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Summary of Dissertation Thesis
(Autoreferát disertační práce)

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Study of pharmacodynamics and pharmacokinetics of potential therapeutic substances in an experimental model of non-alcoholic fatty liver disease and steatohepatitis (NAFLD/NASH)

Studium farmakodynamiky a farmakokinetiky potenciálních terapeutických látek v experimentálním modelu nealkoholického ztukovatění jater a steatohepatitidy (NAFLD/NASH)

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Abstract

Introduction and Aims: Nonalcoholic fatty liver disease and steatohepatitis (NAFLD/NASH) if untreated lead to liver fibrosis, cirrhosis and cancer. Due to the absence of specific pharmacological NASH management, we revolved several molecular targets and compounds potentially influencing glucose, lipid and energy metabolism such as a mammalian target of rapamycin (mTORC1/C2) inhibitor - Ku-0063794 (KU), a sterol regulatory element-binding protein (SREBP1/2) inhibitor - fatostatin (FAT), and a galaninergic system modulator - celastrol (CEL). The purpose of the research was to explore the possible pharmacological benefits of KU, FAT, and CEL in nutritional preclinical models of NAFLD/NASH.

Methods and Results: *In vitro*, KU, FAT, and CEL pre-treatments alleviated palmitic acid-induced lipotoxicity in primary hepatocyte cultures. *In vivo,* the pharmacokinetics of KU was studied first, with higher bioavailability noted after intraperitoneal (i.p.) than after oral administration. For all pharmacodynamics studies, male C57BL/6 mice were fed a high-fat western-type diet (WD) with fructose and glucose (FG) in drinking water for 8-16 weeks to induce NAFLD/NASH and subsequently individual substances were administered to them despite the continued WD/FG diet. KU (5 mg/kg i.p. daily for 3 weeks or 3 times a week for 16 weeks) reduced hepatotoxicity, oxidative stress, liver triglycerides, and TNF-α mRNA but did not fully reverse NASH histopathological progression. FAT (15 mg/kg i.p. every 2-3 days for 4 weeks) and CEL (200 μg/kg i.p. each second day for 4 weeks) decreased glycemia, body, fat tissue and liver weights, serum liver enzymes, cholesterol, liver steatosis, NAFLD activity scores, and some lipogenic and inflammatory genes. However, FAT produced systemic inflammation (as evidenced by increased serum TNF-α) and eczematous symptoms on the skin. On the other hand, CEL up-regulated *Ppargc1α* and *Galr2* genes and reduced lipid peroxidation in the liver without any noticed toxicity.

Conclusion: KU, FAT, and CEL each provided some benefits in alleviating NAFLD-to-NASH progression. KU had limited success, while FAT was highly effective in suppressing metabolic anomalies and histopathological changes in the liver but exhibited skin toxicity. CEL was sufficiently effective and safe, making it as a promising candidate for advanced pharmacological testing in the treatment of NASH and other metabolic dysfunction-related diseases.

Keywords: NAFLD, NASH, Ku-0063794, fatostatin, celastrol, *in vivo, in vitro*

Abstrakt

Úvod a cíle: Nealkoholické ztukovatění jater a steatohepatitida (NAFLD/NASH), pokud nejsou léčeny, vedou k jaterní fibróze, cirhóze a karcinomu. Vzhledem k absenci specifické farmakologické léčby NASH jsme se soustředili na studium několika molekulárních cílů, jakými jsou mTOR („mammalian target of rapamycin“), SREBP („sterol regulatory element-binding protein”) a galaninergní systém, potenciálně ovlivňujících energetický metabolismus glukózy a lipidů. Cílem práce bylo prozkoumat možné pozitivní farmakologické účinky inhibitoru mTORC1/C2 - Ku-0063794 (KU), inhibitoru SREBP1/2 - fatostatinu (FAT) a modulátoru galaninergního systému - celastrolu (CEL) v preklinických modelech NAFLD/NASH.

Metody a výsledky: KU, FAT a CEL zmírnily *in vitro* lipotoxicitu indukovanou kyselinou palmitovou v primárních buněčných kulturách hepatocytů. V pilotní farmakokinetické *in vivo* studii KU vykazoval vyšší biologickou dostupnost po intraperitoneálním (i.p.) než perorálním podání. Ve všech farmakodynamických studiích byli samci myší C57BL/6 krmeni vysokotučnou dietou západního typu (WD) s fruktózou a glukózou (FG) v pitné vodě po dobu 8-16 týdnů, aby se indukovala NAFLD/NASH, a následně jim byly aplikovány jednotlivé látky i přes pokračující WD/FG dietu. KU (5 mg/kg i.p. denně po dobu 3 týdnů nebo 3krát týdně po dobu 16 týdnů) snížil hepatotoxicitu, oxidativní stres, obsah jaterních triglyceridů a TNF-α mRNA, i když zcela nezvrátil histopatologickou progresi NASH. FAT (15 mg/kg i.p. každé 2-3 dny po dobu 4 týdnů) a CEL (200 μg/kg i.p. obden po 4 týdny) snížily: glykémii, hmotnost myší, jaterní a tukové tkáně, dále hladiny jaterních enzymů a cholesterolu v séru, jaterní steatózu, skóre aktivity NAFLD a také některé lipogenní a prozánětlivé geny v játrech. FAT však způsobil systémový zánět (resp. zvýšení sérového TNF-α) a ekzematózní příznaky na kůži. CEL naproti tomu snížil peroxidaci lipidů a zvýšil expresi genů *Ppargc1α* a *Galr2* v játrech bez jakékoli zaznamenané toxicity.

Závěr: Všechny testované látky, KU, FAT a CEL, vykazovaly určitý pozitivní účinek na zmírnění progrese NAFLD do NASH. KU měl však omezený efekt, zatímco FAT byl vysoce účinný při potlačování metabolických anomálií a histopatologických změn v játrech, ale způsoboval kožní toxicitu. Jelikož CEL byl dostatečně účinný a bezpečný, stává se slibným kandidátem pro pokročilé testování farmakologické léčby NASH a dalších onemocnění souvisejících s metabolickou dysfunkcí.

Klíčová slova: NAFLD, NASH, Ku-0063794, fatostatin, celastrol, *in vivo, in vitro*

1. Introduction

Metabolic dysfunction, particularly in conjunction with obesity, elevates the risk of several serious disorders including type 2 diabetes mellitus (T2DM), cardiovascular disease, hypertension, and a silent yet detrimental hepatic condition known as non-alcoholic fatty liver disease (NAFLD) progressing to non-alcoholic steatohepatitis (NASH) (Cao 2014). NAFLD has become a prevalent cause of chronic metabolic liver diseases worldwide, further exacerbating various metabolic disorders in the human body. If left untreated and undiagnosed in its early stages, NAFLD can lead to a cascade of pathological complications. It involves chronic inflammation culminating in simple steatosis (fat accumulation), which can progress to NASH. In severe cases, NASH can advance to liver cirrhosis and fibrosis, and in some instances, even hepatocellular carcinomas (HCCs) over the long term (Vernon, Baranova et al. 2011, Lonardo, Nascimbeni et al. 2017, Kupčová, Fedelešová et al. 2019). Given the lack of specific drugs for treating NASH, there is a pressing need to identify new therapeutic targets and their modulators with the potential for NASH treatment (Pais, Barritt 4th et al. 2016). The nomenclature for the same has lately been upgraded to metabolic dysfunction-associated steatotic liver disease (MASLD) characterized by accumulation of lipids (steatosis) in hepatocytes that progresses to metabolic dysfunction-associated steatohepatitis (MASH) characterized by lipotoxicity augmenting inflammatory and fibrotic pathways in hepatocytes (Kuchay, Choudhary et al. 2020, Rinella, Lazarus et al. 2023).

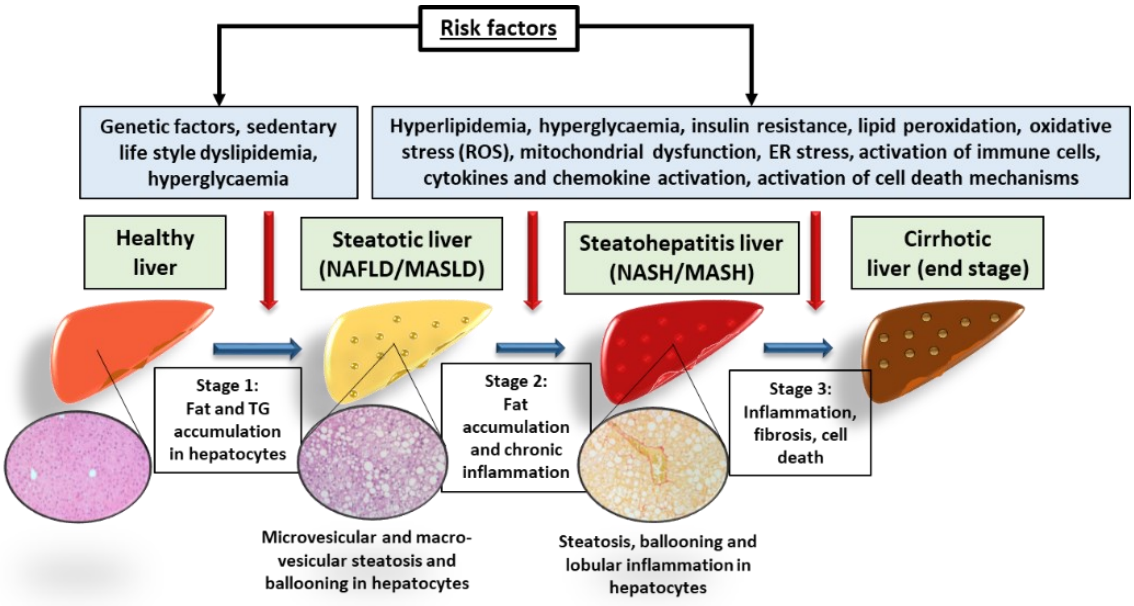


Figure 1: Risk factors and pathogenesis involved in NAFLD/NASH

Numerous factors influence the progression of NAFLD to NASH, encompassing both genetic and non-genetic elements such as dietary patterns, physical inactivity, chronic stress, and sedentary lifestyles (Figure 1). Steatosis entails disruptions in lipid metabolism within hepatocytes, often associated with insulin resistance and hyperglycaemia, leading to elevated serum concentrations of free fatty acids (FFAs). Chronic lipid accumulation within hepatocytes

progresses irreversibly to NASH, characterized by inflammation. Heightened hepatocyte sensitivity leads to further damage, inflammation, and fibrosis, accompanied by significant oxidative stress and activation of cell death mechanisms. Mechanisms of cell death include increased oxidative enzyme induction in the liver, mitochondrial dysfunction, endoplasmic reticulum stress, and imbalances in serum cytokines and adipokines levels. Left undiagnosed and untreated, these processes can culminate in apoptosis, fibrosis, and ultimately end-stage liver diseases such as cirrhosis or HCCs (Schuppan and Schattenberg 2013)

It has been shown that exposing liver cells to high concentrations of FFAs *in vitro* leads to fat overload, triggering inflammation, increased oxidative stress, apoptosis, and the production of fibrogenic cytokines, resembling observations in patients with NAFLD and NASH (Chavez-Tapia, Rosso et al. 2012, Chavez-Tapia, Rosso et al. 2012, Nissar, Sharma et al. 2015). Common *in vivo* dietary rodent models for NAFLD include High Fat Diet (HFD), High Fat/High Cholesterol Diet (HFC), High Fat/High Fructose Diet (ALIOS), High Fat/High Cholesterol/High Fructose Diet (AMLN), and others (Kucera and Cervinkova 2014, Oligschlaeger and Shiri-Sverdlov 2020, Zhong, Zhou et al. 2020).

The lack of FDA-approved treatments for NAFLD/NASH highlighted the urgent need for ongoing research specially when we began the research in year 2020. Targeting NASH is especially vital due to its irreversible inflammatory stage, unlike the reversible steatosis seen in NAFLD. Investigated pharmacotherapies mainly focus on bile acid (BA) signalling, insulin resistance, and liver lipid handling. Obeticholic acid has shown the potential in improving fibrosis in NASH patients (Attia, Softic et al. 2021). In 2024, the US FDA approved resmetirom for NASH treatment. Resmetirom works by enhancing hepatic fat metabolism, reducing lipotoxicity, and affecting liver-specific expression of thyroid receptor β (THR-β), thereby lowering cholesterol and triglyceride (TG) levels, increasing BA synthesis, and promoting fat oxidation (Kokkorakis, Boutari et al. 2024).

The potential therapeutic targets for NASH include also mammalian target of rapamycin (mTOR), the sterol regulatory element-binding protein (SREBP), and galanin receptors, underscoring their significant involvement in lipogenesis and glucose metabolism. mTOR (also known as mechanistic target of rapamycin) is a serine-threonine kinase belonging to the Phosphatidylinositol 3 kinase (PI3K) family. It forms two distinct multi-protein complexes known as mTORC1 and mTORC2, each comprising 6 and 7 subunits respectively (Caron, Richard et al. 2015). mTORC1 has been extensively studied as a target for immunosuppressive and anticancer effects (Wu and Hu 2010, Alalawi, Sharma et al. 2017). mTORC1 is involved in regulating growth factors, energy maintenance, oxygen utilization, and amino acid production within cells. While mTORC2 plays a significant role in activating certain growth factors such as the type I insulin-like growth factor receptor and insulin receptor via mTOR's tyrosine kinase activity, as well as regulating cell survival, metabolism, and cytoskeletal organization, it has not been extensively studied as a therapeutic target for diseases (Yin, Hua et al. 2016). Ku-0063794 is chemically termed (5-(2-((2R,6S)-2,6-dimethylmorpholino)-4-morpholinopyrido[2,3-d]pyrimidin-7-yl)- 2-methoxyphenyl)methanol and comprises one of the earliest reported TORKinibs (Sun 2013). Ku-0063794 (KU) selectively inhibits both mTORC1 and mTORC2 at a concentration of 10 nM without affecting 76 other protein kinases and seven

lipid kinases, such as PI3Ks. It was introduced as a chemical tool for studying the effects of mTOR on cellular processes, including cell proliferation and cell cycle arrest in the G1 phase, with potential applications in anticancer research (García-Martínez, Moran et al. 2009)

SREBP transcription factors are pivotal in regulating numerous metabolic pathways, encompassing insulin resistance, glucose sensing, lipogenesis, and adipogenesis (Soyal, Nofziger et al. 2015). They have garnered attention as promising therapeutic targets for addressing a spectrum of metabolic disorders (Soyal, Nofziger et al. 2015) and certain cancers characterized by aberrant fat synthesis (Xue, Qi et al. 2020). It has 3 isoforms, namely SREBP-1a involved in FA and cholesterol synthesis and SREBP-1c, which mainly activates genomic transcription of the sterols involved in FA and TG synthesis. SREBP-1a and SREBP-1c are inter-related by complex signal transduction pathways. The third form SREBP-2 (encoded by gene Srebf2) is principally associated with cholesterol synthesis. Initially located at the ER membrane, SREBPs are transported to the Golgi apparatus, facilitated by the SREBP cleavage-activating protein (SCAP). Inhibition of SREBP activity has demonstrated efficacy in mitigating metabolic disorders such as NAFLD and HCC. However, the effects of SREBP inhibition on advanced stages like NASH, remain uncertain, warranting further investigation (Xiao and Song 2013, Kawamura, Matsushita et al. 2022). Fatostatin (FAT) is a synthetic small molecule, a diarylthiazole derivative, which inhibits SREBPs-mediated fat synthesis, and it has shown promise in alleviating symptoms of obesity in ob/ob C57BL6J mice (Kamisuki, Mao et al. 2009) and tamoxifen-induced NAFLD (Li, Lu et al. 2022). Beyond metabolic disorders, FAT has garnered attention for its therapeutic potential in various cancers.

Galanin, a neuropeptide, is a fundamental component of the galaninergic system. Despite its long study history (Tatemoto, Rökaeus et al. 1983), numerous biological processes involving galanin remain incompletely understood (Jiang and Zheng 2022, Zhu, Hu et al. 2022). The complexity of galanin-mediated signalling arises from the existence of three G-protein-coupled receptors (GPCRs): GalR1, GalR2, and GalR3, which transduce signals through different pathways (Jiang and Zheng 2022). The galaninergic system plays a crucial role in metabolism, food intake, and obesity, as evidenced by several studies. Galanin's activity in the hypothalamus, particularly through GalR1 stimulation, promotes increased fat intake. This stimulation can lead to a positive feedback loop, contributing to excessive fat intake and obesity (Marcos and Coveñas 2021). Dysregulation of this system may further lead to glucose intolerance, ultimately predisposing individuals to T2DM and/or metabolic syndrome (Fang, Yu et al. 2012). Celastrol, 3-hydroxy-9β,13α-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid, is a pentacyclic triterpenoid (Cascão, Fonseca et al. 2017) isolated from the root of *Tripterygium wilfordii*, which is a plant extensively used in the Chinese medicine, with showed anti-inflammatory and anticancer effects (Lee, Koo et al. 2006). In mice, it demonstrated potency as a leptin sensitizer and anti-obesity agent (Liu, Lee et al. 2015). Studies suggest that celastrol promotes white adipose tissue browning, protects against HFD-induced obesity by activating the hypothalamus-sympathetic axis, and inhibits negative regulators of leptin in the hypothalamus (Wang, Yang et al. 2021). Additionally, celastrol influences mitochondrial metabolism may lead to weight loss by inhibiting expression of galanin and GalR1/GalR3 receptors in the hypothalamus (Abu Bakar, Nor Shahril et al. 2022).

2. Aims and hypothesis

2.1 Aims

The main aim of the project was to evaluate the potential prophylactic and therapeutic effect of modulators of selected signalling molecules (eg. mTOR, SREBP, galanin) in the progression of NAFLD to NASH.

Objectives:

- Establishment of both *in vitro* lipotoxicty and *in vivo* diet-induced NAFLD/NASH models.
- To screen individual experimental substances with potentially antioxidative, anti-inflammatory, and impaired-lipid metabolism modifying properties *in vitro* in palmitic acid-induced lipotoxicity model using primary hepatocyte cell cultures.
- To select candidate modulators from *in vitro* screening to be used *in vivo* at several doses to demonstrate a prophylactic and/or therapeutic effect on NASH progression in a diet-induced animal model of NAFLD/NASH.
- To study pharmacokinetics, pharmacological effects and safety of non-specific mTORC1/C2 inhibitor (Ku-0063794) in a preclinical model of NASH progression.
- To study pharmacodynamic effects (at biochemical, morphological, genetic and histopathological level) and safety profile of SREBP1/2 inhibitor fatostatin in a preclinical model of NASH progression.
- To assess the impact of celastrol treatment on the liver galaninergic system in an experimental mouse model of atherogenic western-type diet-induced obesity and NASH.

2.2 Hypothesis

- According to available researched data (Arora, Kutinová Canová et al. 2022), we have hypothesized that mTORC1 and mTORC2 play a pivotal role in lipid metabolism and adipogenesis that may act as a potential therapeutic target for metabolic diseases like NAFLD/NASH, therefore, we aimed to investigate Ku-0063794, the second generation mTORC1/C2 inhibitor, in managing experimentally-induced NAFLD/NASH.
- SREBPs play a key role in regulating multiple metabolic pathways, such as insulin resistance, glucose sensing, lipogenesis, and adipogenesis. Therefore, we assumed that targeting them by a SREBP1/2 inhibitor, fatostatin, could help alleviate the dysregulation linked to metabolic disorders and be a promising strategy to reduce the severity and progression of NAFLD to NASH.
- Finally, we supposed that celastrol may impact the therapeutic potential in alleviating obesity and related conditions like NAFLD/NASH due to its known inhibitory effect on galanin and galanin receptors in the hypothalamus leading to increased satiety and decreased energy intake. Since the occurrence of members of the galanin family has been described in a number of organs, we hypothesized that celastrol could also favourably affect the galaninergic system directly in the liver.

3. Methodology

In vitro lipotoxicity model was introduced using rat primary hepatocytes, where the cells were pre-treated with selected drugs 8 hr prior inducing lipotoxicity by palmitic acid. After incubation, the cell supernatants were sampled for analyzing cell viability, ALT, nitrites and reactive oxygen species (ROS) levels. To detect PA accumulation in hepatocytes, Oil Red O staining was performed.

First, KU *in vivo* pharmacokinetic (PK) study was performed where KU was administered to mice by oral or intraperitoneal route to compare the bioavailability in serum. The concentration of Ku-0063794 was determined in serum by ultra-performance liquid chromatography-mass spectrometry analysis, the Shimadzu UHPLC Nexera X3 coupled with a Triple Quad 8045 tandem mass spectrometer (Shimadzu, Kyoto, Japan).

Each selected drug was individually assessed in *in vivo* pharmacodynamics (PD) models, for which male C57BL6J mice were arbitrarily assigned into 3 groups. The dietary NAFLD/NASH induction commenced with the feeding of male C57BL/6 mice with a high-fat atherogenic western-type diet (WD) in group 2 and 3 together with fructose and glucose in drinking water (WD/FG) for either 8-16 weeks, aimed at inducing various phases of NAFLD-to-NASH, where each induction represents separate set of experiment, where group 1 always received the standard chow diet with water ad libitum. Alongside their respective diets, group 1 and 2 were treated with respective vehicles and group 3 mice were treated intraperitoneally with either KU for 3 weeks (5 mg/kg daily) or chronically for 3 months (5 mg/kg 3 times a week), FAT (15 mg/kg every 2^{nd}–3^{rd} day) or CEL (200 μg/kg each 2^{nd} day) for next 4 weeks. Ultimately, liver and blood samples were collected for additional analysis.

Determination of the serum concentrations of lipids (total cholesterol, HDL, triglycerides), glucose, albumin, liver enzymes (ALT, AST, ALP), and nitrogen metabolites (urea, creatinine) was performed. The quantitative detection of mouse serum TNF-α was performed with a high-sensitivity ELISA Kit. To determine the severity of liver oxidative stress caused by the disease, total lipid peroxidation was estimated by evaluating conjugated dienes (CDs), thiobarbituric acid reactive substances (TBARS), and nitrites as previously described (Farghali, Cerný et al. 2009). The total amount of protein in homogenates of livers and subsequently also in mitochondria was determined by using the Bio-Rad protein DC assay (Bio-Rad, Prague, Czech Republic). We even estimated the TG content in liver following Triglyceride Quantification Colorimetric/Fluorometric Kit protocol. For the assessment of mitochondrial functions, analyzing the activity of electron transport chain (ETC) complexes activity (I-IV), mitochondrial respiration linked to the ETC complexes I and II, as well as adenosine triphosphate (ATP) production (Ľupták, Fišar et al. 2022). Isolated lobes of livers were used for further qRT-PCR analysis. Relative expression of target mRNA was calculated using ΔΔCt method with *Hprt1* mRNA as an internal control (Lađinović, Pinkas et al. 2020). Further, the

liver samples were homogenized and lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors and assessed for protein expression using western blot of mTOR and p-mTOR. Lastly liver tissues were fixed in 4% paraformaldehyde and then embedded in paraffin, cut into 8 µm thick sections, and stained with either hematoxylin-eosin (HE) or Sirius red (SR) stain. Liver samples were scored for NAFLD and fibrosis by trained histologist who was blinded to the identification of the mice.

For statistical analysis, to compare the differences between two or more groups Student's *t*-test or one-way ANOVA (parametric) with post hoc Bonferroni test, or Kruskal-Wallis test with the post hoc Dunn test was used. respectively. Two-way ANOVA was used to inspect both repeated weighing temporal and group effects. The results for the variables' data are expressed as mean and standard deviation (SD). The differences were considered statistically significant when $p < 0.05$. Statistical analyses and data visualization were performed using GraphPad Prism, version 8.0 for Windows (GraphPad Software, San Diego, California, USA).

4. Results

4.1 Ku-0063794

***In vitro* experiment:** Exposure of the hepatocytes to PA significantly decreased cell viability to 73.0±6.5%, increased ALT, ROS and nitrite production. The pre-treatment with both concentrations of KU (50 and 100 nM) increased cell viability to 83.3±7.9% ($p<0.05$) and 80.6±3.9%, respectively (Fig. 2A). Pre-treatment with KU significantly reduced in the dose-dependent manner the cell culture medium concentrations of ALT exposed to PA (Fig. 2B). Additionally, PA treatment significantly increased both nitrite concentration (as an oxidative stress marker) and overall ROS production, while KU pre-treatment had no effect on these parameters.

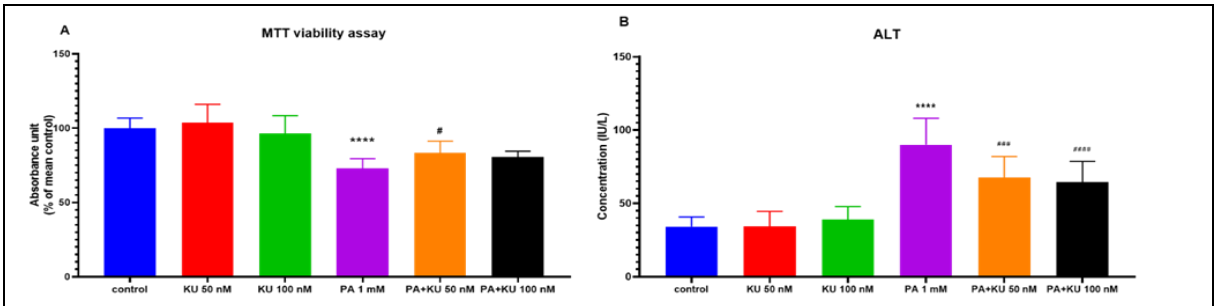


Figure 2: The impact of Ku-0063794 (KU) pre-treatment on palmitic acid (PA)-induced lipotoxicity in the primary hepatocytes.

Pharmacokinetic *in vivo* study: KU is very well and rapidly absorbed both after oral and intraperitoneal dosing. Terminal KU plasma concentrations of mice on WD were among others measured after repeated oral (10 mg/kg) and intraperitoneal (5 mg/kg) routes of dosing to be consequently compared. Daily 3 weeks KU treatment produced an average plasma

concentration of 1736 ng/ml by i.p. route whereas, 193 ng/ml by oral route even at doubled dose as assessed 24 hrs after last KU dose (Fig. 3). These results demonstrate that intraperitoneal application of KU displays higher bioavailability and exposure then oral route of dosing.

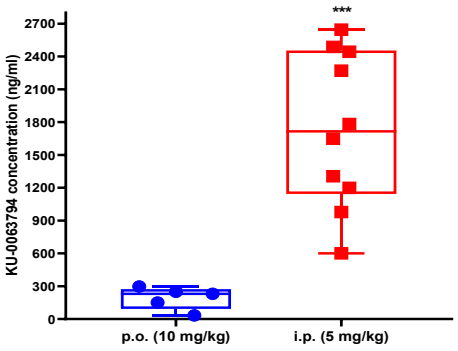


Figure 3: Comparative terminal plasma concentrations on repeated KU-0063794 dosing study when KU given daily by p.o. (n = 5) or i.p. (n = 10) route to mice on WD.

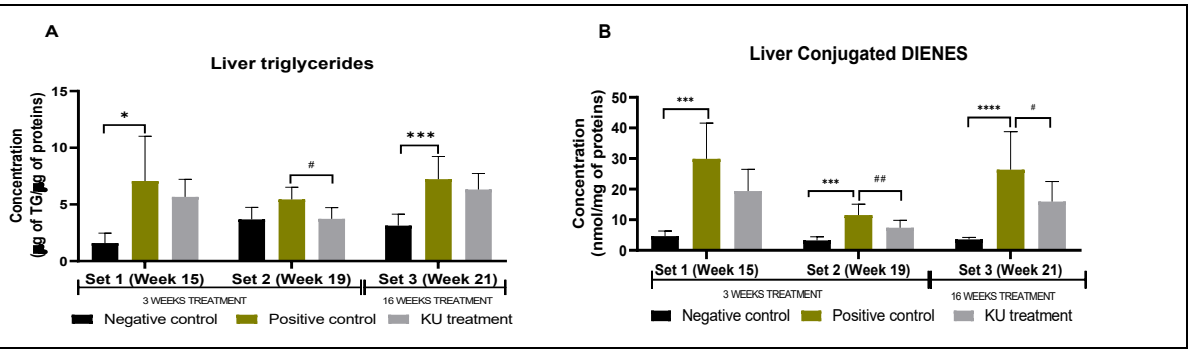
***In vivo* PD experiment** on a diet-induced model of NAFLD/NASH in mice represented by set 1 (acute KU treatment in the onset of NASH), set 2 (acute KU treatment in the progressed NASH), and set 3 (chronical KU prophylactic treatment of NAFLD/NASH).

Biochemical analysis: We found some factors were improved like serum ALT in set 2. In the liver homogenates, KU treatment significantly reduced TGs and oxidative stress markers like conjugated dienes, nitrites and even enhanced ATP mitochondrial production (Fig. 4A-C).

In the gene expression analysis, the follow-up KU treatment in set 1 slightly increased *Sirt1, Nrf2*, and *Cyp2e1* mRNA levels, but did not affect *Nos2*, however, on the contrary, it significantly diminished the expression of *Srebf1*, *Mtor*, *Crtc2*, and *Ppargc1α* genes when compared to positive controls. In set 2, KU treatment normalized significantly decreased expression of *Ppargc1α* and *Crtc2* genes in positive controls (Fig. 4D-G).

Protein analysis: Surprisingly, the absolute amount of phosphorylated mTOR (p-mTOR, an activated form of mTOR) was decreased both in all positive controls (with a significant difference at week 15 only) and significantly in all KU groups compared to respective negative controls. In chronic set 3 experiment, KU significantly further intensified WD/FG-induced down-regulation of p-mTOR (Fig. 4H).

Histopathological analysis: Though the KU treatment did not produce any significant additional effects on liver morphology, it displayed a pattern of slight reduction in liver fibrosis namely in experimental set 1 and set 3.



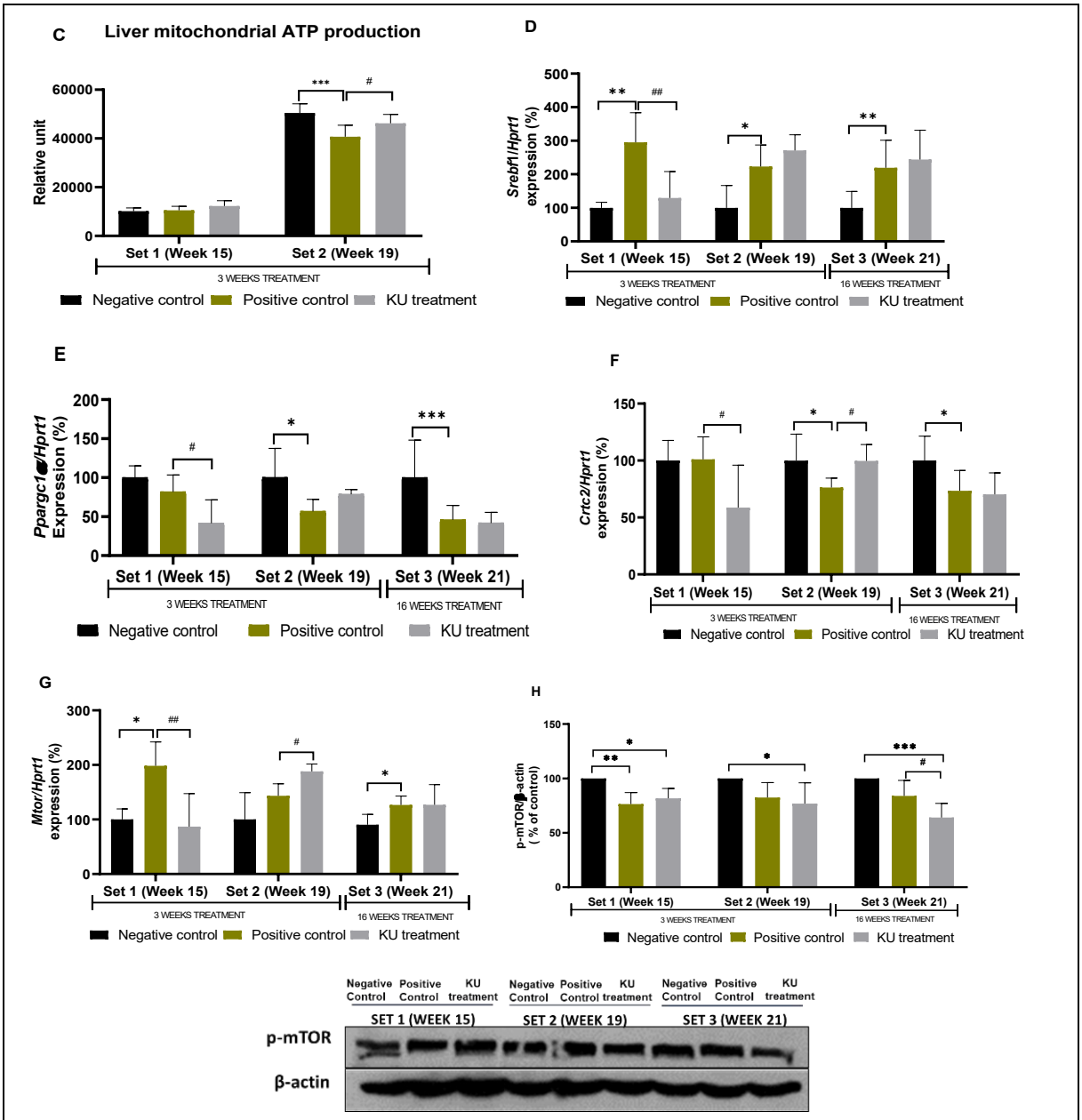


Figure 4: Effect of KU treatment on serum biochemistry, liver markers of oxidative stress, triglycerides, gene and protein expressions, and histopathological analysis in each sets representing 3 time points (week 15, week 19, and week 21) in WD/FG-induced NASH in mice.

Ppargc1a (also Pgc-1α, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), *Crtc2* (CREB, cAMP response element-binding protein, regulated transcription coactivator 2), *Srebf1* (sterol regulatory element binding transcription factor 1), *Mtor* (mechanistic target of rapamycin kinase), *Hprt1* (hypoxanthine phosphoribosyltransferase 1).

4.2 Fatostatin

***In vitro* experiment:** Exposure of the hepatocytes to PA significantly decreased cell viability to 77.0 ± 2.3%, while pre-treatment with both concentrations of FAT (15 and 25 µM) increased cell viability to 81.4 ± 2.9% (n.s.) and 87.0 ± 6.4% ($p < 0.001$), respectively (Fig. 5A). Pre-treatment with FAT at 25 µM significantly lowered the medium concentrations of ALT exposed to PA while only pre-treatment with FAT at 15 µM significantly decreased ROS production but not nitrites (Fig. 5B-D).

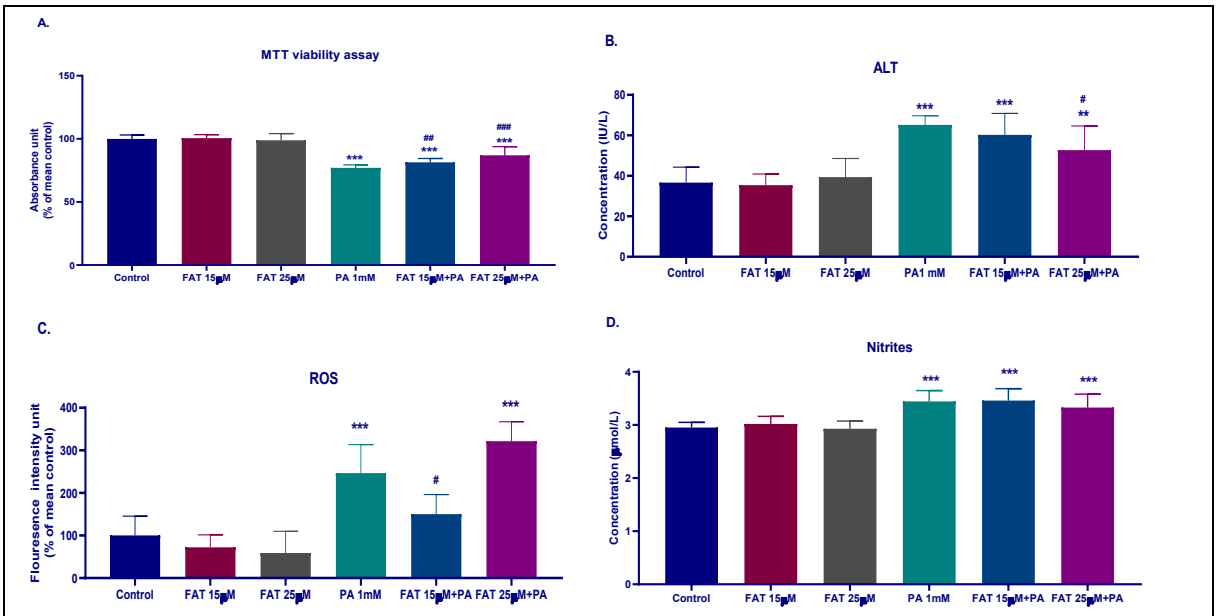
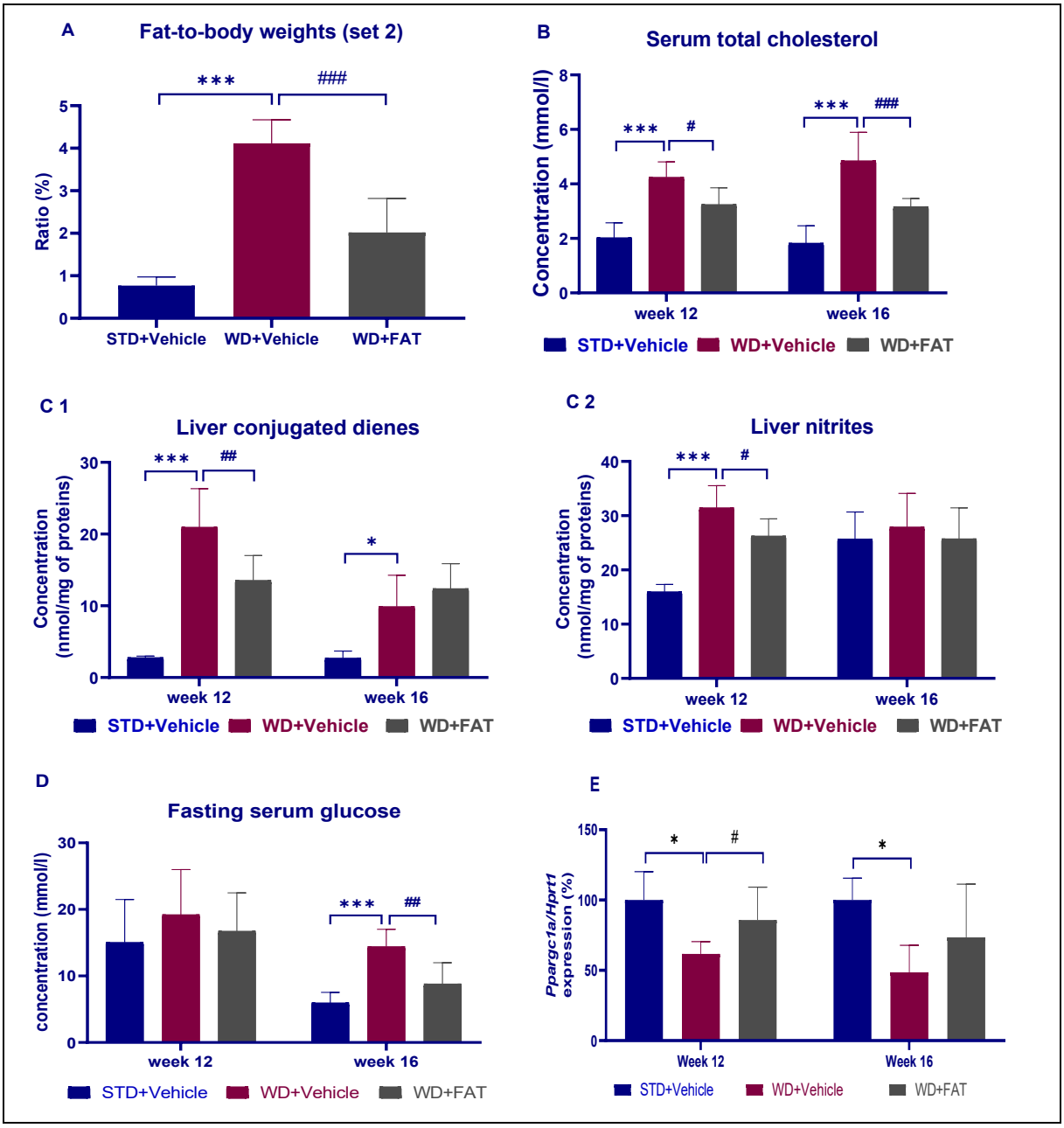


Figure 5: The impact of FAT pre-treatment on palmitic acid (PA)-induced lipotoxicity in the primary hepatocytes.

***In vivo* experiment:**

FAT highly significantly decreased mice body weights after one week of daily treatment or after 2 weeks when treated each second day during the set 1 (representing NAFLD transforming to NASH) or set 2 (progressing NASH) of *in vivo* experiment therefore, FAT dosing was continued every 3 days. Moreover, FAT had significantly reduced fat-to-body weight ratio in the set 2 (Fig. 6A). FAT treatment had significantly reduced the total serum cholesterol (Fig. 6B), HDL, LDL cholesterol and liver TGs (Fig. 6H) at least in one or both sets. FAT treatment produced significant hypoglycaemic effects as evident by oral glucose tolerance test (OGTT) and 12 hour-fasting serum glucose levels (Fig. 6D). The FAT treatment had significantly reduced levels of liver CDs ($p = 0.006$, Fig. 6C1) and nitrites ($p = 0.016$, Fig. 6C2) only at the end of week 12. Metabolic genes like *Ppargc1a* expression coding for PGC-1α was significantly reduced by WD+vehicle in both the experimental sets, while FAT treatment significantly enhanced the expression to baseline at week 12 and similar trend was observed at week 16 (Fig. 6E). The expression of *Srebf1* was extremely ($p < 0.001$) upregulated by WD/FG which was significantly downregulated by FAT treatment at week 16 (Fig. 6F). FAT treatment significantly decreased liver *Tnfa* mRNA levels at week 16 (Fig. 6G). The FAT treatment had

evidently reduced the steatosis score at week 16 ($p = 0.032$) and even significantly decreased the overall NAFLD activity score (NAS) in both the sets (Fig. 6J, K). Surprisingly, FAT treatment significantly enhanced TNF-α serum levels compared to positive controls indicating that FAT precipitates systemic inflammation at week 16 (Fig. 6I). This corroborated with our findings that mice treated with FAT exhibited morphological indicators of skin toxicity characterized by dermatitis spanning their bodies: with swollen nasal passages, inflamed salivary glands, and severe skin flakiness indicative of hair loss, eczema, and itching, predominantly in facial, neck, abdominal, and urogenital regions.



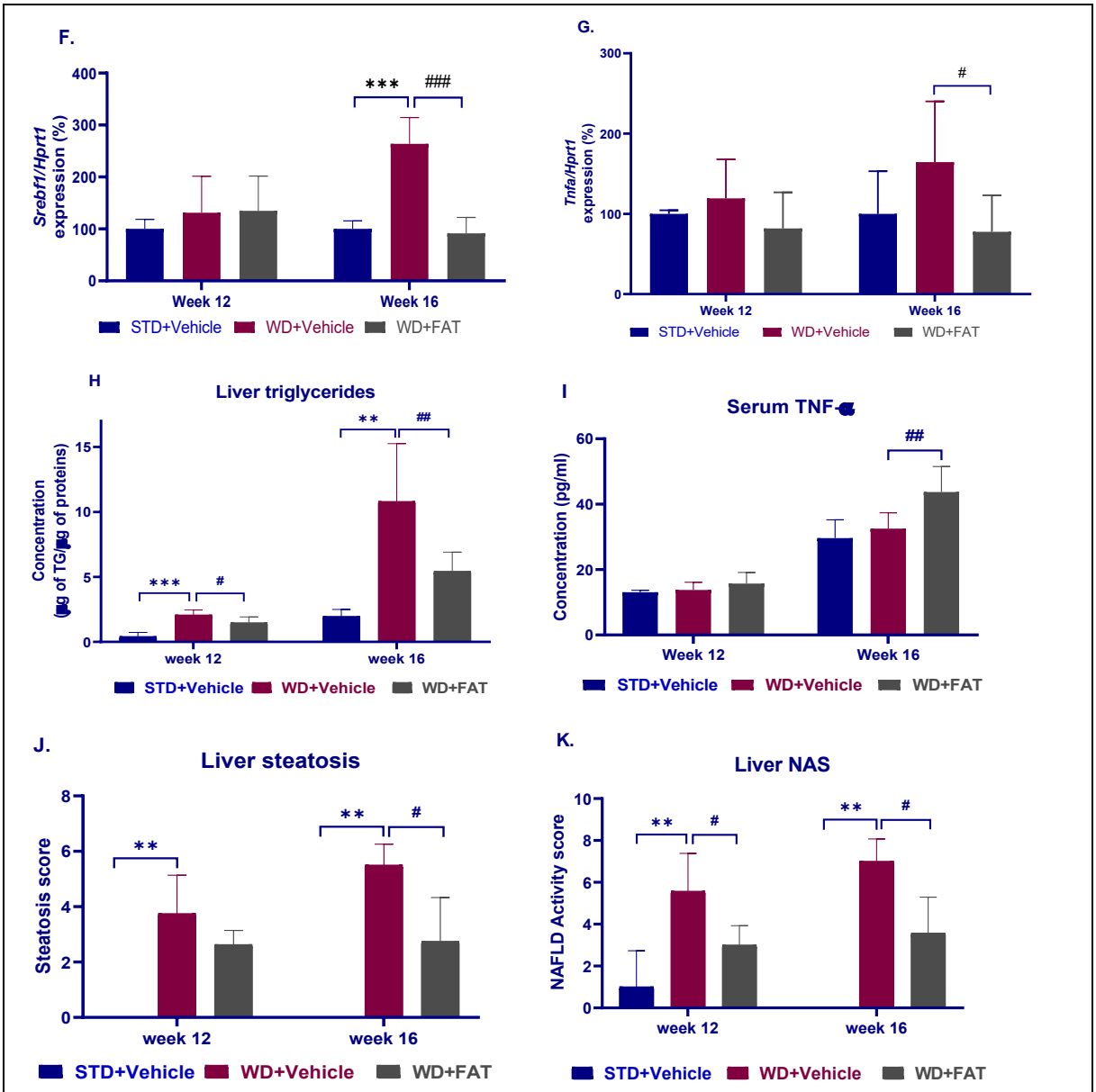


Figure 6: Effect of FAT treatment on serum biochemistry, liver markers of oxidative stress, triglycerides, gene expression, and histopathological analysis in each sets representing 2 time points (week 12 and week 16) in WD/FG-induced NASH mice model.

4.3 Celastrol

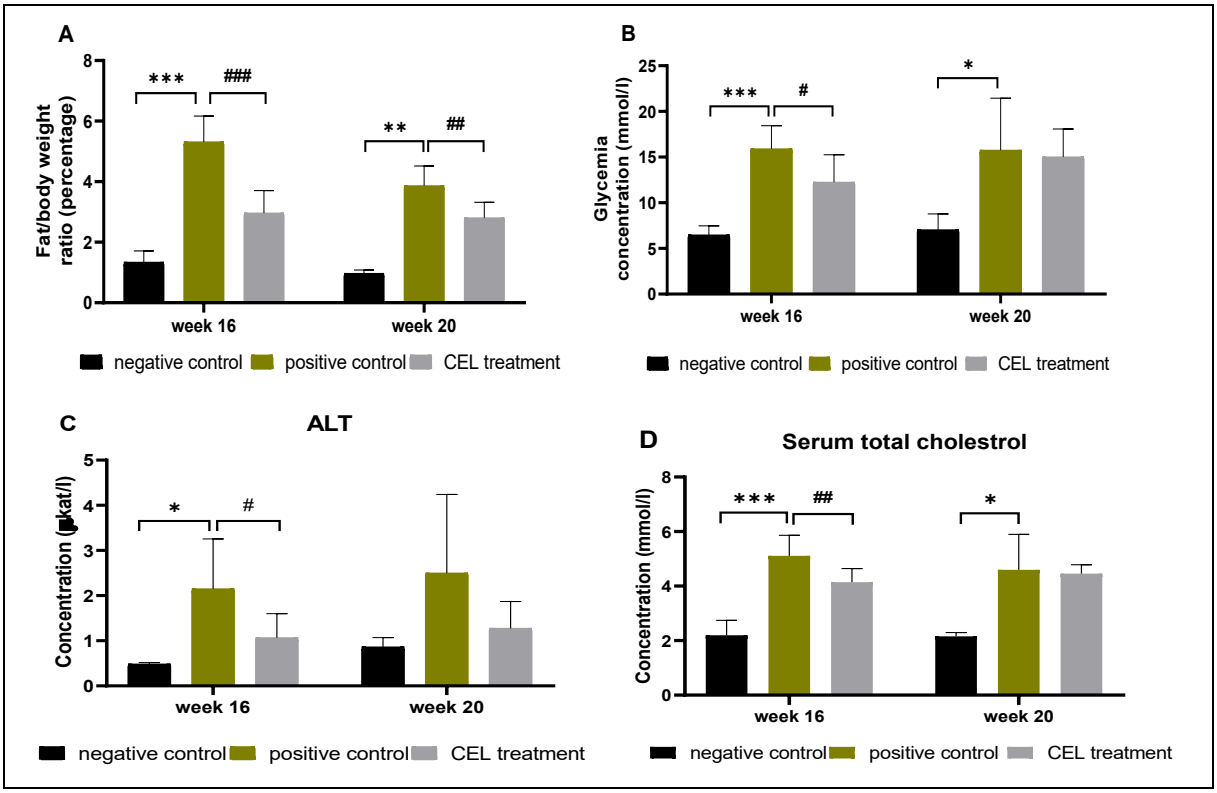
***In vitro* experiment:** The pre-treatment with 1 µM and 5 µM CEL significantly mitigated ROS and nitrite production by hepatocytes, respectively. Moreover, CEL decreased PA-induced accumulation of fat as detected by Oil Red O staining.

***In vivo* experiment:** CEL treatment significantly decreased mice body weights, liver weights and fat-to-body weight ratios (Fig. 7A) when compared to positive control in both sets of experiment (representing set 1 as progressed NASH and set 2 highly progressed NASH). WD/FG elevated glycemia levels in OGTT curve and terminal fasting glucose that was

substantially reduced by CEL treatment (Fig. 7B). CEL treatment significantly decreased serum ALT and lipid markers only at the end of week 16 (Fig. 7C, D). Liver TG content was highly significantly increased in positive controls and, conversely, remarkable ($p < 0.05$) reduced after CEL treatment in both time periods. At the end of week 16, CEL treatment was able to significantly reduced TBARs (Fig. 7E).

Gene expression of *Acaca* (acetyl-Coenzyme A carboxylase alpha) and *Fasn* (fatty acid synthase) that code for important lipogenic enzymes promoting *de novo* lipogenesis and adipogenesis were induced in mice livers of positive controls of both periods. However, CEL treatment normalized Acaca and Fasn mRNA levels only at the end of week 16 (Fig. 7F, G). For evaluation of inflammatory pathway, we assessed liver expression of genes coding TNF-α and IL-1β cytokines. *Tnfa* mRNA levels were increased (n.s.) by WD/FG and significantly downregulated by CEL at week 16(Fig. 7J). Gene expression of *Il1b* was significantly up-regulated by a atherogenic diet during both periods and CEL treatment significantly reversed it only at the end of week 16 (Fig. 7H). In mice liver tissue, only *Galr2* gene expression could be quantitatively evaluated because the expression of other genes (i.e. *Gal*, *Galr1*, and *Galr3*) was very low or undetectable. Interestingly, *Galr2* gene expression in the mice liver of the set 1 was affected at completely different manner: it was significantly down-regulated by the WD/FG, highly significantly increased by CEL and unchanged by FAT when compared to positive controls (Fig. 7I).

At the end of week 16, CEL significantly reduced liver steatosis, inflammation and total NAS (Fig. 7K, L) and ameliorate liver morphology. CEL treatment displayed a similar pattern of slight reduction in all histological scores including liver fibrosis and overall liver morphology at the end of week 20.



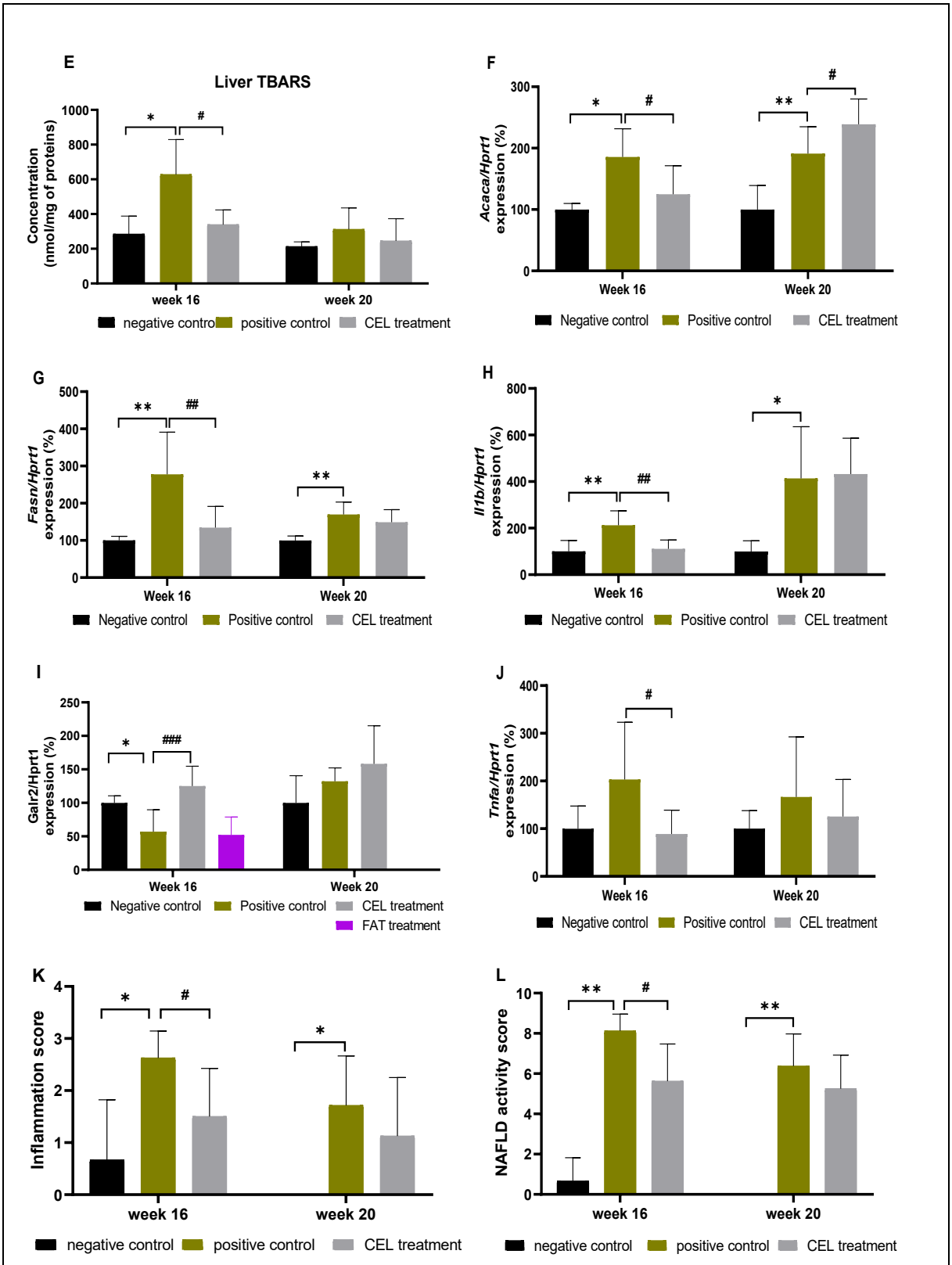


Figure 7: Effect of CEL treatment on serum biochemistry, liver markers of oxidative stress, triglycerides, gene expression, and histopathological analysis in each sets representing 2 time points (week 16 and week 20) in WD/FG-induced NASH mice model.

5. Discussion

In our research journey, we encountered a group of compounds known as mTOR inhibitors, which have strong experimental evidence of delivering beneficial effects in NAFLD/NASH, as discussed in our prior review on mTOR inhibitors (Arora, Kutinová Canová et al. 2022). Therefore, we embarked on a preclinical three-stage pharmacological investigation (*in vitro*, and *in vivo* PK and PD) with Ku-0063794, a second-generation mTOR inhibitor, to validate the effects of mTORC1 and mTORC2 inhibition specifically in NASH. The primary hepatocytes exposed to PA treatment showed reduced viability, confirming the lipotoxic properties of the fatty acid (Chen, Li et al. 2018, Fang, Wu et al. 2019). KU decreased PA-induced lipotoxicity and lipid accumulation in our experiment and furthermore, KU at the tested concentrations did not induce any toxicity to intact cells. Studies supporting our findings suggest both a suppression of downstream mTORC1 regulators and protection against PA-induced cell death in hepatocytes (AML12 and HepG2 cells) when mTORC1 is inhibited, as shown by some first-generation mTOR inhibitors like rapamycin and Torin-1 (Wang, Li et al. 2016, Chen, Griffiths et al. 2020). Moreover, a rapamycin-containing nanoparticle formulation may significantly inhibit oleic acid-induced lipid accumulation in HepG2 cells (Zhao, Zhu et al. 2020).

Based on the measured KU concentrations, we established that KU is highly and rapidly absorbed both after oral and intraperitoneal dosing in mice. However, bioavailability after intraperitoneal dosing was approximately 160% of that after oral dosing, potentially due to extended absorption, therefore, we selected i.p. dosing for further studies. In the PD study with KU, we replicated a model of progressing NASH induced by a high-fat western-type diet (WD/FG) in mice, characterized by typical metabolic abnormalities and histological findings associated with obesity, hyperglycemia, and atherogenic-like lipid profile (Staňková, Kučera et al. 2020, Lastuvkova, Faradonbeh et al. 2021, Staňková, Kučera et al. 2021). NASH induced by a HFD is known to be linked with a specific type of insulin resistance (Li, Wu et al. 2020). Interestingly, both short-term and long-term treatment with KU did not result in high blood sugar levels, which is a well-known side effect of mTOR inhibitors (Nguyen, Vautier et al. 2019). Various studies have demonstrated the effectiveness of rapamycin (Brown, Stefanovic-Racic et al. 2007, Chang, Chiu et al. 2009), everolimus (Chang, Hou et al. 2021), and even some new generations of PI3K-Akt-mTOR inhibitors (Liu, Han et al. 2016) in reducing lipid accumulation, *de novo* lipid biosynthesis, and adipogenesis, as confirmed by the genetic expression of downstream regulators of the mTOR pathway critical in lipid metabolism. But in our NASH mouse model, KU neither increased nor decreased serum lipid levels, indicating its cardiovascular safety. Fortunately, KU treatment reduced oxidative stress markers, indicating its potential antioxidant effects, particularly beneficial in diseases like NASH. Studies have shown how rapamycin treatment effectively reduces oxidative stress at the mitochondrial level, enhances antioxidant capacity, and promotes autophagy through mTOR (Sapp, Gaffney et al. 2014, Martínez-Cisuelo, Gómez et al. 2016, Oaks, Winans et al. 2016). A decrease in hepatic mitochondrial ATP levels was observed in NASH but not in NAFLD, consistent with our findings in sets 1 and 2 (Karkucinska-Wieckowska, Simoes et al. 2022), representing a transition stage of NAFLD to NASH in experimental set 1 and progressed NASH in set 2.

Interestingly, KU treatment improved ATP production at week 19, highlighting mTOR's role in mitochondrial function. Furthermore, specific investigations outlined in the review (Speca, Giusti et al. 2012) have highlighted the involvement of mTOR in regulating autophagy, inflammation, and fibrosis. However, some researchers argue that long-term use of mTORC1 inhibitors may exacerbate inflammation, fibrosis, and even tumor formation despite transiently reducing liver steatosis (Umemura, Park et al. 2014, Love, Mudasir et al. 2017). This contrasts with our PD experiments, where systemic inflammation, as indicated by significantly increased serum TNF-α levels and upregulated liver TNF-α gene expression, was efficiently downregulated by KU in long-term treatment without worsening fibrosis or triggering liver tumor formation. WD/FG effectively induced NASH, characterized by high levels of micro- and macro-vesicular steatosis, inflammation, and fibrosis until week 15, but demonstrated an aggressive worsening of hepatic condition by weeks 19 and 21. Some studies using mTOR inhibitors have shown effective improvement in steatosis under similar dietary conditions and models (Love, Mudasir et al. 2017). However, none of our sets with intraperitoneal KU application showed improvement or worsening of highly progressed NASH histopathology.
However, we supposed that mTOR inhibition may be beneficial in liver diseases as supported by numerous studies (Okuno, Kakehashi et al. 2018, Gosis, Wada et al. 2022). On the contrary, another study supports the activation of mTOR as a protective mechanism in NASH, however, undesirable in NAFLD (Uehara, Sostre-Colón et al. 2022). Even KU was previously demonstrated to potently inhibit the phosphorylation of mTORC1 at Ser2448 *in vitro* in HEK-293 cells in a dose- and time-dependent manner (García-Martínez, Moran et al. 2009), we proved the same *in vivo* after chronic treatment with KU (experimental set 3) only. We can speculate that it could be caused by unpredictable high background reduction of mTOR phosphorylation/activation by WD/FG throughout all sets of our *in vivo* experiments and that there must be mechanisms *in vivo* that compensate for mTORC1/C2 loss. Only recently, after the completion of our *in vivo* experiments, the research work on an animal dietary model of NAFLD/NASH has been published showing that mTORC1 activity varies depending on the progression of the disease: it is higher in the NAFLD stage, and significantly decreases in the NASH stage with further progression (Uehara, Sostre-Colón et al. 2022). These correspond with our results and may explain KU's beneficial effects on *in vitro* PA-induced lipotoxicity (resembling NAFLD/NASH) together with the relative inefficiency of KU to prevent and reverse the progression of NASH *in vivo*. We may speculate that simultaneous inhibition of mTORC2 activity by KU (García-Martínez, Moran et al. 2009) could neutralize detrimental effects of NASH-decreased mTORC1 activation produced by WD/FG through inhibition of negative feedback of the mTORC2-AKT-FOXO-SREBP1c lipogenesis axis (Uehara, Sostre-Colón et al. 2022). In this context, and because of the previous finding that mTORC2 expression was increased in the liver of patients with cirrhosis and tumor arising from NAFLD (Guri, Colombi et al. 2017),
We also found that SREBP, acting as a secondary messenger in the mTOR signaling pathway, holds a significant role in lipogenesis and adipogenesis. Therefore, targeting SREBP alone could be a critical step in altering lipid and fat metabolism, with its inhibition potentially effective in treating metabolic diseases like obesity, NAFLD, or NASH. With this in mind, we

selected FAT, which effectively inhibits SREBP activation by blocking its translocation protein SCAP, ultimately preventing further adipogenesis (Kamisuki, Mao et al. 2009). Scutellarin, an mTOR modulator, suppressed protein SREBP-1c and effectively reduced PA lipotoxicity in HepG2 cells (Luan, Huo et al. 2020). After 30 minutes of pretreatment with FAT and ilexgenin A, the concentration of palmitate was considerably reduced in a 24-hour culture of primary mouse hepatocytes with palmitate and oleic acids (Lu, Ma et al. 2022). A study similar to ours supports these findings, indicating that FAT treatment for 28 days reduces total cholesterol, HDL cholesterol, and cholesterol profile, while increasing TGs, VLDL, FFAs, and ketones in ob/ob mice (Kamisuki, Mao et al. 2009). Conversely, no change in serum TGs and cholesterol was observed with FAT treatment of BALB/c male mice daily for 3 days (Ma, Murakami et al. 2022). In the similar study, serum levels of ALT, AST, creatinine, and urea persisted without any alteration upon treatment with FAT, supporting that acute treatment with FAT is not related to liver or kidney toxicity (Mesquita, Ferreira et al. 2020). FAT (30 mg/kg i.p. daily for 28 days) has also been reported in early studies to be a hypoglycemic agent, reducing body weight and epididymal fat considerably, thus acting as an anti-obesity agent in ob/ob mice (Kamisuki, Mao et al. 2009). In another study examining FAT for its anti-inflammatory effects in rheumatoid arthritis, significant reductions in body weight on animals treated with FAT (0.6 mg in 80 µl of DMSO, approximately 24 mg/kg) via intraperitoneal route for three days daily were observed (Ma, Murakami et al. 2022). However, a recent study, presented after the finalization of our study on FAT, found that giving FAT to C57/Bl6J mice at a dose of 30 mg/kg for 21 days resulted in significantly decrease in the body weight as a consequence of FAT treatment at higher dose, while organ and pathophysiological weight remained same (Zhu, Shi et al. 2024). The hypoglycemic effects was observed at the genetic level, as FAT treatment normalized the expression of the *Ppargc1a* gene. PGC-1α plays a crucial role in carbohydrate metabolism and is essential for insulin-mediated suppression of hepatic glucose production (Besse-Patin, Jeromson et al. 2019). The lower blood cholesterol levels seen in our study may be explained by the downregulation of *Srebf1* gene expression by FAT, which was consistent with the FAT research by Kamisuki et al. (Kamisuki, Mao et al. 2009). Similarly, FAT even downregulated the expression of the liver genes *Acaca* and *Fasn*, which may be connected to decreased TG serum levels and liver TG content, reduced fat production, and steatotic score in histopathological analysis. Furthermore, FAT's suppression of the SREBP2-LDLR pathway in mice allowed it to successfully prevent tamoxifen-induced NAFLD (Li, Lu et al. 2022). All of these findings demonstrate how effective FAT therapy is at lowering hepatic steatosis. Additional research has demonstrated that FAT therapy inhibited TNF-mediated activation of genes involved in the cholesterol pathway in addition to SREBP2 genes, and that TNF-α stimulation is inhibited in skin wound healing and peritonitis models by SREBP2 ablation (Kusnadi, Park et al. 2019). Only the liver *Tnfa* gene expression in set 2 of our investigations showed a substantial downregulation following FAT therapy. Surprisingly, the severe systemic inflammation and dermatitis-like skin toxicity symptoms appeared after FAT treatment. Nevertheless, we are the first to show that administration of FAT after a cumulative dosage schedule (approximately 90-100 mg/kg) is linked to skin toxicity, including dermatitis in C57Bl6J mice that are maintained on WD/FG for the duration of the trial. Atopic dermatitis is

linked to decreased ceramide levels (Imokawa, Abe et al. 1991). Moreover, it is well-known that SREBP-2 plays a significant role in controlling the synthesis of phospholipids, FAs, and cholesterol in mouse cultured keratinocytes. These phospholipids are then further used in the manufacture of ceramides. Palmitate, a precursor for ceramides that is produced during the synthesis of FAs and is indirectly controlled by SREBP-2, is needed (Elias 2005).

In our research, we are showcasing the positive impacts of celastrol on diet-induced obesity and the progression of NAFLD to NASH in mice by reducing inflammation, boosting antioxidant defence, and controlling lipid and glucose metabolism. Other studies have also noted similar beneficial effects of CEL on HFD-induced obesity and fatty liver in mice (Zhang, Geng et al. 2017, Li, Xie et al. 2022). CEL has been shown to produce its metabolic benefits, among others, by activating PGC-1α, which enhances energy expenditure and browning of white adipose tissue (WAT) by regulating mitochondrial function and biogenesis (Fang, He et al. 2019, Li, Xie et al. 2022). Interestingly, changes in food behaviours under challenging HFD and glucose intolerance have also been described for *Gal*-KO mice (Ahrén, Pacini et al. 2004, Adams, Clapham et al. 2008) suggesting the involvement of galanin and GalR1 in adjusting food intake and metabolic responses to variations in dietary fat, while also influencing glucose regulation in mice (Zorrilla, Brennan et al. 2007). Moreover, a substantial up-regulation of lipogenic genes such as *Acaca* and *Fasn* was observed in conjunction with an increase in liver TG content, steatosis score, adipose tissue weight, and serum total cholesterol that was caused by WD/FG. In the set 1 experiment, CEL dramatically reduced both genes and all of these lipid metabolism markers, which is consistent with prior animal investigations (Zhang, Geng et al. 2017, Hu, Li et al. 2018).The hepatic expression of the *Galr2* gene, the sole identified member of the galanin family with detectable mRNA levels in the liver of mice, exhibited variability during our investigation. Recent research has shown that individuals with NAFLD had higher levels of serum galanin. Additionally, a 5-week treatment with CEL has been found to improve HFD/high cholesterol-induced NASH in mice, as demonstrated by (He, Huang et al. 2023). Furthermore, the study demonstrated that murine macrophages express GalR2, suggesting a novel function for galanin in suppressing the pro-inflammatory characteristics of macrophages and promoting their M2-polarization (He, Huang et al. 2023). Remarkably, the same scientists found that galanin suppresses the profibrogenic properties of primary rat hepatic stellate cells by activating the GalR2 receptor (He, Li et al. 2016). Therefore, we can speculate that CEL could exert its anti-inflammatory, lipid, and NAS-lowering effects in the liver through increased expression of the *Galr2* gene, namely in the set 1 of our experiments. Interestingly, fatostatin did not affect liver *Galr2* gene expression, which was also accompanied by significantly decreased liver *Tnfa* mRNA, but unchanged both *Il1b* gene expression and inflammatory score. The involvement of galanin in liver fibrosis and inflammation is multifaceted, with varying research findings (He, Huang et al. 2023). Moreover, CEL was histologically demonstrated to be safe in major organs including the liver, spleen, lungs, kidneys, and brain of HFD-induced obese mice (Fan, Zhao et al. 2022). Another review primarily outlined the favourable impacts of CEL on cardiovascular disease derived from *in vitro* and *in vivo* preclinical research and potential underlying mechanisms including CEL´s inhibitory effect on the central galaninergic system (Li, Zhang et al. 2022).

6. Conclusion

Our *in vitro* study with PA-induced lipotoxic model effectively worked for screening of drugs for NAFLD/NASH model, in lieu KU, FAT, and CEL emerged as a promising candidate drugs for further *in vivo* studies in mice.

Further, we would like to conclude that WD/FG was highly aggressive in producing NASH with inflammation and fibrosis that closely mimicked the dietary progression of NASH in humans. In the adopted dietary mouse model of NAFLD/NASH, we found that the longevity of the diet induction firmly affected the severity of the progression of NASH as assessed by important markers such as serum biochemical markers (liver enzymes and lipid profile), oxidative stress parameters, and inflammatory markers and by the expression of crucial antioxidative and metabolic genes, and the liver histopathological assessment.

In summary of *in vivo* PK studies, we found the i.p. route of KU application to be more effective than the oral one to achieve effective plasma concentrations. Our intraperitoneal application of KU (5 mg/kg) for 3 weeks and KU (8 mg/kg, 3 times weekly) for 16 weeks did not exert any serious toxic effects as manifested for other mTOR inhibitors rendering it safe and beneficial in some critical NASH parameters. Though KU seemed to improve some oxidative stress parameters, mitochondrial biogenesis, and gene expression of inflammatory marker, KU was not able to completely reverse the progression of NAFLD to NASH in mice. We support more pronounced and elaborative studies with KU in the early phase of NAFLD, as we found NASH irreversibly and severely progressed till 19 to 21 weeks, which may not be fully alleviated by KU treatment. Our study demonstrates the complexity of mTOR signaling regulation as KU acts by modulating the mTORC1/C2 signaling pathway and its downstream regulators depending upon the longevity and severity of the disease that suggests stratified therapeutic management throughout the disease.

It can be conclusively stated that fatostatin treatment improved features of the late NAFLD to early NASH progression as evidenced by: decreased body weight, fat, glycaemia levels, serum cholesterol and liver triglycerides; normalized expression of liver metabolic gene expressions like *Acaca*, *Fasn*, *Srebf1*, *Nrf2* and *Tnfa*. FAT even lowered steatosis and NAFLD activity scores in the histopathological assessment. Concerning its safety profile, FAT seemed to be safe for kidney and liver as per biochemical results. However, FAT therapy induced skin toxicity, such as eczema, and even enhanced systemic inflammation (i.e. serum TNF-α) in mice in the presence of WD/FG rendering poor health quality. We believe that SREBP1/2 is a potential target for metabolic diseases and its inhibition by selective hepatic SREBP1/2 inhibitors could be valuable in the treatment of NAFLD/NASH without skin toxicity. Though, further detailed advanced studies would be required to unwind the potential role of SREBPs in ceramide and FA synthesis in different organs to understand the mechanism of skin toxicity linked with FAT on repetitive dosing regimen.

Summarizing our results associated with celastrol, along with reducing fat intake, weight gain, and amelioration of NAFLD to NASH progression in mice, CEL also affected the expression of inflammatory, lipogenic and galanin receptor 2 genes in the liver, which may be involved in

the regulation of energy metabolism, oxidative stress, inflammation, and fibrosis. Therefore, it can be supposed that CEL may have a beneficial effect on the galaninergic system modulation in the liver of obese mice on the western-type atherogenic diet. However, the exact mechanisms and implications of celastrol's action on the galaninergic system are not fully understood and require further investigation.

In conclusion, CEL due to its effectiveness and safety could be a potential therapeutic agent for metabolic dysfunction-associated disorders like obesity and NAFLD/NASH in humans.

Concluding highlights of the dissertation work:

- Although the tested agents hit different molecular targets, all were beneficial to some extent in reducing NASH severity.
- Ku-0063794, a mTORC1/2 inhibitor, was least effective, but unlike other selective mTOR inhibitors (e.g. rapamycin), it was safe.
- Fatostatin, a SREBP1/2 inhibitor, corrected metabolic abnormalities and slowed the histopathological progression of NASH; however, it induced eczema-like skin toxicity.
- Since celastrol, a galaninergic system modulator, demonstrated sufficient efficacy and safety, it emerges as a promising candidate for further testing in the case of metabolic dysfunction-associated disorders, including NASH.

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8. List of publications

8.1 Publications related to the topic of the dissertation

- **Arora, M**., Kutinová Canová, N., and Farghali, H., 2022. mTOR as an eligible molecular target for possible pharmacological treatment of nonalcoholic steatohepatitis. European Journal of Pharmacology, 921, p.174857. **IF (2021) 5.195**, Q1 *(A review)*

- **Arora, M.**, Pavlíková, Z., Kučera, T., Kozlík, P., Šopin, T., Vacík, T., Ľupták, M., Duda, M., Slanař, O., and Kutinová Canová, N., 2023. Pharmacological effects of mTORC1/C2 inhibitor in a preclinical model of NASH progression. Biomedicine & Pharmacotherapy, 167, p.115447 **IF (2022) 7.5**, D1, Q1 *(An original paper)*

- Njeka Wojnarova, L., Kutinova Canova, N., **Arora, M.,** and Farghali, H., 2023. Differentiated modulation of signaling molecules AMPK and SIRT1 in experimentally drug-induced hepatocyte injury. Biomedical Papers of the Medical Faculty of Palacky University in Olomouc, 167(1). **IF (2022) 1.2**, Q3.

- **Arora, M.**, Šopin, T., Pavlíková, Z., Kučera, T., Vacík, T., Slanař, O., Kutinová Canová, N., Pharmacodynamic effects of SREBP1/2 inhibitor fatostatin in a preclinical model of MASH progression. European Journal of Pharmacology. *(An original paper, under submission)*

- Kutinová Canová, N., Šípková J, **Arora, M.**, Pavlíková, Z., Kučera, Šeda O, Šopin, T., Vacík, T., Slanař O., Effect of celastrol on heart and liver galanin system expression in mice model of western-type diet-induced obesity and metabolic dysfunction-associated steatohepatitis. Biomedicine & Pharmacotherapy (*An original paper, under submission)*

8.2 Publications not related to the topic of the dissertation

- Farghali, H., Kutinová Canová, N., and **Arora, M**., 2021. The potential applications of artificial intelligence in drug discovery and development. Physiological Research, 70 (Suppl 4), p.S715. **IF (2020) 1.881,** Q 3 *(A review)*

- Hlušička, J., **Arora, M.**, Brůha, R., and Žák, A., 2022. Statins and liver. Casopis lekaru ceskych, 161(2), pp.80-83. A peer-reviewed journal *(A review)*

- Pozniak J, Ryšánek P, **Arora M,** Das D, Šíma M, Slanař O. Ivacaftor pharmacokinetics and lymphatic transport after enteral administration in rats. Frontiers in Pharmacology. 2024 Feb 20;15:1331637. **IF (2023) 5.6, Q1** *(An original paper)*

- Arya A.K and **Arora M**: Evaluation of in-vitro anti-inflammatory activity of hydroethanolic extract of Delosperma cooperi plant. International Journal of Pharmacognosy 2020; 7(11): 334-37, E- ISSN: 2348-3962, P-ISSN: 2394-5583. A peer reviewed journal *(An original paper)*

- Arya A.K, Singh M.F, and **Arora M**. Pharmacological activity of dried kernels of *Juglans regia* on ovotoxicity induced post-menopausal complications in female rats. International Journal of Pharmacy and Pharmaceutical Research, 2020, Vol.17, Issue 4, 399-429. ISSN 2349-7203, **SJIF IF (2019) 6.64.** *(An original paper)*

- Arya, A.K., **Arora, M.,** and Singh, M.F. A review on pharmacological activity of Juglans regia. International Journal of Pharmacognosy 2020, Vol.7(1):1-11. ISSN (Online): 2348-3962, ISSN (Print): 2394-5583. A peer reviewed journal**,** *(A review)*

- Bisht M., **Arora M**., Tiwari V., Tiwari A. Evaluation of *in vitro* Antiangiogenesis Activity on Methanolic Extract of Clematis Buchaniana Plant. Asian Journal in Pharmaceutical and Clinical Research, 2017, 10(2): 227-229, Online ISSN: 2455-3891 Print ISSN: 0974-2441. A peer reviewed journal**,** *(An original paper)*

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