2015.05.465
Tushuizen ME, Bunck MC, Pouwels PJ, van Waesberghe JH, Diamant M, Heine RJ (2006): Incretin mimetics as a novel therapeutic option for hepatic steatosis. Liver Int. **26,** 1015-1017
https://doi.org/10.1111/j.1478-3231.2006.01315.x
Valdecantos MP, Ruiz L, Pardo V, Castro-Sanchez L, García-Monzón C, Lanzón B, Rupérez J, Barbas C, Naylor J, Trevaskis

JL, et al. (2018): Differential effects of a glucagon-like peptide 1 receptor agonist in non-alcoholic fatty liver disease and in response to hepatectomy. Sci. Rep. **8,** 16461 https://doi.org/10.1038/s41598-018-33949-z

Vanni E, Bugianesi E, Kotronen A, De Minicis S, Yki-Järvinen H, Svegliati-Baroni G (2010): From the metabolic syndrome to NAFLD or vice versa? Dig. Liver Dis. **42,** 320-330 https://doi.org/10.1016/j.dld.2010.01.016

Vilsbøll T, Christensen M, Junker AE, Knop FK, Gluud LL (2012): Effects of glucagon-like peptide-1 receptor agonists on weight loss: systematic review and meta-analyses of randomised controlled trials. BMJ **344,** d7771 https://doi.org/10.1136/bmj.d7771

Vilsboll T, Agerso H, Krarup T, Holst JJ (2003): Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic patients and healthy subjects. J. Clin. Endocrinol. Metab. **88,** 220-224 https://doi.org/10.1210/jc.2002-021053

Zhang Y, Yang X, Shi H, Dong L, Bai J (2011): Effect of α-linolenic acid on endoplasmic reticulum stress-mediated apoptosis of palmitic acid lipotoxicity in primary rat hepatocytes. Lipids Health Dis. **10,** 122 https://doi.org/10.1186/1476-511X-10-122

Received: September 1, 2018
Final version accepted: April 26, 2019
First published online: June 26, 2019