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TARGETING IL-1β AND ASSESSING SEX SPECIFIC MOLECULAR DIFFERENCES IN MOUSE MODELS OF NON-ALCOHOLIC STEATOHEPATITIS

PhD thesis

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LIST OF ABBREVIATIONS

11β-HSD1	11β-hydroxysteroid degydrogenase type 1
ACC	acetyl-CoA carboxylase
AGE	advanced glycation endproducts
AFP	α-fetoprotein
ALT	alanine aminotransferase
Anti-IL-1β	anti-interleukin-1β monoclonal antibody
ASK	apoptosis signal-regulating kinase
AST	aspartate aminotransferase
AT_2R	angiotensin type 2 receptor
CANTOS	Canakinumab Antiinflammatory Thrombosis Outcome Study
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CDAA	choline deficient L-amino acid defined
CON	control
CPT	carnitine palmitoyltransferase
CTGF	connective tissue growth factor
CTLA4	cytotoxic T cell antigen 4
CXCL	chemokine (C-X-C) motif ligand
DAMP	damage-associated molecular pattern
DGAT	diacylglycerol acyltransferase
DNL	de novo lipogenesis
DPP-4	dipeptidyl dipeptidase 4
ECM	extracellular matrix
EMA	European Medicine Agency
EV	extracellular vesicle
E/e’	ratio of early mitral inflow velocity and mitral annular early diastolic velocity
FA	fatty acid
FAS	fatty acid synthase
FDA	Food and Drug Administration
FGF	fibroblast growth factor

FXR	farnesyl X receptor
GI	gastrointestinal
GCS	global circumferential strain
GIP	gastric inhibitory polypeptide
GLP1	glucagon-like peptide 1
GLS	global longitudinal strain
GPC3	glypican 3
HCC	hepatocellular carcinoma
hs-CRP	high-sensitivity C-reactive protein
HRT	hormone replacement therapy
HSC	hepatic stellate cell
HMG- CoA	3-hydroxy-3-methylglutaryl coenzyme A
HSP	heat shock protein
IL	interleukin
Iso CON	isotype control
IFN	interferon
K-18	keratin-18
LB	lobular
LDL	low-density lipoprotein
LOXL2	lysyl oxidase-like 2
LVEDV	left ventricular end-diastolic volume
LVPWT	left ventricular posterior wall thickness
MMP	matrix-metalloproteinase
MKI67	marker of proliferation Ki-67
MPC	mitochondrial pyruvate carrier
MPO	myeloperoxidase
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD Activity Score
NASH	Non-alcoholic steatohepatitis
NET	nuclear extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3

PAMP	pathogen-associated molecular pattern
PCSK9	proprotein convertase subtilisin/kesin type9
PCNA	proliferating cell nuclear antigen
PD-1	programmed cell death 1
PDGF	platelet-derived growth factor
PD-L1	programmed cell death ligand 1
PPAR	peroxisome proliferator-activated receptor
Pro-C3	Pro-collagen III
PRR	pattern recognition receptor
PV	periportal
ROS	reactive oxygen species
SAT	subcutaneous adipose tissue
SCD1	steroyl-CoA desaturase 1
SGLT2	sodium-glucose co-transport 2
SrE	early diastolic strain rate
TC	total cholesterol
TG	triglycerides
TGF-β	tumor growth factor-β
THR-β	thyroid hormone receptor-β
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
VAT	visceral adipose tissue
VLDL	very-low-density lipoprotein
WAT	white adipose tissue

1. INTRODUCTION

1.1. The stages of non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide (1), causing significant healthcare and socio-economic burden (2, 3). NAFLD is characterized by a spectrum of stages: steatosis, non-alcoholic steatohepatitis (NASH), cirrhosis, that eventually may progress into hepatocellular carcinoma (HCC).

1.1.1. *Steatosis*

NAFLD usually develops with a background of metabolic dysregulation such as obesity, type 2 diabetes, dyslipidemia, hypertension and metabolic syndrome (4), thus NAFLD is often referred to as hepatic manifestation of metabolic syndrome. However, certain other causes may also lead to steatosis such as medications (amiodarone, glucocorticoids, estrogens, tamoxifen, rifampicin, antiretroviral drugs), chronic alcohol consumption, toxin exposure (e.g. ochratoxin A), or viral infections (e.g. hepatitis viruses, human immunodeficiency virus). In the past two-decades the global welfare substantially increased, leading to unhealthy lifestyle changes (western diet, sedentary lifestyle). Epidemiological studies show that the prevalence of NAFLD is increased in parallel with the prevalence of obesity (5). Steatosis is a reflection of misbalance of hepatic energy metabolism. Excess energy, in form of fats and carbohydrates, is delivered to the liver, while hepatocytes are unable to oxidize and/or export it, leading to storage of the excess energy as fats. Overconsumption of processed food and soft drinks, often called western diet, with high-level of carbohydrates, fats, while low intake of dietary choline promotes NAFLD (6-9). Sugars, cholesterol and other lipids promote lipid accumulation in hepatocytes (for further detail see reviews [(9, 10)], while choline is essential in very-low-density lipoprotein (VLDL) export and mitochondrial β-oxidation. Steatosis is further aggravated by insulin resistance. Insulin resistance in skeletal muscles and white adipose tissue funnels glucose and fatty acids (FAs) into the liver, respectively (11, 12). Meanwhile, hepatic insulin resistance impairs glycogenesis and induces de novo lipogenesis (**Figure 1**) (13, 14). Triglycerides are not considered directly toxic; however, indirectly they may induce endoplasmic reticulum stress (15) and their products of metabolism (e.g. ceramides) may interfere with insulin signaling and induce cell injury (16), thus promoting a vicious cycle.

Hepatosteatosis is reversible. If the unhealthy lifestyle is ceased, then steatosis may reverse over time. Otherwise, if the unhealthy lifestyle is maintained for a prolonged time, then it is estimated that over 25% of patients with NAFLD may progress into the second stage, called non-alcoholic steatohepatitis (NASH) (5, 17).

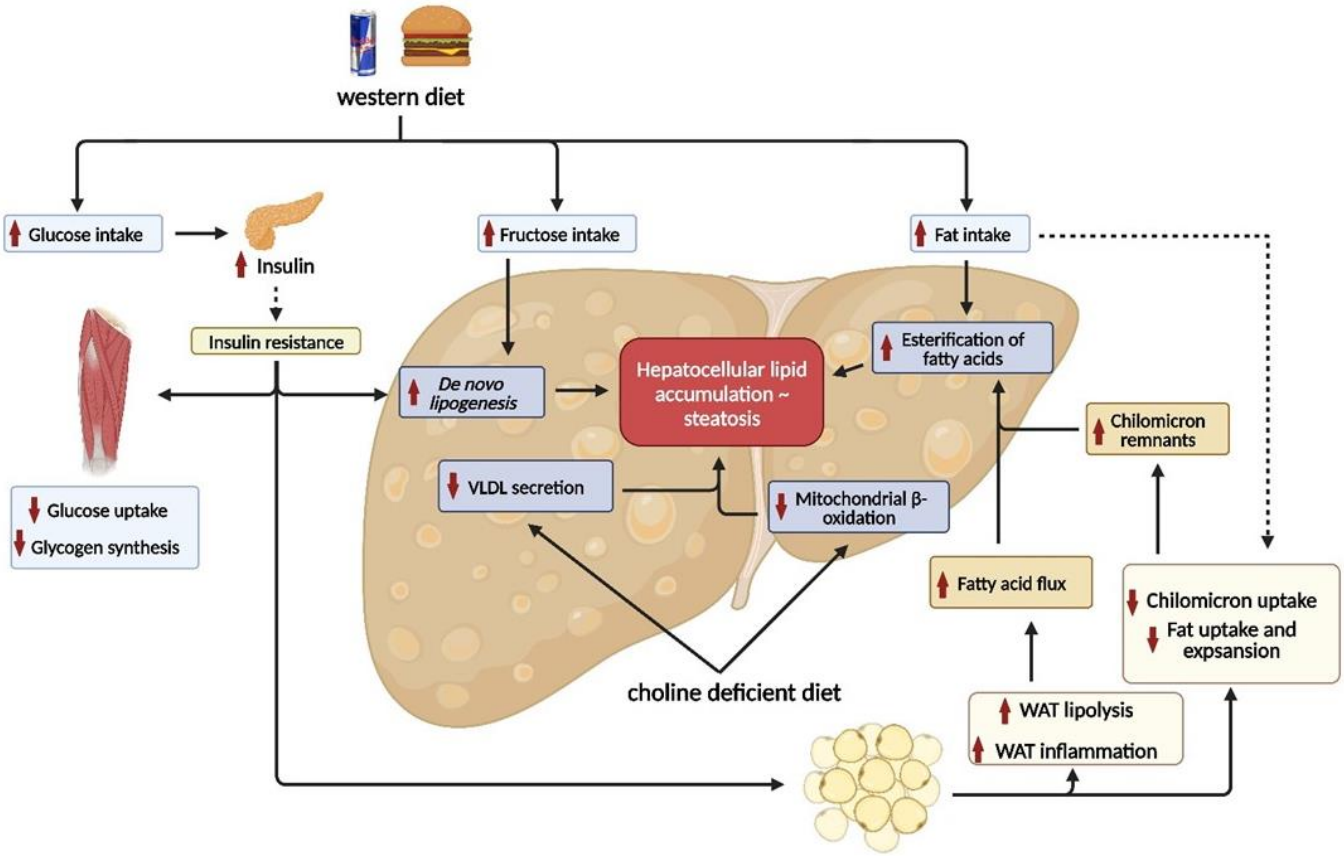

Figure 1 – Contributing factors of hepatic lipid accumulation – steatosis.

Unhealthy food and soft drink consumption increases the intake of carbohydrates in form of glucose, fructose and sucrose, and increases the intake of fats. Decades of noxious lifestyle may lead to the development of metabolic syndrome: dyslipidemia, insulin resistance, obesity. Muscular insulin resistance will prevent the uptake of glucose in to myocytes and glycogenesis will be greatly hindered. Hepatic insulin resistance and increase fructose consumption may promote de novo lipogenesis. Acetate derived from fructose metabolism will provide ample substrate for cholesterol and fatty acid synthesis. Consumption of food with insufficient choline may hinder the molecular processes needed for VLDL secretion and mitochondrial β-oxidation. Increased fat consumption and white adipose tissue (WAT) insulin resistance may funnel the liver with lipid excess. All these mechanisms promote the development of hepatosteatosis. *(Summary figure has been made in accordance to references cited in the main text.)*

1.1.2. Non-alcoholic steatohepatitis

It is widely accepted that NAFLD is a progressive disease, where the liver pathology progresses consecutively through different stages. According to the classical two-hit theory of NAFLD (18), steatosis is considered to be the first hit. However, as previously stated, NAFLD patients are often affected by co-morbidities such as type 2 diabetes, hypertension, dyslipidemia, obesity or the constellation of these diseases, called metabolic syndrome. All of these pathologies and the advanced age of patients contribute to significant systemic inflammation. Concomitant presence of systemic inflammation may further burden the steatotic liver (for example by contributing to hepatic insulin resistance). Thus in this scenario steatosis and systemic inflammation afflict liver damage at the same time, formulating the "multiple-hit" theory (19). However, some studies even suggest that inflammation may precede steatosis and is the main driver of progression to NASH (20, 21). However, it is generally accepted that hepatic inflammation occurs after cellular damage due to steatosis. Disease progression and/or development is not fully understood, and these aspects further proves the complexity of NAFLD. Whatever the case might be, inflammation is a major factor for both NAFLD and NASH.

During NASH a myriad of events occur. First, we are going to detail the intrahepatic factors:

1.1.2.1. Intrahepatic factors of NASH

1.1.2.1.1. Hepatocytes

Damaged fat-laden hepatocytes may undergo apoptosis, necrosis and/or pyroptosis (22) releasing damage-associated molecular patterns (DAMPs). Additionally, liver cells release hepatokines (e.g. fetuin A, FGF-21, selenoprotein P, angiopoietin like 4) and extracellular vesicles (EVs). All these secreted molecules, apoptotic bodies and cellular debris act on several types of cells (e.g. macrophages, monocytes, neutrophils, dendritic cells, hepatic stellate cells) through pattern-recognition receptors (PRRs) and cytokine receptors and promote steatosis (23, 24), insulin resistance (25), cell death (26), inflammation and fibrosis (**Figure 2**).

1.1.2.1.2. Macrophages

One of the most relevant cells in response to hepatocellular damage are resident macrophages, aka. Kupffer cells. The aforementioned factors polarize Kupffer cells into M1 phenotype and activate them to release reactive oxygen species (ROS), pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-18, TNFα, etc.) and chemokines (e.g. CCL2, CCL5, CXCL10 etc.). Macrophage polarization changes over time, thus after a certain period (or maybe depending on whether the insult is still present or not) macrophages tend to polarize into the M2 phenotype (27). M2 macrophages secrete anti-inflammatory cytokines (e.g. IL-10) and they are even able to induce apoptosis in pro-inflammatory M1 macrophages (28), thus they may introduce a balance to the inflammatory processes or even suppress it. However, M2 macrophages also secrete pro-fibrotic cytokines such as TGF-β and by doing so, they might participate in tissue repair and remodeling resulting "wound healing". However, M2 macrophages increases the risk of overt fibrosis and deterioration of NASH (29). Kupffer cells recruit monocytes, neutrophils further aggravating the tissue damage and inflammation (**Figure 2**). For more detail see ref (30).

1.1.2.1.3. Monocytes

Infiltration of monocyte to the liver is mostly regulated by the CCR2-CCL2 axis (31). CCL2 is secreted by a wide variety of cells (e.g. resident macrophages, hepatocytes, endothelial cells, etc.), while CCR2 is highly expressed on circulating monocytes. Recruited monocytes differentiate into distinctive macrophage subpopulation with pro-inflammatory, pro-fibrotic and pro-angiogenetic attributes (32).

1.1.2.1.4. Neutrophils

Hepatic neutrophils infiltration occurs in the early phases of NASH, where they contribute with several mechanisms to NASH pathophysiology: promotion of inflammation by secreting pro-inflammatory cytokines, ROS, myeloperoxidase (MPO) and neutrophil extracellular traps (NETs,) promotion of fibrogenesis by stimulating hepatic stellate cells (HSC) to differentiate, to proliferate and to release proteases. Initially, it was presumed that neutrophils contribute to anti-inflammatory processes simply by undergoing apoptosis. However, similarly to macrophages, neutrophils also have a distinctive "pro-

resolution" subpopulation that inhibit the production of inflammatory molecules and/or enzymatically degrade them (33) or deliver EVs laden with anti-inflammatory miR-223 to hepatocytes (34) and/or macrophages (35) (**Figure 2**). For more detail see review (36).

1.1.2.1.5. Hepatic stellate cells (HSCs)

Quiescent HSCs are stimulated by both DAMPs, pathogen-associated molecular patterns (PAMPs) (see later) and pro-inflammatory cytokines. HSCs are capable to phagocytize hepatocytes and leukocytes (37). Both molecular triggers and phagocytic activities results in differentiation of HSCs into myofibroblasts and, subsequently, to produce collagen, to rearrange the extracellular matrix by secreting matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), to secrete pro-inflammatory and pro-fibrotic cytokines (38). Loss of hepatocytes also trigger the release of growth factors such as CTGF, PDGF and TGF-β, thus inducing myofibroblast proliferation, further sustaining pro-fibrotic events (**Figure 2**). For further detail see review (39).

1.1.2.2. Extrahepatic factors of NASH (inflamm-aging, adipose tissue dysfunction, loss of intestinal barrier)

As for extrahepatic factors, systemic inflammation and insulin resistance were already mentioned above. In addition, inflamm-aging, a systemic, chronic, low-grade inflammation associated with advanced age, is linked to multiple organ malfunction, including the liver's (40-43).

Adipose tissue dysfunction characterized by infiltrating macrophages, where they initiate phagocytosis (forming crown-like structures) and release of cytokines and chemokines, further contributing to chronic systemic inflammation and insulin resistance (44). Additionally, adipocytes secrete a wide variety of adipokines (e.g. adiponectin, leptin, IL-6, TNF) that promote NASH.

Loss of intestinal permeability might contribute to leaking gut microbiota-derived products into the portal system showering the liver with PAMPs facilitating hepatic and systemic inflammation (45).

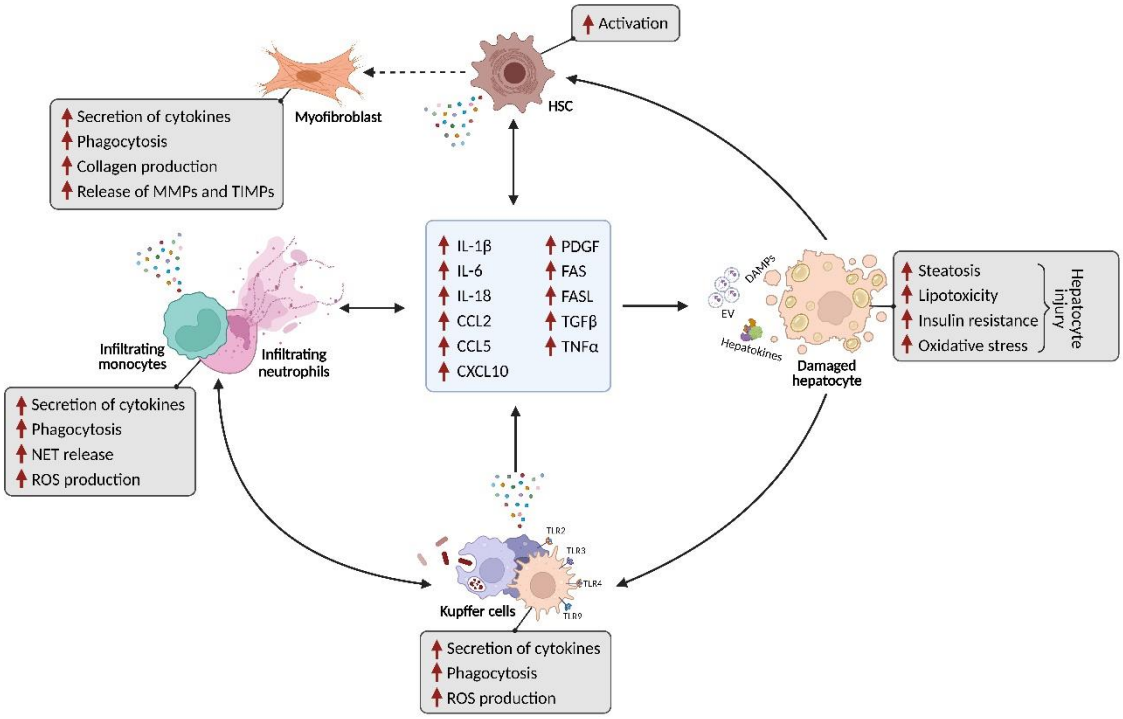

Figure 2 – Cellular contributors of NASH progression.

Prolonged accumulation of lipids in hepatocytes will ultimately cause cellular damage. Stressed and/or damaged hepatocytes will release hepatokines and EVs, while dying cells will bud off apoptotic bodies and DAMPs, thus resulting the activation of macrophages, monocytes, neutrophils and hepatic stellate cells. Activated Kupffer cells will proceed to engulf cells that go through apoptosis and simultaneously start releasing pro-inflammatory -, pro-fibrotic cytokines and various growth factors. HSCs will initiate differentiation to myofibroblasts that will, subsequently, rearrange the extracellular matrix (ECM). Infiltrating monocytes and neutrophils will further contribute to inflammation, fibrosis and oxidative stress. *(Summary figure has been made in accordance to references cited in the main text.)*

1.1.3. Advanced fibrosis and cirrhosis

Fibrosis is the end-stage of chronic liver diseases (**Figure 3**). The cellular and molecular microenvironment of NASH (e.g. sustained loss of hepatocytes, release of pro-inflammatory and pro-fibrotic cytokines, polarization of hepatic epithelial and immune cells to a pro-inflammatory and pro-fibrotic phenotype) is profoundly characterized with initiation of fibrosis and its maintenance, out of which chronic activation of HSCs is the main driver of liver fibrosis (46, 47). Fibrogenesis is characterized by accumulation of fibrotic proteins within the space of Disse, resulting loss of capillarization and microvilli of hepatocytes. Battle between ECM deposition and degradation determines whether scar formation or scar healing would unfold. During NASH, HSCs differentiate into myofibroblasts, which continuously release components of ECM and, in parallel, regulate the ratio of released MMPs and TIMPs. MMPs degrade the proteins of ECM, while TIMPs inhibit MMPs. During transition from NASH to advanced fibrosis and/or cirrhosis, the ratio of MMPs/TIMPs is low, resulting accumulation of fibrotic proteins, thus the liver eventually loses its architecture and function (48). It is estimated that 40% of patients with NASH progress 1 fibrosis stage per decade (5). Fibrosis is the most relevant predictive factor for long-term outcomes of NASH, including hepatocellular carcinoma (49).

1.1.4. Hepatocellular carcinoma

Live cancer is the fifth most common cancer and is the second leading cause of cancer-related death (50). An American population-based study concluded that NAFLD or NASH has become the most important risk factor for HCC, 59% of HCC patients has NASH as the primary etiologic factor for HCC development (51). While a study from Northern England revealed that NAFLD is the main cause of 35% of HCC cases (52). The number of patients with NASH-associated HCC increases with 2.6% every year (53). The mechanism of NASH-to-HCC transition is not fully understood. Several coinciding events may contribute to the development of HCC with a steatohepatitic background. These events can be classified as:

- **metabolic:** altered metabolic program (54), accumulation of oncometabolites (e.g. fumarate, succinate, 2-hydroxyglutarate, lactate, polyamines) (55)

- **intracellular:** DNA damage (56) and the subsequent response to it (57), dysregulation of autophagy (58), ER stress (59)
- **immunologic:** hepatic infiltration of immunosuppressive and/or cytotoxic leukocytes, upregulation of anti-inflammatory immune checkpoints and cytokines (60)
- **other:** compensatory hepatocellular proliferation (22), ROS derived from metabolic and immunologic events (61, 62).

For more comprehensive review see ref (63).

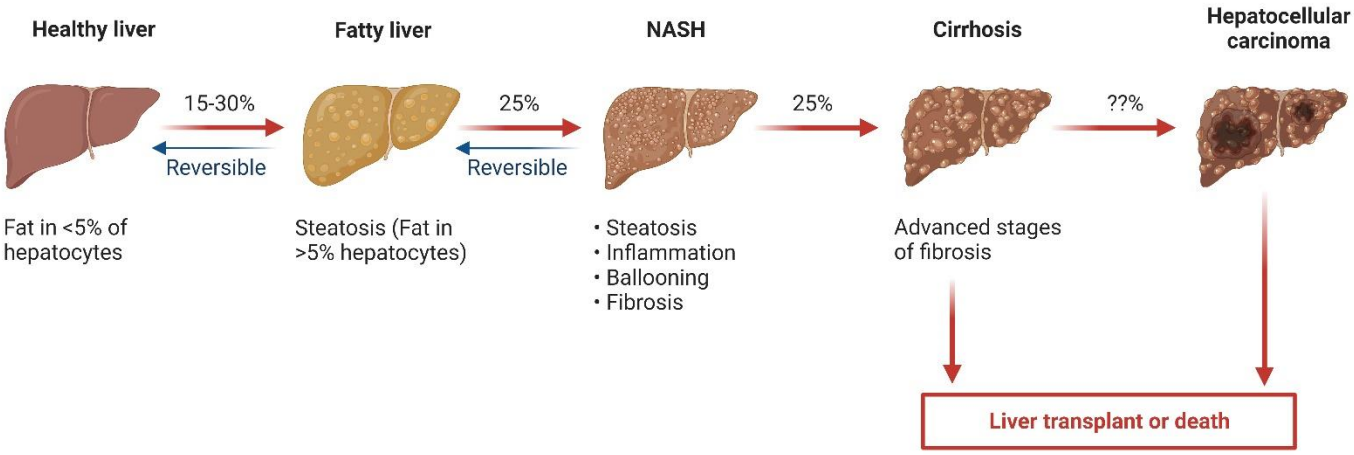

Figure 3 – Stages of non-alcoholic fatty liver disease.

NAFLD is a progressive disease, starting with benign and reversible steatosis. If this state is not alleviated within a reasonably time period, then hepatocyte may succumb due to the prolonged stress, thus progressing to steatohepatitis. This stage is also considered reversible, but hepatic inflammatory and fibrotic events manifests itself. The continuous loss of hepatic architecture will lead to advanced fibrosis and cirrhosis. At this stage, the damage is beyond resolution and the risk of hepatocellular carcinoma or end-stage liver failure is substantially increased. *(The figure has been made in accordance to references cited in the main text.)*

1.2. Sex and age-dependent differences in NASH

Modern healthcare community's interest grew in personalized therapeutics over the past decade (64, 65). The goal of precision medicine is to improve diagnostics, prevention, treatment and cure by using genetic, molecular and environmental measurements to account for every possible contributing factor. As such, biological sex and advanced age are important factors (66, 67). Several diseases show sex-dependent differences, and NAFLD is no exception. Epidemiologic studies reported that the prevalence of NAFLD is higher in males (68-70). Although premenopausal women have lower incidence of NAFLD than age-matched men, this disparity is lost following menopause (71).

1.2.1. Sex differences of NASH

1.2.1.1. Sex differences in NASH-related co-morbidities

Lonardo A. *et* Trande P. reported for the first time that glycaemia and central fat distribution predicts fatty liver in women, thus suggesting, for the first time, that co-morbidities of NASH may impact differently both sexes (72).

Postmenopausal women and men have higher risk to develop metabolic syndrome (**Figure 4**), than premenopausal women (73). Similarly, premature ovarian insufficiency increases the risk for both metabolic syndrome and insulin resistance (74).

Regarding hypertension, it was shown that higher proportion of men have hypertension (75); however, estradiol is necessary to maintain basal renin level (76). The depressor effect of AT_2R in females is lost with age (77), but it may be restored with hormone replacement therapy (78).

Although obesity is more prevalent in women (79), premenstrual women are relatively protected from the potential cardiometabolic consequences, meanwhile men are not (**Figure 4**). In the 2010s, it was suggested that one possible reason for this disparity is that estrogens modulate the expression cortisol activating enzymes in the liver and in adipose tissue resulting hypercortisolism, which may contribute to NAFLD development in males, but not in females (80-82).

Fat distribution also differs between sexes. Men tend to accumulate fat in the visceral adipose tissue (VAT) causing an "apple shape" form of obesity (**Figure 4**), which is associated with higher level of postprandial insulin and lipid levels. Meanwhile women

predominantly have more subcutaneous adipose tissue (SAT) and it is distributed mostly into the gluteal-femoral region resulting in a “pear shape” form of obesity, which is considered to have lower risk for metabolic diseases (83, 84). Additionally, SAT and VAT differ in their rate of lipolysis. VAT has a higher rate of lipolysis releasing free FA into the portal system and has a more pro-inflammatory profile, consequently it burdens the liver more (85). Estradiol itself further contributes to alleviate the burden of the liver by decreasing the rate of lipolysis and by improving insulin sensitivity in adipose tissue (86, 87).

Oophorectomy in young women due to ovarian cancer is associated with the development of type 2 diabetes and hypercholesterolemia and greatly increases the risk of NAFLD (88).

1.2.1.2. Sex differences in hepatic inflammation and fibrosis

Postmenopausal women and men possess higher risk for advanced fibrosis and NASH (89, 90), in women it is independent of metabolic risk factors (91). Additionally, premature menopause and long-standing estrogen deficiency increases the risk for NAFLD and severe liver fibrosis (92-94).

As detailed above, lack of estradiol in women has deleterious consequences on liver health, but these effects are mitigated and/or reversed by hormone replacement therapy (HRT) (95). However, reproductive young women and consumers of oral contraceptives are not completely free of NAFLD development and, indeed, they dispose more severe hepatocellular injury and lobular inflammation than postmenopausal women or men (96) (**Figure 4**). Several immune cells are known to express sex hormone receptors, consequently sex hormones influence immune functions as well (97). It was suggested that not estrogens, but rather progesterone is responsible for the aforementioned pro-inflammatory effects (96).

1.2.1.3. Sex differences in lipid metabolism

Dietary choline is necessary for VLDL release (as it is the precursor of phosphatidylcholine, a component of VLDL) and mitochondrial β-oxidation (8). Choline deficiency hinders these processes resulting hepatic steatosis. Young women require less dietary choline (98), but after menopause, the decline of estrogen levels entails a

decreased supply of endogenous choline prompting increased dietary choline demand. Accordingly, men and postmenopausal woman are more susceptible to develop NAFLD due to choline deficiency than premenopausal woman (99).

Regarding lipid homeostasis, women have lower VLDL and LDL plasma concentration, due to lower hepatic FA influx (see above) and increased muscular clearance (100). Intramuscular buildup of lipids, nevertheless, is not associated with muscle insulin resistance in woman, but it is in men (101). Woman have triglyceride-richer VLDLs (102), while men produce apoB-richer VLDL particles (103). For more details, see reviews (102, 104).

PCSK9 is an important regulator of serum level of LDL particles. Circulating PCSK9 level is higher in women (105), independently of age (106). Postmenopausal women, however, have even higher PCSK9 concentrations, compared to premenopausal women (107) (**Figure 4**). It was observed that PCSK9 level changes with the menstrual cycle, and showing an inverse relationship: PCSK9 level is lowest at ovulation (108). Besides cardiovascular disease risk, PCSK9 was also associated with steatosis severity in NAFLD patients (109). Data about sex differences of PCSK9 in NASH is scarce, further studies are required to elucidate whether there is a sexual difference, and if there is, then how it will impact our knowledge of NASH pathophysiology and treatment strategies.

1.2.1.4. Sex differences in disease outcome and mortality

The main causes of death of NAFLD/NASH are cirrhosis, cardiovascular, non-hepatic cancer and HCC. Significantly more men die due to NASH-related HCC, than women (90). Women have lower risk for cardiovascular disease irrespectively of estrogen level (in contrary to NAFLD development) (110). Mortality of women with NAFLD steeply increased in a survey between 2007-2016 (90).

Cardiovascular risk modifying co-morbidities (hypertension, hyperlipidemia, obesity etc.) are usually present in patients with NAFLD, thus it is no wonder that these patients have worse cardiovascular outcome. As such, a question arises: “Does NAFLD and/or NASH independently contribute to cardiovascular mortality?” In 2015 VanWagner L. B. *et al.* published a population-based study, where they have associated NAFLD with subclinical myocardial remodeling and dysfunction (111). In this study, patients with NAFLD had increased heart weight, elevated LVEDV and E/e’ suggesting increased left

ventricular filling pressure. Interestingly, ejection fraction was normal. These data might suggest that NAFLD might contribute to development of heart failure with preserved ejection fraction. For further information about this topic see ref (112). As of yet, there is no information that sex affects the relationship of the liver and the heart.

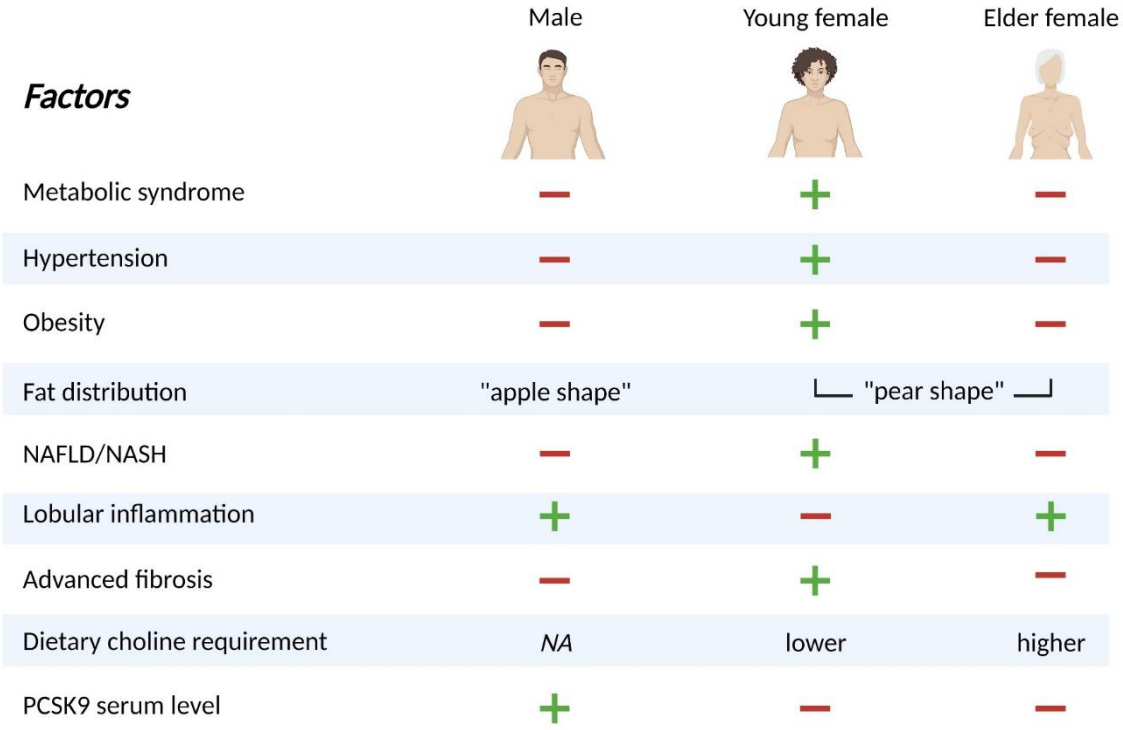

Figure 4. Major sex differences of NASH and its main risk factors.

The most important risk factors of NASH (obesity, hypertension, metabolic syndrome) and NASH itself show sexual disparity: premenopausal women are more protected than men and postmenopausal woman. Young females, in general, have stronger immune response, thus hepatic inflammation might be more severe is this population. Daily dietary need of choline inversely proportion of serum estrogen level. In general, PCSK9 level is lower in men, while in women PCSK9 level increases with age. Green plus sign means protection, red minus sign means less protection, NA abbreviates not available. *(Summary figure has been made in accordance to references cited in the main text.)*

1.3. Therapeutics of NASH

Despite the substantial healthcare and socio-economic burden of NAFLD and subsequent NASH (113), no effective treatment is approved by the FDA nor the EMA. Vitamin E and pioglitazone have shown mild efficacy in NASH (114). The use of both agents in NASH became controversial, preventing the widespread use for this indication. For vitamin E, risk of stroke and prostate cancer was raised (115, 116). Long-term use of pioglitazone, a PPAR-γ agonist, might increase the risk of bladder cancer (117).

Drug candidates for NASH can be categorized by their targets in to two main groups (118): targeting lipid and/or carbohydrate metabolism, targeting inflammation and fibrosis.

The major clinical trials conducted for NASH to date and their outcome are summarized in **Table 1-2** and **Figure 5**.

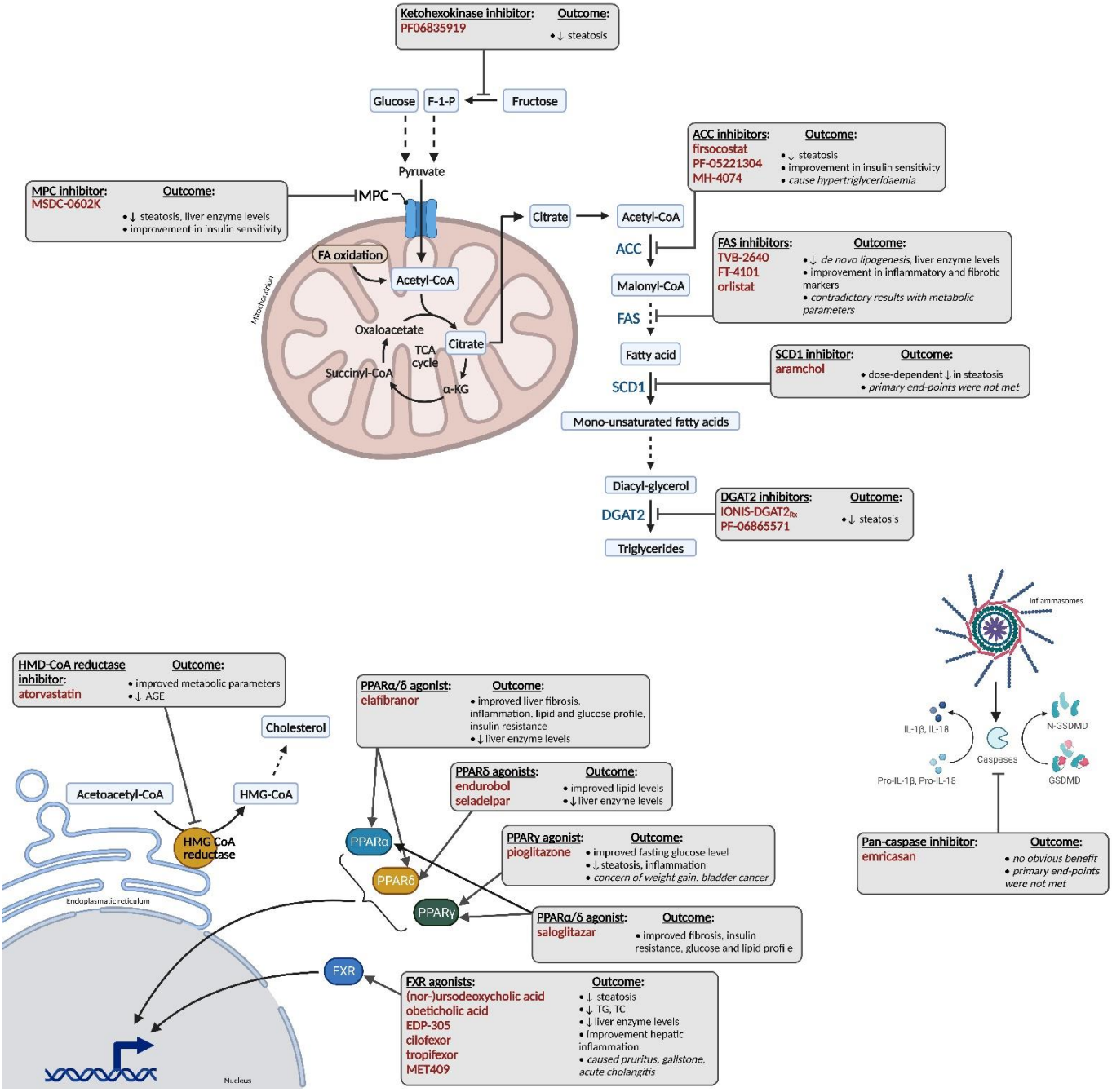

Figure 5 – Summary of major drug candidates for NASH.

Summary of clinical trials that targeted key enzymes of DNL, nuclear receptors that regulate metabolism and caspases that promote inflammation and inflammatory cell death, pyroptosis. *(Summary figure has been made in accordance to references cited in* ***Table 1 and 2****.)*

Table 1 – Lipid and/or glucose metabolism targeting clinical trials for NASH

Drug classes	***Drug candidates***	***Main results***	***Ref***
ACC inhibitors	firsocostat, PF-05221304, MK-4074	Improvement of hepatic steatosis and insulin sensitivity, *but hypertriglyceridemia occurred*	(119-121)
FAS inhibitors	TVB-2640	Reduced DNL, steatosis, ALT	(122, 123)
	FT-4101	Reduced DNL, steatosis Hepatic, glucose-lipid metabolism markers did not change	(124)
	orlistat	Mild improvements	(125)
SCD1 inhibitor	aramchol	Dose-dependently decreases hepatic fat content	(126)
		Primary end-points were not met, while secondary end-point promising improvements	(127)
DGAT2 inhibitors	IONIS-DGAT2$_{Rx}$	Reduced steatosis No hypertriglyceridemia	(128)
	PF-06865571	Reduced steatosis	(129)
HMG-CoA reductase inhibitor	atorvastatin	Metabolic parameters improved, *but glucose parameters did not change* AGE decreased	(130)
FXR agonists	ursodeoxycholic acid	Reduced hepatic steatosis Decreased level of LDL, TG, TC Amelioration of inflammation	(131, 132)
	obeticholic acid	Fibrosis regression Reduced hepatic steatosis Improved insulin sensitivity	(133-135)
	EDP-305, cilofexor, tropifexor, MET409	Reduced ALT and steatosis	(136-139)
11β-HSD1 inhibitor	RO5093151	Reduced steatosis, body weight, ALT	(140)
THR-β agonists	resmetirom (MGL-3196), VK2809	Reduced NASH Acceleration of NASH Decreased LDL, TG	(141-143)
FGF19 analog	aldafermin	Reduced steatosis Improvement in fibrosis and NASH, *but LDL increased*	(144, 145)
FGF21 analogs	pegbelfermin, efruxifermin	Reduced steatosis Improved glucose and lipid levels Improved histology	(146, 147)
PPARα agonist	pemafibrate	Did not achieve its primary end-point	(148)
PPARδ agonists	endurobol, seladelpar	Improved lipid levels	(149, 150)
PPARα/δ agonist	elafibranor	Improved liver fibrosis, inflammation, enzymes, lipids and glucose profile	(151, 152)

PPARα/γ agonist	saroglitazar	Improved insulin resistance, fibrosis, lipid and glucose	(153, 154)
PPARγ agonist	pioglitazone	Reduced steatosis and inflammation Improved fasting glucose level Greater resolution of NASH *Concern of weight gain, bladder cancer*	(155, 156)
PPARα/δ/γ agonist	lanifribranor	Reduced steatosis, fibrosis, inflammation, liver enzymes Adverse effects limit their further use (collectively true for all PPAR agonists)	(157)
GLP agonists	exenatide, liraglutide, semaglutide	Reduced steatosis, liver enzymes Improved blood pressure, glycemia, inflammation Decreased body weight	(158-167)
GIP/GLP dual agonist	tirzepatide	Decreased level of ALT, AST, K-18, Pro-C3 Increased adiponectin level	(168, 169)
Glucagon/GLP agonist	cotadutide	Reduced steatosis, body weight and ALT/AST levels Improved fibrosis	(170)
DPP-4 inhibitors	sitagliptin, linagliptin, saxagliptin, alogliptin	Improvement of HbA_{1C}, did not improve key feature of NASH	(171-173)
SGLT2 inhibitors	empagliflozin, dapagliflozin, canagliflozin, ipragliflozin	Reduced steatosis, body weight, liver enzymes, fibrosis Improved glycemic control, blood pressure Increased adiponectin level	(174-184)
MPC inhibitor	MSDC-0602K	Reduced steatosis, liver enzymes Improvement in parameters of glycemia (insulin sensitivity) *Did not meet the primary endpoint*	(185)
Ketohexokinase inhibitor	PF-06835919	Reduced steatosis *No effect on insulin sensitivity*	(186)

Table 2 – Inflammation and fibrosis targeting clinical trials for NASH

Drug classes	***Drug candidates***	***Main results***	***Ref***
Caspase inhibitor	emricasan	*No obvious benefit was observed, and may even worsened fibrosis*	(187-191)
Galectin 3 inhibitor	belapectin	*No improvement, did not meet the endpoints*	(192, 193)
CCR2/CCR5 inhibitor	cenicriviroc	Improvement in fibrosis Decreased level of inflammatory biomarkers *No improvement in key NASH features, program terminated*	(194, 195)
ASK1 inhibitor	selonsertib	*Primary end-points were not met, program terminated*	(196, 197)
LOXL2 inhibitor	simtuzumab	Improvement in fibrosis *Trial terminated due to lack of efficacy*	(198)
TNFα inhibitor	pentoxyfylline	*Contradictory effects in NASH*	(199, 200)

2. OBJECTIVES

Most clinical trials tested drug candidates that interfere with lipid and/or glucose homeostasis, while clinical investigations that directly target inflammatory processes are relatively few in numbers. Numerous preclinical studies attempted to evaluate potential anti-inflammatory medications, but with little-to-no success. Additionally, clinical data suggest a sex- and age-dependent variation in major NASH risk factors, metabolism, outcome and, most importantly, NASH pathophysiology as well. However, description of molecular sex differences in NASH is still lacking. As the global trend of unhealthy lifestyles is increasing, the burden of NASH increases in parallel, thus studies that fill the gaps of knowledge about NASH pathophysiology and effective treatment is urgently needed.

Therefore, in this work we set the following aims:

1. To investigate the cardiac and hepatic effects of an Interleukin-1β binding monoclonal antibody in an aged mouse model of NASH
2. To assess sex-specific expression of genes-related to cholesterol metabolism, inflammation and fibrosis in a middle-aged mouse model of NASH

3. METHODS

3.1. Experimental animals, diets, treatments and ethical approval

All experimental animals were purchased from Oncological Research Center, Department of Experimental Pharmacology, Budapest, Hungary. Mice were maintained under 12–12 light–dark cycle under controlled environment (20–24°C and 35–75% relative humidity) in individually ventilated cages, holding 2–4 mice per cage. Standard chow diet and tap water were available ad libitum.

Control diet (CON, E 15668–04) and choline deficient L-amino acid defined diet (CDAA, E15666–94) was purchased from SSNIFF GmbH (Soest, Germany).

Anti-IL-1β monoclonal antibody (BE0246) and the corresponding isotype control (BE0091) were purchased from BioXCell, USA.

All experimental procedures were done in accordance with the Guide for Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication No. 85–23, revised 1996), with the EU Directive (2010/63/EU), and in compliance with the ARRIVE guidelines, and was approved by the National Scientific Ethical Committee on Animal Experimentation (PE/EA/1912–7/2017, Budapest, Hungary).

3.2. Non-alcoholic steatohepatitis model

This work constitutes of two subprojects:

In the first, we used 24 months old male C57Bl/6J mice. Mice were randomized by body weight and assigned to CON diet-fed group (n = 10) or CDAA diet-fed group (n = 10) and were treated with anti-IL-1β Mab (n = 9) or isotype control (n = 10) for 8 weeks. The reason why aged male mice were used is that older males show a higher susceptibility to frailty and inflamm-aging-derived cardiac decline (201). The animals were treated two times per week with a dose of 50 μg/mouse (202). Before termination, echocardiographic evaluation was done to assess cardiac function.

In the second subprojects, 10 months old female and male C57Bl/6J mice were randomly assigned to the CON diet-fed group (n = 10) or CDAA diet-fed group (n=10).

In both projects, the mice were sacrificed after 8 weeks of diet, tissue and blood samples were collected for analyses.

3.3. Echocardiography and strain analysis with 2D speckle-tracking

Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance), cardiac functions were assessed with the Vevo 3100 high-resolution in vivo imaging system (Fujifilm VisualSonics, Toronto, Canada) with a MX400 transducer. The obtained images were used for both conventional echocardiographic measurements and strain analysis with speckle-tracking. For specific details see (203).

3.4. Histology

Liver and heart samples were fixed in neutral buffered formalin for 24 h, then dehydrated and embedded in paraffin. Five μm thick sections were cut with a microtome and used later on. All staining was visualized and captured with Leica LMD6 microscope (Wetzlar, Germany). In case of liver samples, the specimens' entire area was scanned and analyzed with 6.3 × magnification, while, in case of heart samples, 5 microphotographs were captured from endocardial regions.

3.4.1. Hematoxylin and eosin staining

Paraffin embedded liver sections were deparaffinized, hydrated, and stained with hematoxylin and counterstained with eosin. H&E staining was used to assess morphologic changes, the area of lipid droplets and inflammatory clusters using ImageJ software.

3.4.2. Immunohistochemistry

Liver sections underwent antigen retrieval (citrate buffer pH = 6 or Tris buffer pH = 9) for 15 min. Endogenous peroxidase was blocked by 3% H2O2 in PBS. Afterwards, sections were blocked with 2.5% goat or horse serum and 2% milk powder or bovine serum albumin. Primary antibodies – Iba1, marker of macrophages (019–19741, Wako Pure Chemical Industries, Japan); Clec4/Clecsf13 for Kupffer cells (MAB2784, R&D Systems, Minneapolis, MN, United States); MPO, marker of neutrophils (AF3667, R&D Systems, USA); CD3e for T cells (D7A6E, Cell Signaling Technology, Danvers, MA, United States); E-cadherin, marker of epithelial-mesenchymal transition (610181, BD Biosciences, USA); PCNA, marker of proliferation (13110S, Cell Signaling Technology,

USA – were diluted (1:2000, 1:200, 1:200, 1:2000, 1:200, 1:4000, respectively) in goat or horse serum and were incubated overnight at 4°C. Sections were washed three times with PBS, then the specimens were incubated with the following secondary antibodies: anti-rabbit IgG HRP (8114S, Cell Signaling Technology, USA), anti-goat IgG HRP (MP-7405, Vector Laboratories, USA), anti-mouse HRP (MP-2400, Vector Laboratories, USA), then were washed and signals were developed with diaminobenzidine (ImmPact DAB EqV Peroxidase (HRP) Subrate, Vector Laboratories, Burlingame, CA, United States).

3.4.3. Lectin histochemistry

Wheat germ agglutinin (WGA-FITC – marker of cell membrane, 1:50, Sigma Aldrich, L4895) and with isolectin B4 (ILB4-DyLight 594 – marker of cardiac endothelial cells, 1:50, Invitrogen, L32473) were used to assess cardiomyocyte cross-sectional area and capillary density.

3.5. qRT-PCR

Total RNA was isolated from liver, heart, kidney, small intestine, and adrenal samples with the isopropanol/chloroform precipitation method. Results were calculated with the $2^{-\Delta\Delta Cp}$ evaluation method. For more detail see (203, 204).

3.6. Western blot

Frozen liver samples were homogenized in RIPA lysis buffer. Twelve μg of protein was loaded onto 4–20% polyacrylamide gel. After gel electrophoresis, proteins were transferred onto PVDF membranes (BioRad, Hercules, CA, United States). The membranes were blocked with bovine serum albumin, then primary antibodies against IL-1β (ab9722, Abcam, Cambridge, MA, United Kingdom, 1:1000), NLRC4 (D5Y8E, Cell Signaling Technology, Danvers, MA, United States, 1:2500 dilution), and NLRP3 (D4D8T, Cell Signaling Technology, Danvers, MA, United States, 1:2500 dilution) were incubated overnight at 4°C. After washing, secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit, 7074, Cell Signaling Technology, Danvers, MA, United States, 1:5000 dilution) were incubated at room temperature. Band intensity was

evaluated using the Image Lab Software (BioRad, Hercules, CA, United States). For more detail see (204).

3.7. ELISA

Serum protein concentration of PCSK9 was measured according to the manufacturer's instructions. For more details see (204).

3.8. Serum triglyceride and cholesterol level measurement

Triglyceride and total cholesterol content were measured from serum using a colorimetric method (Diagnosticum, Budapest, Hungary) according to the manufacturer's instructions (204).

3.9. Data and statistical analysis

All values are presented as mean ± standard error of mean (SEM). In the first subproject, we two-way ANOVA followed by Fisher's LSD post hoc test, while in the second subproject two-way ANOVA followed by Tukey's post hoc test. Contingency analysis was evaluated by Fisher's exact test. The statistical analyses were performed with the GraphPad Prism software. $*P < 0.05$ was considered significant.

4. RESULTS

4.1. Targeting inflammation in NASH

Interleukin-1β is a major pro-inflammatory cytokine with key importance in the pathophysiology of NASH. Both hepatic and immune cells are capable to secrete IL-1β, which may exert its effect locally (by promoting steatosis, hepatic insulin resistance, fibrosis) and systemically (by promoting neutrophil infiltration or by interfering with other organs' function). Circulating low levels of IL-1β derived from chronic systemic inflammation may contribute to deterioration of cardiac function and it is well known factor in several cardiometabolic disorders (e.g. chronic and acute heart failure, atherosclerosis).

As its importance is hard to overestimate, affecting this cytokine might prove to be an adequate target in NASH and inflammation-driven cardiovascular diseases as well.

4.1.1. Interleukin-1β inhibition improves cardiac diastolic function

To assess cardiac function, to attain volumetric, diametric and geometric analysis of the chambers, we performed conventional echocardiography. Mitral inflow velocity and annular velocity was measured to obtain data for diastolic function. Furthermore, we performed 2D speckle tracking echocardiography, a more sensitive measurement of cardiac muscle fiber torsion **(FIGURE 6A)**.

In our aged model of NASH, systolic function did not change in the group that was fed with a choline deficient diet (CDAA), and anti-IL-1β treatment did not show a cardiac deterioration nor improving effect on systolic function, as indicated by the preservation of ejection fraction, GLS and GCS.

At first glance, left ventricular filling pressure was not affected by the NASH-inducing diet, indicated by no change in E/e' ratio, an indirect marker of diastolic function. However, the ratio of early mitral inflow velocity-to-early diastolic strain rate (E/SrE) deteriorated upon CDAA diet, and the treatment was able to significantly improve it **(FIGURE 6A)**.

To assess cardiac remodeling, we carried out lectin histochemistry. No difference was observed in cross-sectional area nor in capillary density **(FIGURE 6B)**.

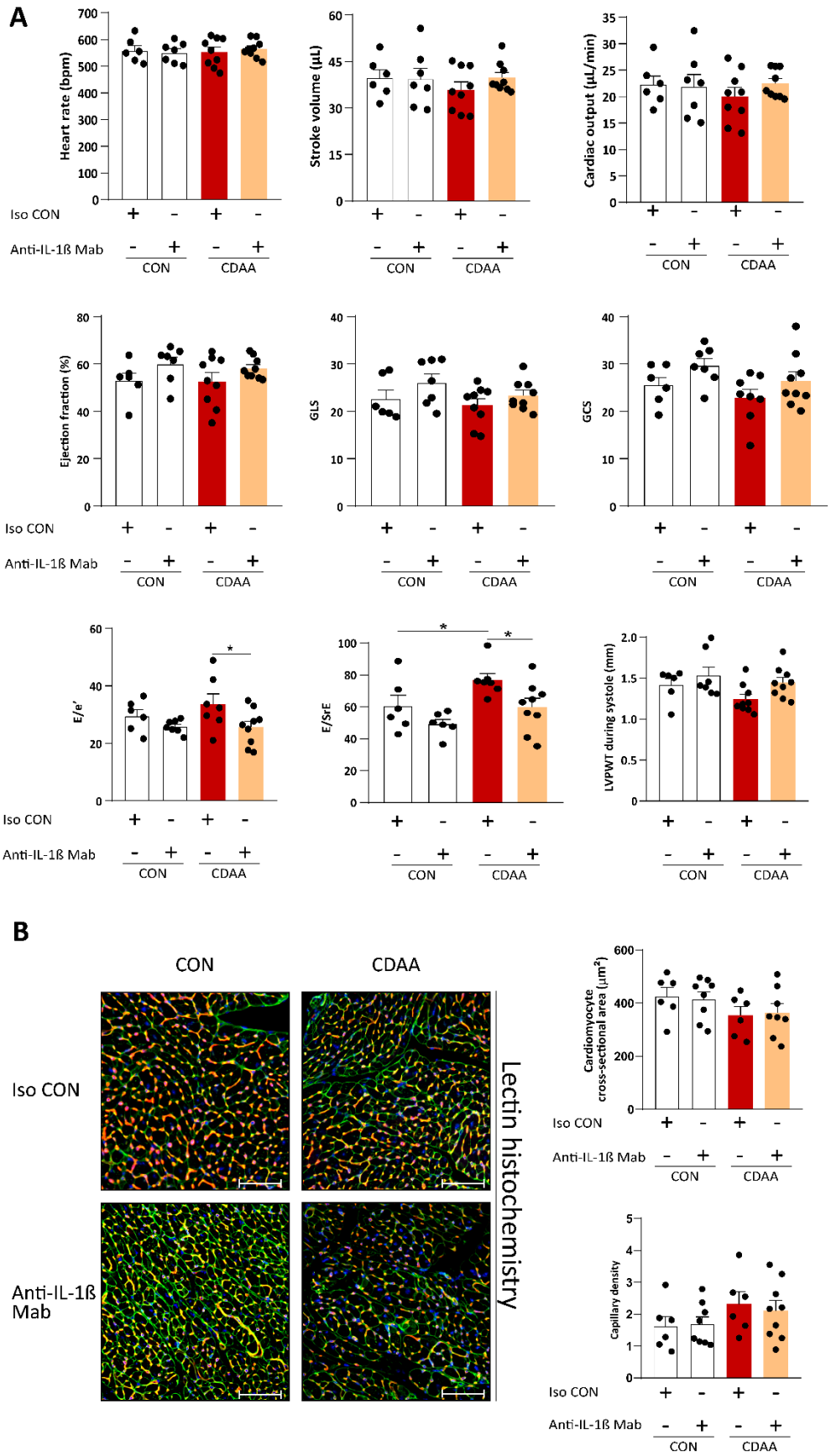

Figure 6. Cardiac function and remodeling in an aged NASH mouse model.

Conventional and 2D speckle tracking echocardiography (**A**). Cardiac lectin histochemistry (**B**). Two-way ANOVA, Fisher's LSD post hoc test, n = 6-9/group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (203).

4.1.2. Interleukin-1β inhibitor decreases the expression of fibrotic genes, while does not impact overall fibrosis

As mentioned above, fibrosis is a major determinant of NASH outcome. Eight weeks feeding of CDAA diet induced significant fibrosis. IL-1β neutralization decreased the expression of *Col1a1* and *Col3a1*. However, the overall quantification of fibrosis did not change macroscopically **(FIGURE 7)**.

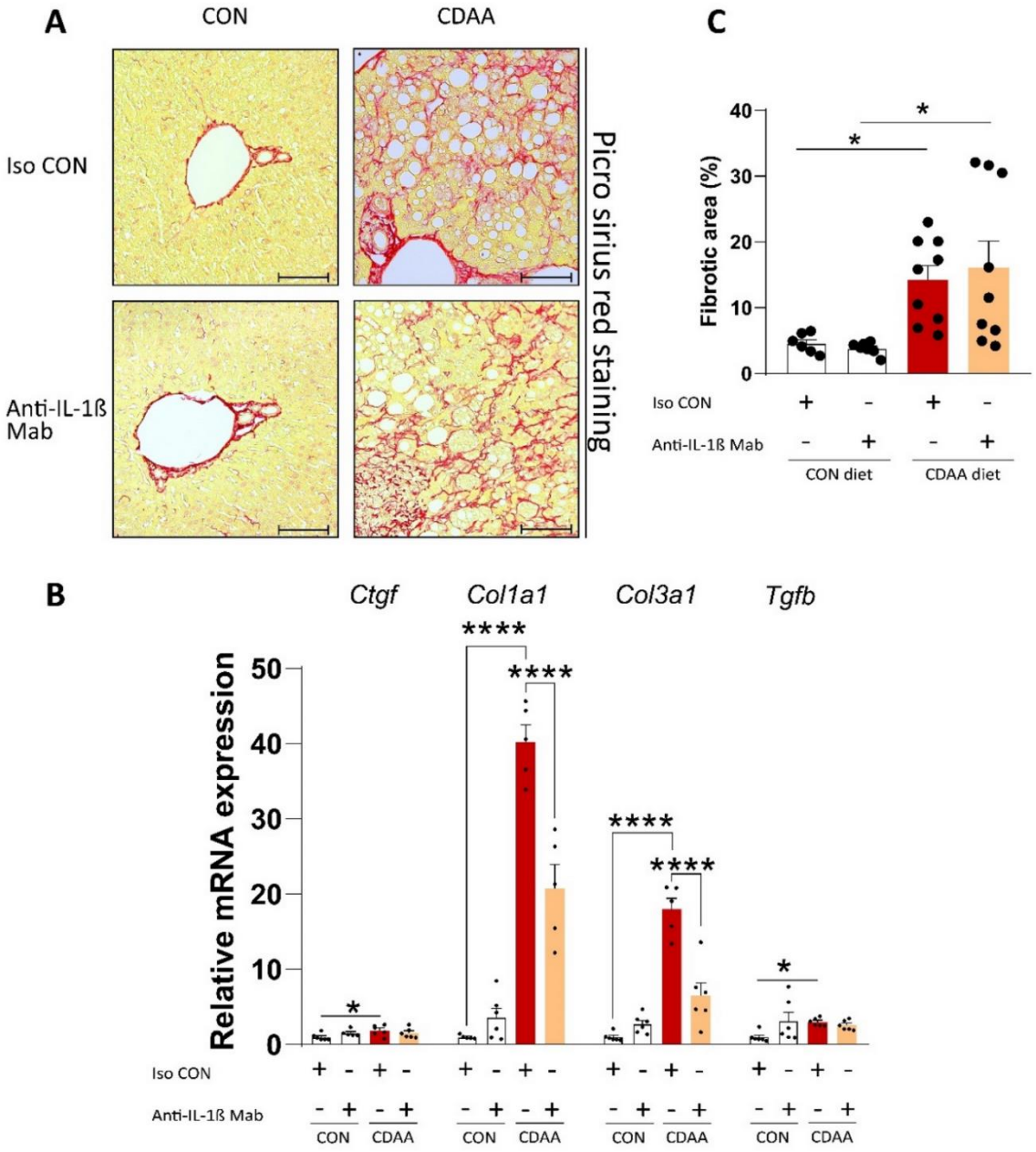

Figure 7 – Histological and molecular analysis of hepatic fibrosis.

Microscopic evaluation of hepatic fibrosis by picrosirius-red staining, n = 6-9/group (**A**).

Quantitative RT-PCR analysis of major pro-fibrotic genes, (n = 5–6/group) (**B**). Quantification of overall hepatic fibrosis (**C**). Scale bar shows 100 µm. Two-way ANOVA, Fisher's LSD post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (203).

4.1.3. Interleukin-1β inhibition does not improve steatosis or inflammatory cell infiltrations of NASH

The choline deficient diet caused extensive hepatic steatosis and inflammatory infiltrations. NAFLD Activity Score is a comprehensive scoring system, which shows disease severity in regard of steatosis, hepatocyte ballooning and inflammation, which was not affected by the treatment. IL-1β inhibiting monoclonal antibody achieved to reduce the number of periportal infiltrations with small area (<100 µm^2), while other features and parameters of NASH remained unaffected (**FIGURE 8**).

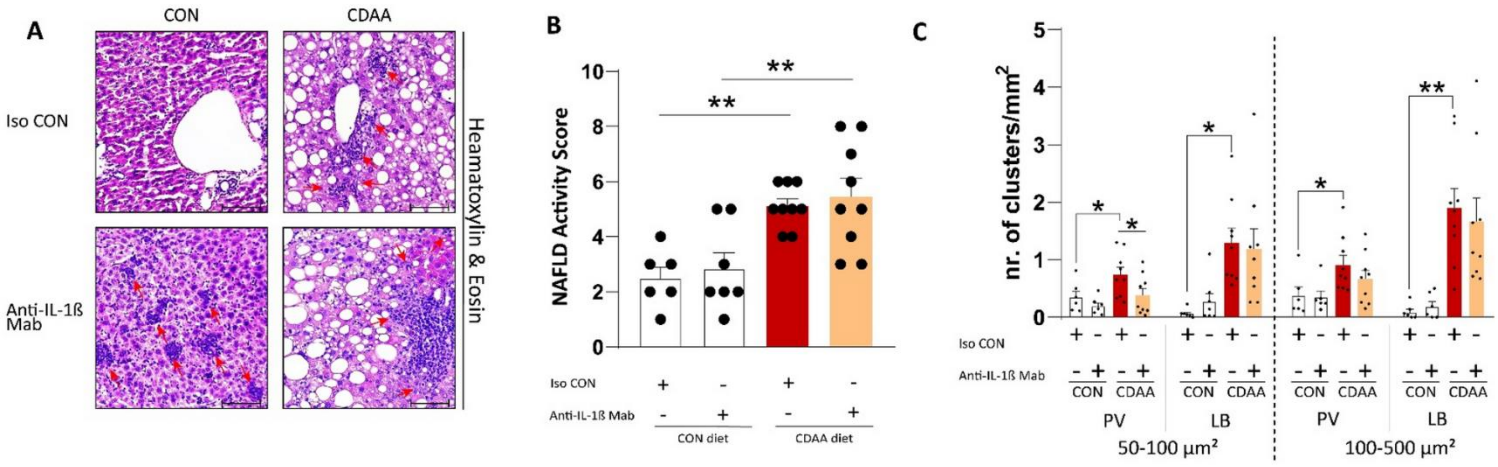

Figure 8 – Investigation of hepatic inflammatory cell infiltrations and NAS.

Macroscopic evaluation of hepatic inflammatory foci on hematoxylin-eosin stained sections (**A**). NAFLD Activity Score (NAS) (**B**). Quantitative and areal assessment of inflammatory infiltrations, n = 6-9/group (**C**). Two-way ANOVA, Fisher's LSD post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (203).

4.1.4. Interleukin-1β binding monoclonal antibody affected key inflammatory mediators

Macrophages (both resident and infiltrating) and neutrophils are important cellular participants of initial immunologic events of NASH. Initially both cell types acquire a pro-inflammatory phenotype, contributing to further damage. However, as detailed above both of them may switch over time to a restorative phenotype. IL-1β serves as an activator and a chemokine for both monocytes/macrophages and neutrophils. Therefore, its inhibition might serve as a protector of the hepatocellular microenvironment. Iba1 staining of macrophages showed no change due to neither the diet nor the treatment (**Figure 9A**). Surprisingly, however, by inhibiting IL-1β the number of neutrophils significantly increased (**Figure 9B**).

Next, we analyzed key M1 and M2 markers. The most relevant chemokine's expression in NASH pathophysiology, CCL2, increased due to the diet, while the treatment was able to decrease it, but unfortunately, not significantly. Interestingly, groups that were administered the IL-1β inhibitor showed a compensatory increase of *Il1b* in hepatocytes (**Figure 9C**). The pro-tumorigenic $CD163^{+}$ macrophages were depleted in CDAA-fed and treated animals.

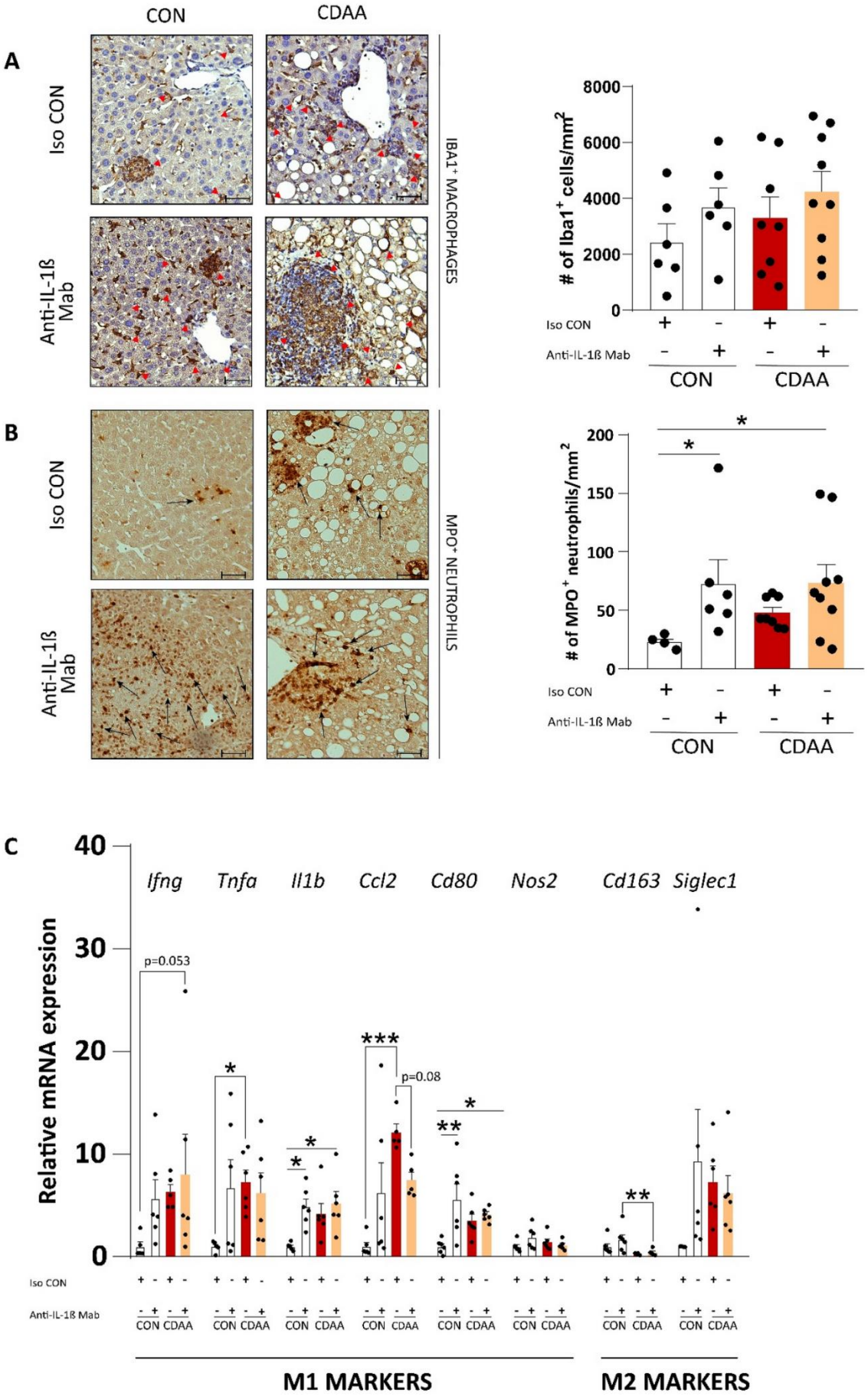
A
CON
CDAA
Iso CON
Anti-IL-1ß Mab
IBA1+ MACROPHAGES
# of Iba1+ cells/mm2
8000
6000
4000
2000
0
Iso CON
Anti-IL-1ß Mab
CON
CDAA
B
Iso CON
Anti-IL-1ß Mab
MPO+ NEUTROPHILS
# of MPO+ neutrophils/mm2
200
150
100
50
0
*
*
C
Relative mRNA expression
40
30
20
10
0
Ifng
Tnfa
Il1b
Ccl2
Cd80
Nos2
Cd163
Siglec1
p=0.053
p=0.08
*
**
***
Iso CON
Anti-IL-1ß Mab
CON
CDAA
M1 MARKERS
M2 MARKERS

Figure 9 – Assessment of pro-inflammatory cells and mediators.

Immunohistochemical evaluation of $Iba1^+$ macrophages staining (n = 6-9/group) (**A**) and MPO^+ neutrophils (n = 4-9/group) (**B**). Transcriptomic analysis of major M1 and M2 genes, n = 5-6/group (**C**). Scale bar shows 100 μm. Two-way ANOVA, Fisher's LSD post hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (203).

4.1.5. NASH and IL-1β blockade might promote a pro-tumorigenic microenvironment

Hepatocellular apoptosis, fibrosis and inflammation are main drivers of compensatory liver cell proliferation, which if goes uncontrolled it might give rise to malignant alterations. PCNA staining revealed marked hepatocellular proliferation. IL-1β neutralization had no effect in this regard. Next, we checked the expression of major oncogenes. *Myc*, *Gpc3*, *Mki67* and *Pcna* were significantly increased in the CDAA-fed groups. Furthermore, the expressions of key immune checkpoints were greatly affected. The expression of *Pd-l1* was increased by the diet, while the treatment induced the up-regulation of *Pd-1* and *Ctla4* (**FIGURE 10**).

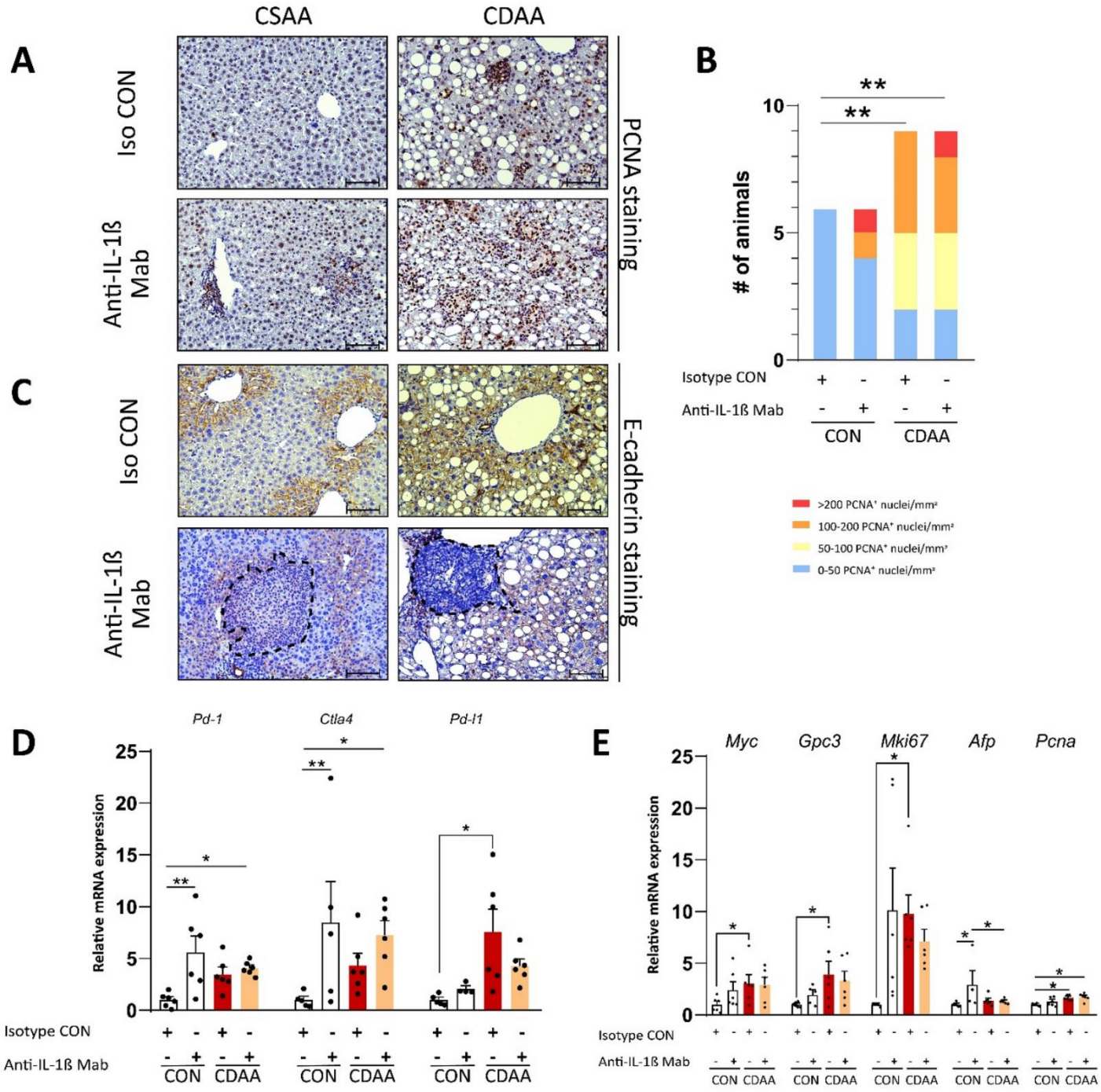

Figure 10 – Histological and molecular analysis of hepatic microenvironment.

Representation of hepatocellular proliferation by PCNA staining (n = 6-9/group) (**A**) and quantification of PCNA positivity (**B**). Assessment of invasiveness by E-cadherin staining (**C**). Transcriptomic analysis of major immune checkpoints (n = 6-9/group) (**D**) and proto-oncogenes (n = 4-6/groups) (**E**). Scale bar shows 100 µm. Two-way ANOVA, Fisher's LSD post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (203).

4.2. Sex-specific differences of inflammation, fibrosis and cholesterol metabolism

In order to increase the success of NASH treatment, we must consider the potential dissimilarities of the population that might arise from sex and age. To do so, first, we must understand key molecular contributors of NASH pathophysiology. As such, we designed a NASH model with the aforementioned CDAA diet with middle-aged animals. Ten months old male and female C57Bl/6J mice were used. This age in mice is considered perimenopausal age in females (205).

4.2.1. Elevated cholesterol level in females with NASH

As previously shown, CDAA diet recaptures key feature of NASH (steatosis, hepatomegaly). Although total cholesterol level in control animals is lower in females, but CDAA feeding caused elevation in cholesterol level in females, compared to males with NASH and healthy females alike (**FIGURE 11**).

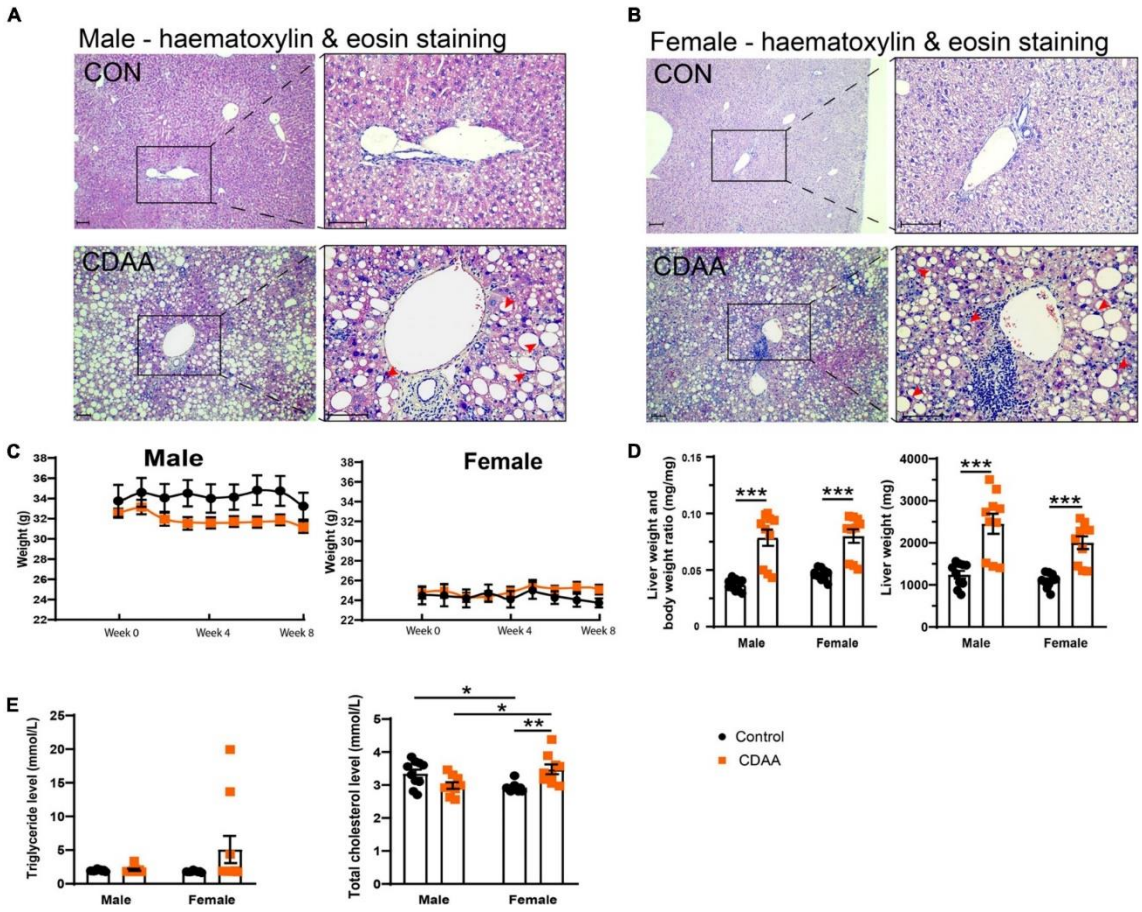

Figure 11 – Sex-specific differences in hepatic architecture and serum lipid levels.

Depiction of steatosis in males (**A**) and females (**B**) on heamatoxylin-eosin staining. Body weight of male and female mice throughout the study (**C**). Liver weight (**D**). Triglyceride and total cholesterol serum level (**E**). Scale bar indicates 100 μm. n = 10/group. Two-way ANOVA followed by Tukey's post hoc test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (204).

4.2.2. Expression of major cholesterol level regulator differs in sex-dependent manner

PCSK9 profoundly affects serum LDL level, which has been exploited to reduce LDL-cholesterol level and, subsequently, risk of cardiovascular diseases. Hepatic expression of *Pcsk9* is increased in control females compared to control males, while females with NASH had decreased expression of this gene. This finding was further supported with ELISA measurement, which showed a similar pattern of serum PCSK9 level. Interestingly, *Ldlr*, the main target of PCSK9, had a similar pattern of expression as PCSK9 itself. Expression of *Cd36*, the second major target of PCSK9, showed elevation in CDAA-fed females, compared to diet-matched males. The renal expression pattern of *Ldlr* was similar to the hepatic expression pattern, while the renal transcription level of *Cd36* was decreased in females regardless of diet (**FIGURE 12**).

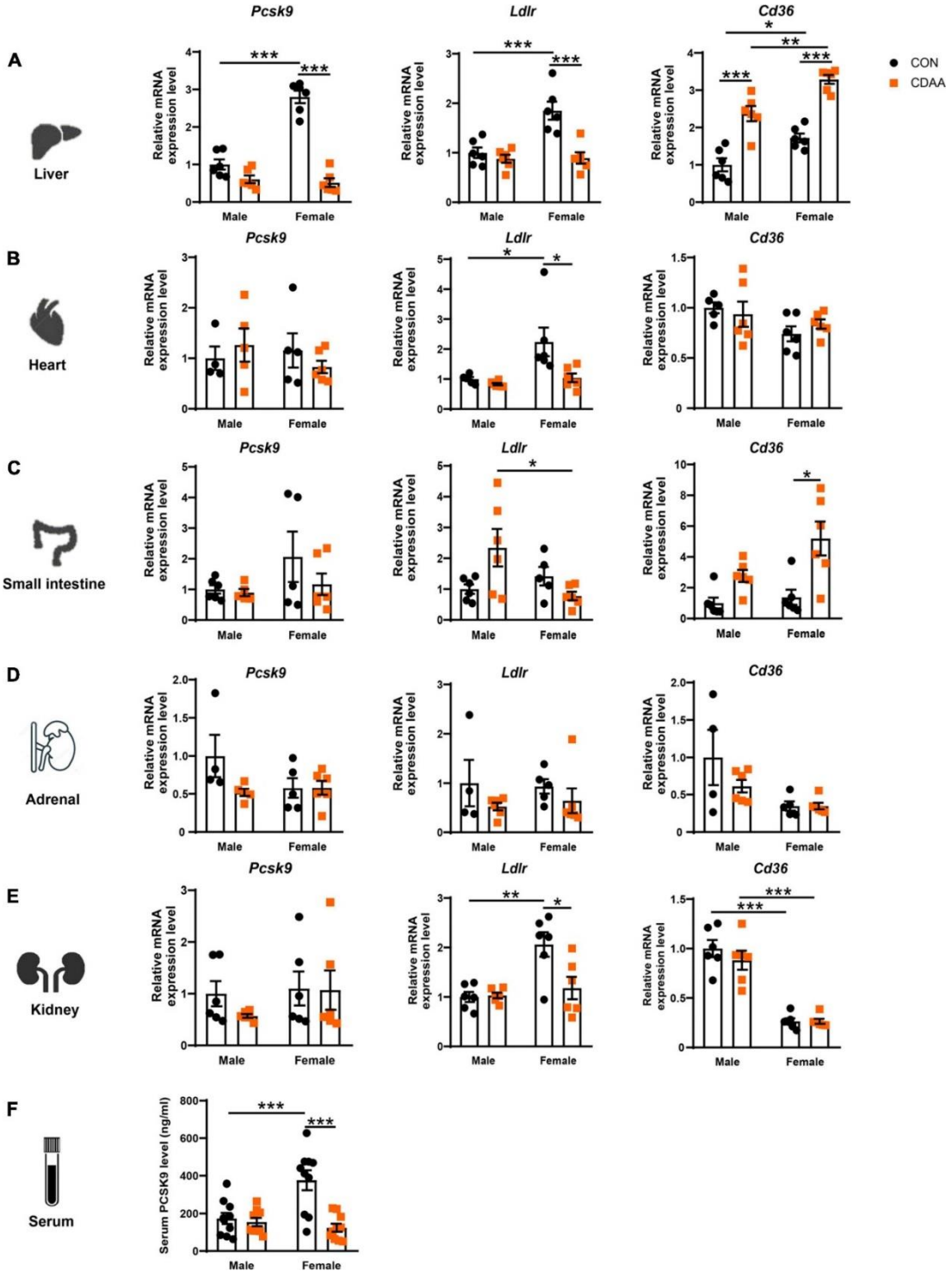

Figure 12 – Sex-dependent gene expression differences of Pcsk9 and its major targets.

Assessment of *Pcsk9*, *Ldlr* and *Cd36* gene expression in the liver (**A**), heart (**B**), small intestine (**C**), adrenal glands (**D**) and kidneys (**E**), n = 6/group. Serum level of PCSK9, n = 10/group (**F**). Two-way ANOVA followed by Tukey's post hoc test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (204).

4.2.3. Males with NASH are characterized by profound fibrosis

As previously mentioned, the extent of fibrosis is a dominant prognostic marker for disease progression. Quantification of overall fibrosis showed that males have substantially higher level of fibrosis after 8 weeks of CDAA feeding. The gene expression of CTGF also showed a sex-specific difference, significantly elevated in males with NASH compared to females. However, the expression of collagen types I and III did not differ between the sexes (**FIGURE 13**).

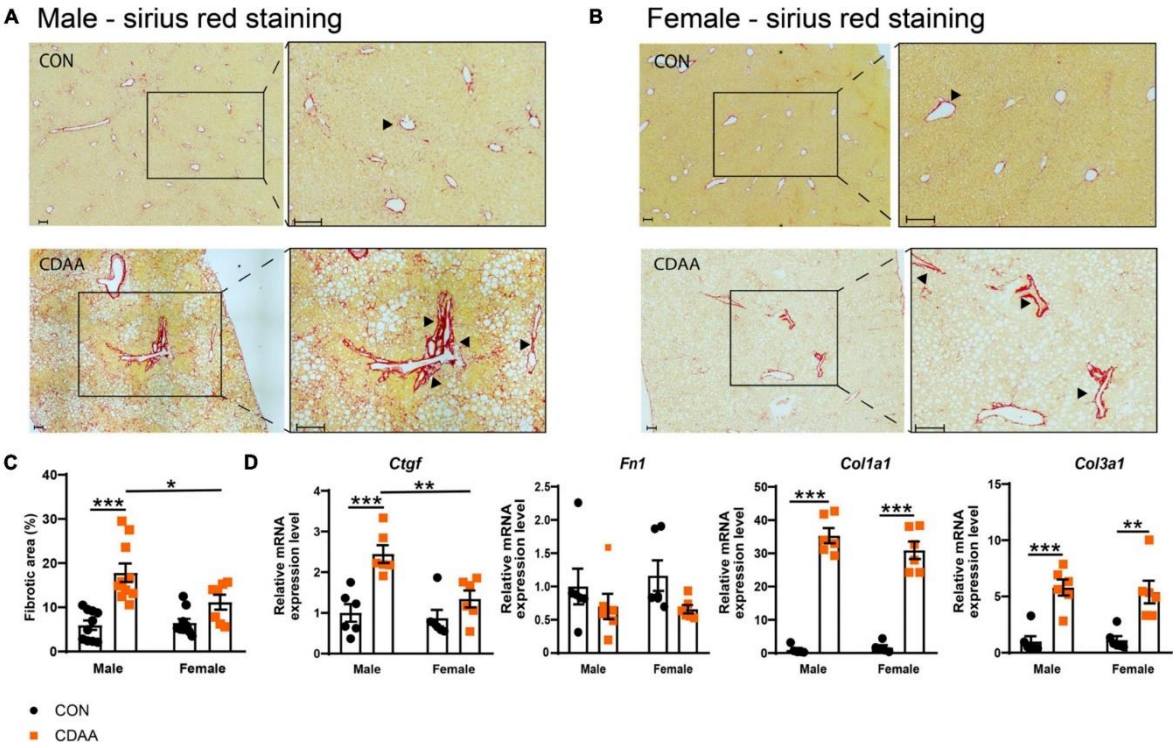

Figure 13 – Sex differences in hepatic fibrosis.

Depiction of hepatic fibrosis on pricrosirius-red stained sections in males (**A**) and (**B**). Quantification of overall fibrosis, n = 10/group (**C**). Transcriptomic analysis of major pro-fibrotic genes, n = 6/group (**D**). Two-way ANOVA followed by Tukey's post hoc test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (204).

4.2.4. The hepatic immune cell repertoire is slightly different in females

As described in the introduction, complex inflammatory events occur during NASH, in which multiple type of immune cells participate. Myeloid cells are the first responders at the dawn of pro-inflammatory processes in NASH. Accordingly, we performed immunohistochemistry to evaluate the potential sex differences of these cells in NASH pathophysiology. First we stained for macrophages with the pan-macrophage marker, Iba1. We did not observe a difference between the sexes. Next, we were interested in resident macrophages, thus we continued with Clec4f staining, which revealed a higher number of Kupffer cells in CON female mice compared to males. Upon NASH, the number of these cells declined in females (**Figure 13**).

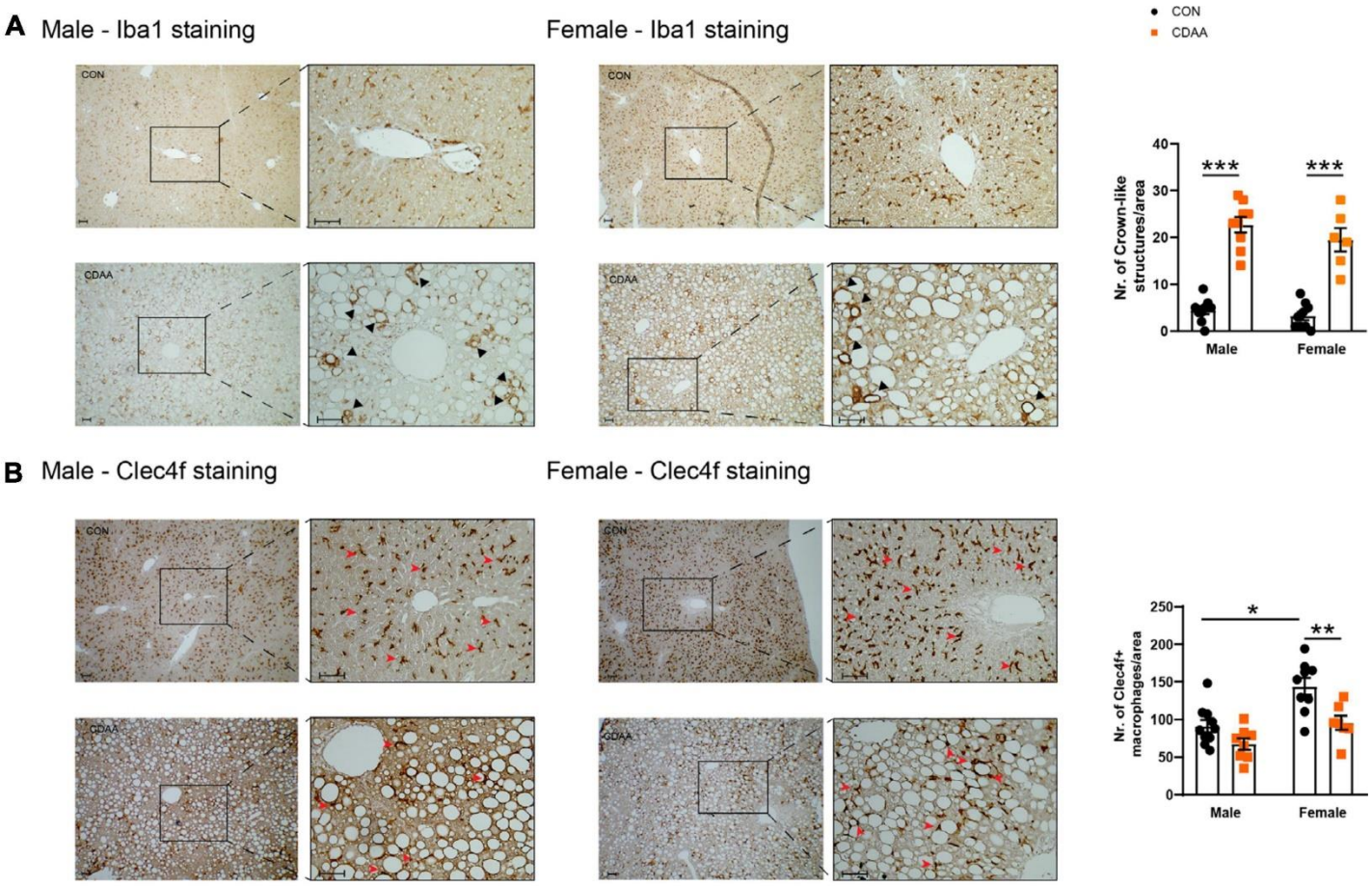

Figure 14 – Immunohistochemical staining of macrophages.

Iba1 (**A**) and Clec4f (**B**) immunostaining of male and female hepatic sections and its respective quantifications. Two-way ANOVA followed by Tukey's post hoc test, n = 10/group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (204).

Neutrophils (as mentioned before) and T cells are also present in the immunologic events of NASH (206). Following immunostaining for MPO^+ neutrophils and $CD3^+$ T cells, we did not observe any sex-specific disparity **(FIGURE 15)**.

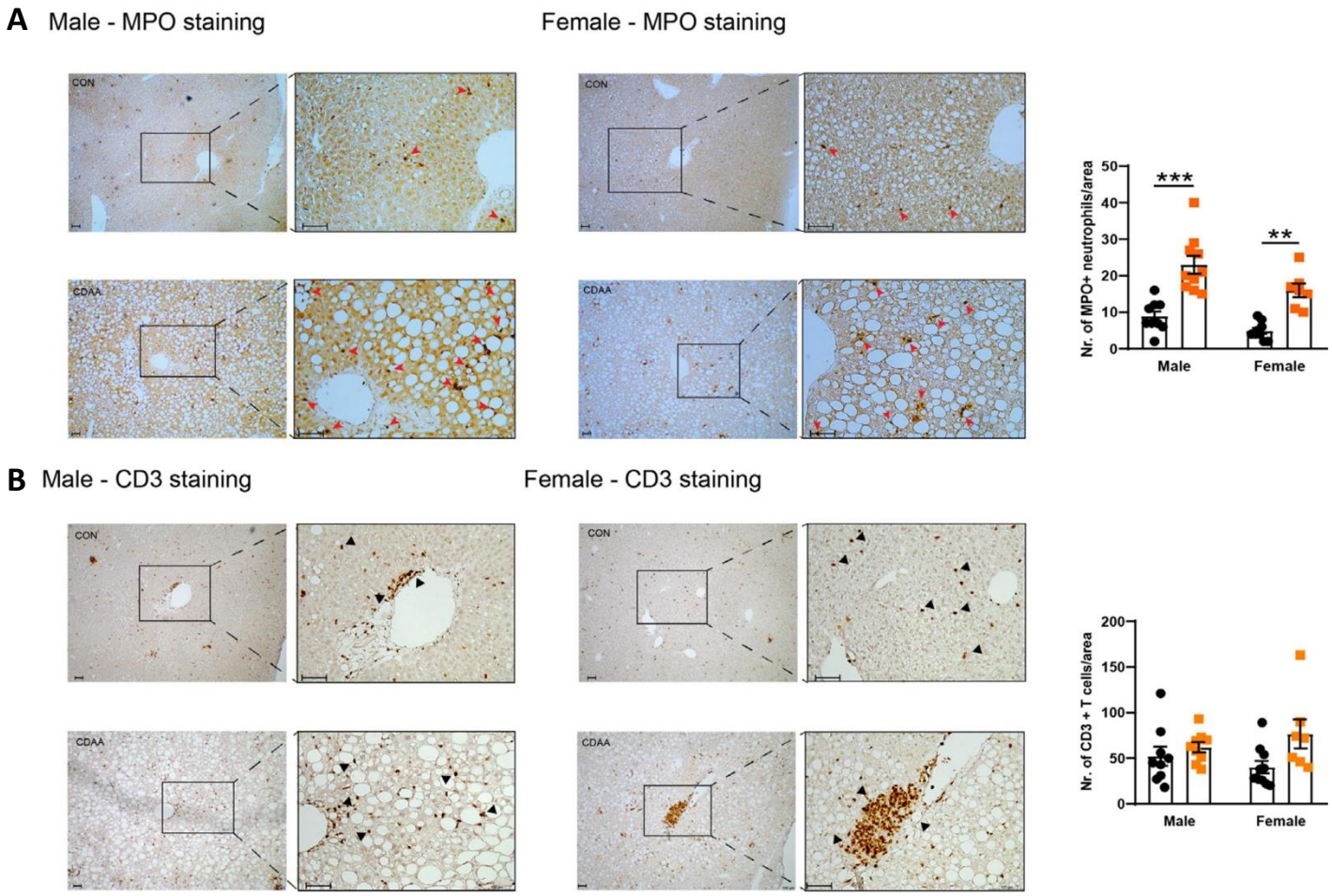

Figure 15 – Immunostaining of neutrophils and T cells

Histological and quantitative analysis of MPO^+ neutrophils (**A**) and $CD3^+$ T cells (**B**). Two-way ANOVA followed by Tukey's post hoc test, n = 10/group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (204).

4.2.5. Females are characterized with a more pro-inflammatory phenotype

As discussed in the previous work that inflammation is also considered as a major participants of NASH pathomechanism and a major contributor to disease progression. Therefore, we wished to assess whether these cytokines have a sex-dependent expression profile in NASH. Genes of IL-1β and IFN-γ were significantly higher expressed in the liver of female mice with NASH. This was further confirmed with Western Blot analysis, showing increased protein level of cleaved IL-1β in females with NASH. The responsible inflammasome for the enzymatic cleavage of IL-1β has been revealed to be NLRP3. The expression of *Tnfa*, *Ccl2*, *Ccr1* and *Cd68* were increased independently of sex, while *Cd163* was downregulated (**FIGURE 16**).

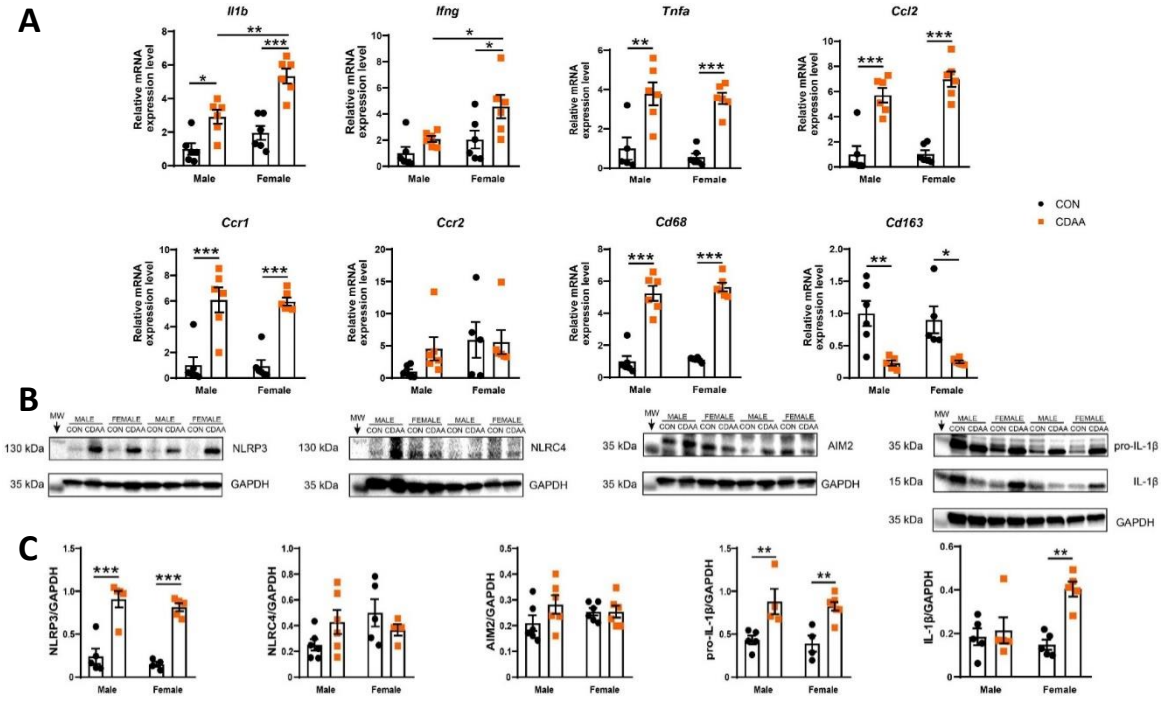

Figure 16 – Sex-dependent differences in hepatic inflammation.

Gene expression analysis of major pro-inflammatory cytokines (**A**), n = 6/group. Western blot analysis of major inflammasomes and IL-1β, n = 4/group (**B**, **C**). Two-way ANOVA followed by Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (204).

5. DISCUSSION

Non-alcoholic fatty liver disease is a major concern for global healthcare systems and is considered a significant socio-economic burden (2, 3). As such, it is paramount to develop a safe and effective medication as soon as possible. In order to achieve this challenging task, we need to fill in the missing details about the pathomechanism of NASH, and elucidation of the potential factors that might contribute to disease progression or define factors that might affect treatment success is crucially important.

In this work we aimed to answer the following questions: Is directly targeting a major pro-inflammatory cytokine beneficial in a cardiometabolic disease like NASH? Is there any molecular difference between sexes during NASH?

In our task to answer these questions, we decided to use a dietary model of NASH, where choline is deficient. CDAA diet is able to model key features (macroscopic and microscopic steatosis, inflammation, fibrosis) of NASH within 8 weeks. The disadvantage of this model is that it lacks essential clinical and metabolic traits of NASH, such as insulin resistance and, most importantly, obesity. Both obesity and insulin resistance are relevant drivers of meta-inflammation, a type of systemic inflammation derived from metabolic dysregulation, thus causing systemic burden spanning multiple organs, such as the heart and liver (207). We have chosen this model for precisely this reason. We wished to isolate the burden of these systemic factors in order to investigate the sex differences specifically in NASH and the effects of anti-IL-1β monoclonal antibody specifically in NASH.

We planned that the subprojects of this work to run simultaneously, because the animals for the aged model needed to age for the desired age. According to Kane A. E. *et al.* (201) frailty is associated with maladaptive cardiac changes in aged male mice. Alterations of cardiac geometry in aged males were correlated with plasma concentration of several pro-inflammatory cytokines (201). Thus, we decided to use male mice to test the cardiac and hepatic effects of canakinumab mimicking antibody. We choose to target Interleukin-1β, because of its extensive role in NASH pathomechanism (208). Additionally, canakinumab - a human monoclonal antibody against circulating IL-1β – has been proven to effectively decrease mortality in patients with history of myocardial infarction and high level of hs-CRP. CANTOS trial was the first to prove that targeting cardiometabolic

inflammation improves cardiac outcome; however, such therapy risks upper respiratory tract infections (209).

In this subproject, strain analysis revealed increased in E/SrE ratio in CDAA-fed animals. A previously reported mouse model of HFpEF supports that strain rate analysis is a more sensitive method to measure subtle myocardial functional alteration (210). In our study, an improvement of diastolic dysfunction has been seen upon IL-1β inhibition, which was observable only with speckle tracking echocardiography. This finding is in line with studies, where it was shown that IL-1β signaling may interfere with the transduction of β-adrenergic receptors, causing impairment in cardiac function (211-213).

Unfortunately, IL-1β blockade, in our study, failed to improve key features of NASH in aged mice. Significant hepatic fibrosis developed upon CDAA diet. Hepatic fibrosis is mainly driven by hepatic stellate cells, which produces collagen upon activation by IL-1β and hepatocellular debris (47). Interestingly, IL-1β blockade decreased the transcription of *Col1a1* and *Col3a1*; however, macroscopic quantification of fibrosis showed no overall change in anti-IL-1β monoclonal antibody treated mice. We may assume that IL-1β inhibition might induce pro-resolution processes at molecular level, but that does not manifest macroscopically within 8 weeks of treatment. During hepatic wound healing, fibrosis might resolve both by hepatic stellate cell apoptosis and degradation of fibrotic proteins, resulting in reduction of ECM deposition and degradation of ECM (214). Previous reports showed that upstream blockade of cleavage of IL-1β by inhibition of NLRP3 inflammasome or caspase-1 reduced hepatic fibrosis, proving that targeting mechanism that subsequently decreases IL-1β maturation in NASH may ameliorate pro-fibrotic events (215, 216). It is reasonable to assume, that it is likely that 8 weeks of treatment may not be sufficient to meaningfully alter fibrosis in our model.

Next, we investigated whether inflammatory foci are affected by IL-1β inhibition and we observed a possible halt of small immune cluster progression into large ones. Interestingly, a tendency of increase in macrophage population was seen in mice with NASH treated with IL-1β blocker, while transcription of *Ccl2* gene was marginally diminished by the treatment. NF-κB, the downstream transcription factor of IL-1β, regulates the secretion of CCL2. In a model with an atherogenic diet, NLRP3 inhibition resulted significant down-regulation of *Ccl2* (217). Furthermore, a trend-like increase was visible in the expression of IFN-γ in CDAA-fed mice treated with the IL-1β blocking

antibody. Hart K. *et al.* recognized that elevated levels of IFN-γ, a potent polarizing factor for M1 macrophages and a main driver for Th1 commitment, can be protective in NASH (29). A surprising finding was that IL-1β blockade increased the number of infiltrating neutrophils into the liver. This finding of ours is in contrast to previous publications, where NLRP3 inhibitor-treated mice with NASH showed decreased hepatic neutrophil count (217). A possible explanation for this contradiction might be the direct inhibition of inflammasomes and/or caspase-1 decreases the maturation of both IL-1β and IL-18, thus the unaffected IL-18, in our study, is free to act as an activating agent for neutrophils as reported by Leung B. P. *et al.* (218).

We continued to assess hepatocyte proliferation in settings of IL-1β inhibition. We report no change in this regard. Compensatory proliferation of hepatocytes has the role to regenerate the liver's damaged architecture in order to restore the lost parenchymal cell due to various forms of cell death (219). Patients with NASH have a higher rate of hepatocellular apoptosis (thus it is considered as a key contributor to disease progression), the subsequent compensatory proliferation might drive malignant transformation (220-222). As expected, we observed marked proliferation in aged males fed with CDAA diet. Inflammatory cell death, pyroptosis, occurs due to caspase-1 activation resulting the release of IL-1β, triggering a vicious cycle of inflammatory cell death by further promoting positive-feedback of pyroptosis. Although clinical trials of caspase inhibitors did not meet their primary endpoints against NASH (189), we hypothesized that interference with IL-1β's vicious cycle, then we might be able to halt further loss of liver cells. However, anti-IL-1β treatment, in our study, failed to meaningfully affect hepatocyte proliferation. NASH is a one of the possible etiology of liver cancers, such as hepatocellular carcinoma (223). HCC is classically considered to be a radio- and chemotherapy-resistant malignancy (224). Immunotherapies has emerged as potential treatment options for HCC. Immune checkpoint inhibitors (ICIs) are approved in advanced HCC (225); however, the immune microenvironment of HCC is highly relevant to achieve efficacy. ICIs were proven effective in viral HCC, but they did not show improvement in NASH-induced HCC (226). Extensive investigations are currently underway to develop therapeutic options for NASH-related HCC. Accordingly, we investigated how IL-1β blockade affects the transcription of immune checkpoints. We report that microenvironment of NASH is characterized by increased expression of *Pd-*

l1; similarly, to the findings of Zong Z *et al.* who showed that IL-1β might induce PD-L1 expression on malignant liver cells (227).

Although IL-1β is a pro-inflammatory cytokine, increased levels IL-1β may possess a crucial immunosuppressive role in different tumors, thus its inhibition might prove to be legitimate (209, 228-230). However, we observed that IL-1β blockade increased the expression of *Ctla4* and *Pd-1*, which may suggest a microenvironment with immunosuppressive attributes. This was observed in clinical studies, where patients diagnosed with liver cancer had poor prognosis, if their pro-inflammatory cytokine profile was suppressed, including of IL-1β (231).

Interestingly our model is characterized by immunosuppressive molecular pattern; however, analysis of major macrophage markers shows a clear M1 polarization, which are generally thought to worsen disease outcome during NASH by promoting steatosis and inflammation (232), but monocytes/macrophages that infiltrate into tumor microenvironment and differentiate into M1 phenotype have anti-tumor potential. Simply put: M1 macrophages during NASH contribute to disease progression, while during hepatic malignant events they might prove to be beneficial. Similarly, M2 macrophages are also blessed with dual role in NASH. First, M2 macrophages may initiate apoptosis of M1 macrophages (28), thus contributing to disease resolution. Meanwhile $Cd163^{+}$ M2 macrophages are considered pro-tumorigenic, in contrast to anti-tumorigenic $Siglec1^{+}$ cells (233). In our project, we report that *Cd163* expression is down-regulated, and *Siglec1* expression, although not significantly, is increased in mice with NASH. All in all, we can say that our model is characterized by an anti-tumorigenic niche at cellular level, while immunosuppressive microenvironment is visible at molecular level.

As mentioned before IL-1β has a wide variety of effects. The literature describes significant contribution to cancer-promoting inflammation in a wide variety of malignancies (234). Adversely, IL-1β may also possess anti-tumorigenic attributes. Consequently, interference with IL-1β may negatively impact diverse cancers. IL-1β activate Th9 cells, which previously showed propensity to effectively target melanoma cells (235). Thus, interruption of IL-1β signaling in patients with melanoma might prove deleterious for disease progression, would be a sound argument. It was shown that IL-R1 deficiency on neutrophils drive CRC progression, further proving that IL-1β inhibition is not optimal (229). Nasopharyngeal carcinoma releases IL-1β which may act upon tumor-

associated neutrophils resulting a tumorlytic effect, thus IL-1β blockade might prevent this beneficial effect of neutrophils (236).
It is clear that IL-1β has a wide range of effects in different cancers and cardiometabolic diseases as well, thus future treatments with IL-1β blockers should take into consideration the aforementioned adverse possibilities.

Beside local microenvironment and age-related systemic inflammation, sex is also a determining factor that should be considered for future treatments of NASH. In our second subproject, we highlighted major molecular and cellular differences between male and female mice in the aforementioned CDAA diet-based NASH model, however, in this case we used middle-aged (10 months old) animals. The reason for this, firstly, is that this age is considered perimenopausal for female mice (205). Secondly, the younger age (compared to our first subproject) may decrease the burden of age-related systemic inflammation, so we are able to describe NASH at a time point where major hormonal changes occur without the impact of inflamm-aging.
As detailed above, major sex-dependent differences have been described in the clinical cardiometabolic field, but key molecular differences are still missing, that would help understand the sex-dependent pathomechanisms of NASH or molecular entities that might eventually prove to be a potential therapeutic target against NASH.
Prevalence of NAFLD, as mentioned before, differ with sexual status between women, and between men and women. In premenopausal women, sex hormones not only govern hepatic metabolism to meet the demands of reproduction (237), but also modulate immune responses augmented by genes coded on X chromosomes. Consequently, women tend to develop a stronger immune response to antigens, which results in more effective pathogen clearance, but this may lead to increased immune-related pathologies, such as autoimmune or inflammatory diseases (238). Inflammation is a major driver of steatosis-to-steatohepatitis progression, fibrosis, and even hepatocellular carcinoma (208).
After the discovery of PCSK9, it has become a molecular entity with huge interest, especially in cardiometabolic diseases. Targeting PCSK9 proved to be a powerful tool to reduce LDL-cholesterol level. Some evidence might suggest that PCSK9 has a role in NASH pathophysiology (239).

With the facts above in mind, we aimed in our second subproject to evaluate PCSK9- and inflammation-related sex-differences.

We showed that middle-aged female mice with NASH display reduced hepatic *Pcsk9* gene expression and reduced serum protein level. This might seem contradictory, when considering the beneficial effects of PCSK9 inhibition. Nonetheless, Lai *et al.* published similar results, where they proposed that the transcription factor E2F1, which is a key regulator of hepatic PCSK9 expression, might induce downregulation of PCSK9 in order to prevent excessive cholesterol accumulation in hepatocytes (240). PCSK9 knock-out mice fed with high-fat diet resulted in more severe steatohepatitis (241).

The literature and our data suggest that PCSK9 deficiency could promote steatosis, especially in female mice. This could be a real concern of pharmacological inhibitors of PCSK9, which was not investigated in NASH patients. Additionally, new cholesterol lowering drugs, such as mipomersen and lomitapide, showed propensity to cause hepatosteatosis (242, 243). Postmenopausal women and men have higher risk for severe fibrosis (89), but in our model with perimenopausal mice revealed pronounced fibrosis in males.

Altogether, we might say that females would benefit more of anti-steatotic and/or anti-inflammatory treatment, while males with anti-fibrotic strategy.

6. CONCLUSIONS

In this work we showed that, although interleukin-1β is a major contributor to systemic inflammation, to cardiovascular diseases (e.g. myocardial infarction, heart failure) and to metabolic diseases, such as non-alcoholic steatohepatitis, targeting this pro-inflammatory cytokine is not a viable option to treat NASH. Anti-interleukin-1β monoclonal antibody improved diastolic dysfunction of mice with NASH; however, it failed to beneficially alter key features of NASH and even promoted the formation of an immunosuppressive microenvironment that might, subsequently, give rise to non-benign alterations.

Our work demonstrated that middle-aged males develop profound fibrosis, while females suffered a more intensive hepatic inflammation.

In conclusion we might say that interleukin-1β is not a viable target in treating NASH. Additionally, it is likely that males would benefit more of anti-fibrotic treatment, while females may require anti-inflammatory treatment to achieve higher success rate.

7. SUMMARY

Non-alcoholic fatty liver disease and subsequent steatohepatitis is global healthcare concern with no effective treatment on the market. This cardiometabolic disease, which is in fact the hepatic manifestation of metabolic syndrome, shows relevant sex-dependent differences in humans; however, data about key molecular perpetrators in this regards are still missing.

Therefore, we aimed in this work to investigate the cardiac and hepatic effects of anti-interleukin-1β monoclonal antibody and to investigate sex-specific molecular differences in fibrosis, inflammation and cholesterol metabolism.

In a choline deficient diet-based aged model, we showed mild diastolic dysfunction in animals with NASH, which improved upon the treatment. At molecular level, the monoclonal antibody was able to ameliorate hepatic fibrosis, but overall fibrosis assessment did not change. NAFLD Activity Score, consist of grading inflammation, steatosis and hepatocyte ballooning, revealed no improvement by the treatment. Hepatic microenvironment showed signs of immunosuppression and potential pre-malignant alterations.

Similar model was used to investigated molecular differences in NASH, but with middle aged male and female mice. Female mice with NASH were characterized by higher serum cholesterol level than males. Serum level and hepatic expression level of PCSK9 is reduced in females fed with CDAA diet. Males with NASH had higher overall fibrosis level, while females showed elevated expression of hepatic *Il1b* and higher rate of IL-1β maturation level.

8. REFERENCES

1. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol. 2018;15(1):11-20.
2. Younossi ZM, Blissett D, Blissett R, Henry L, Stepanova M, Younossi Y, Racila A, Hunt S, Beckerman R. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. Hepatology. 2016;64(5):1577-86.
3. Younossi ZM, Zheng L, Stepanova M, Henry L, Venkatesan C, Mishra A. Trends in outpatient resource utilizations and outcomes for Medicare beneficiaries with nonalcoholic fatty liver disease. J Clin Gastroenterol. 2015;49(3):222-7.
4. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018;67(1):328-57.
5. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64(1):73-84.
6. Ouyang X, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, Johnson RJ, Abdelmalek MF. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. J Hepatol. 2008;48(6):993-9.
7. Liang JQ, Teoh N, Xu L, Pok S, Li X, Chu ESH, Chiu J, Dong L, Arfianti E, Haigh WG, Yeh MM, Ioannou GN, Sung JJY, Farrell G, Yu J. Dietary cholesterol promotes steatohepatitis related hepatocellular carcinoma through dysregulated metabolism and calcium signaling. Nat Commun. 2018;9(1):4490.
8. Corbin KD, Zeisel SH. Choline metabolism provides novel insights into nonalcoholic fatty liver disease and its progression. Curr Opin Gastroenterol. 2012;28(2):159-65.
9. Herman MA, Samuel VT. The Sweet Path to Metabolic Demise: Fructose and Lipid Synthesis. Trends Endocrinol Metab. 2016;27(10):719-30.
10. Yki-Järvinen H, Luukkonen PK, Hodson L, Moore JB. Dietary carbohydrates and fats in nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol. 2021;18(11):770-86.

11. Flannery C, Dufour S, Rabøl R, Shulman GI, Petersen KF. Skeletal muscle insulin resistance promotes increased hepatic de novo lipogenesis, hyperlipidemia, and hepatic steatosis in the elderly. Diabetes. 2012;61(11):2711-7.
12. Couillard C, Bergeron N, Prud'homme D, Bergeron J, Tremblay A, Bouchard C, Mauriège P, Després JP. Gender difference in postprandial lipemia : importance of visceral adipose tissue accumulation. Arterioscler Thromb Vasc Biol. 1999;19(10):2448-55.
13. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev. 2018;98(4):2133-223.
14. Irimia JM, Meyer CM, Segvich DM, Surendran S, DePaoli-Roach AA, Morral N, Roach PJ. Lack of liver glycogen causes hepatic insulin resistance and steatosis in mice. J Biol Chem. 2017;292(25):10455-64.
15. Lebeaupin C, Vallée D, Hazari Y, Hetz C, Chevet E, Bailly-Maitre B. Endoplasmic reticulum stress signalling and the pathogenesis of non-alcoholic fatty liver disease. J Hepatol. 2018;69(4):927-47.
16. Pierantonelli I, Svegliati-Baroni G. Nonalcoholic Fatty Liver Disease: Basic Pathogenetic Mechanisms in the Progression From NAFLD to NASH. Transplantation. 2019;103(1):e1-e13.
17. Diehl AM, Day C. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. N Engl J Med. 2017;377(21):2063-72.
18. Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. QJM. 2010;103(2):71-83.
19. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 2016;65(8):1038-48.
20. Zhao L, Zhong S, Qu H, Xie Y, Cao Z, Li Q, Yang P, Varghese Z, Moorhead JF, Chen Y, Ruan XZ. Chronic inflammation aggravates metabolic disorders of hepatic fatty acids in high-fat diet-induced obese mice. Sci Rep. 2015;5:10222.
21. Tilg H, Adolph TE, Moschen AR. Multiple Parallel Hits Hypothesis in Nonalcoholic Fatty Liver Disease: Revisited After a Decade. Hepatology. 2021;73(2):833-42.

22. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology. 2014;147(4):765-83.e4.
23. Negrin KA, Roth Flach RJ, DiStefano MT, Matevossian A, Friedline RH, Jung D, Kim JK, Czech MP. IL-1 signaling in obesity-induced hepatic lipogenesis and steatosis. PLoS One. 2014;9(9):e107265.
24. Stienstra R, Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, Staels B, Kersten S, Müller M. Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxisome proliferator-activated receptor alpha activity. Hepatology. 2010;51(2):511-22.
25. Jager J, Grémeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology. 2007;148(1):241-51.
26. Knorr J, Wree A, Feldstein AE. Pyroptosis in Steatohepatitis and Liver Diseases. J Mol Biol. 2022;434(4):167271.
27. Jindal A, Bruzzì S, Sutti S, Locatelli I, Bozzola C, Paternostro C, Parola M, Albano E. Fat-laden macrophages modulate lobular inflammation in nonalcoholic steatohepatitis (NASH). Exp Mol Pathol. 2015;99(1):155-62.
28. Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, Pecker F, Tran A, Gual P, Mallat A, Lotersztajn S, Pavoine C. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology. 2014;59(1):130-42.
29. Hart KM, Fabre T, Sciurba JC, Gieseck RL, 3rd, Borthwick LA, Vannella KM, Acciani TH, de Queiroz Prado R, Thompson RW, White S, Soucy G, Bilodeau M, Ramalingam TR, Arron JR, Shoukry NH, Wynn TA. Type 2 immunity is protective in metabolic disease but exacerbates NAFLD collaboratively with TGF-β. Sci Transl Med. 2017;9(396).
30. Kazankov K, Jørgensen SMD, Thomsen KL, Møller HJ, Vilstrup H, George J, Schuppan D, Grønbæk H. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Nat Rev Gastroenterol Hepatol. 2019;16(3):145-59.

31. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313-26.
32. Krenkel O, Puengel T, Govaere O, Abdallah AT, Mossanen JC, Kohlhepp M, Liepelt A, Lefebvre E, Luedde T, Hellerbrand C, Weiskirchen R, Longerich T, Costa IG, Anstee QM, Trautwein C, Tacke F. Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. Hepatology. 2018;67(4):1270-83.
33. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhöfer D, Frey B, Lell M, Manger B, Rech J, Naschberger E, Holmdahl R, Krenn V, Harrer T, Jeremic I, Bilyy R, Schett G, Hoffmann M, Herrmann M. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nat Med. 2014;20(5):511-7.
34. He Y, Rodrigues RM, Wang X, Seo W, Ma J, Hwang S, Fu Y, Trojnár E, Mátyás C, Zhao S, Ren R, Feng D, Pacher P, Kunos G, Gao B. Neutrophil-to-hepatocyte communication via LDLR-dependent miR-223-enriched extracellular vesicle transfer ameliorates nonalcoholic steatohepatitis. J Clin Invest. 2021;131(3).
35. Calvente CJ, Tameda M, Johnson CD, Del Pilar H, Lin YC, Adronikou N, De Mollerat Du Jeu X, Llorente C, Boyer J, Feldstein AE. Neutrophils contribute to spontaneous resolution of liver inflammation and fibrosis via microRNA-223. J Clin Invest. 2019;129(10):4091-109.
36. Hwang S, Yun H, Moon S, Cho YE, Gao B. Role of Neutrophils in the Pathogenesis of Nonalcoholic Steatohepatitis. Front Endocrinol (Lausanne). 2021;12:751802.
37. Canbay A, Taimr P, Torok N, Higuchi H, Friedman S, Gores GJ. Apoptotic body engulfment by a human stellate cell line is profibrogenic. Lab Invest. 2003;83(5):655-63.
38. Zisser A, Ipsen DH, Tveden-Nyborg P. Hepatic Stellate Cell Activation and Inactivation in NASH-Fibrosis-Roles as Putative Treatment Targets? Biomedicines. 2021;9(4).
39. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. Nat Rev Gastroenterol Hepatol. 2017;14(7):397-411.

40. Hunt NJ, Kang SWS, Lockwood GP, Le Couteur DG, Cogger VC. Hallmarks of Aging in the Liver. Comput Struct Biotechnol J. 2019;17:1151-61.
41. Sheedfar F, Di Biase S, Koonen D, Vinciguerra M. Liver diseases and aging: friends or foes? Aging Cell. 2013;12(6):950-4.
42. Kim IH, Xu J, Liu X, Koyama Y, Ma HY, Diggle K, You YH, Schilling JM, Jeste D, Sharma K, Brenner DA, Kisseleva T. Aging increases the susceptibility of hepatic inflammation, liver fibrosis and aging in response to high-fat diet in mice. Age (Dordr). 2016;38(4):291-302.
43. Zakai NA, Katz R, Jenny NS, Psaty BM, Reiner AP, Schwartz SM, Cushman M. Inflammation and hemostasis biomarkers and cardiovascular risk in the elderly: the Cardiovascular Health Study. J Thromb Haemost. 2007;5(6):1128-35.
44. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D, Coussieu C, Basdevant A, Bar Hen A, Bedossa P, Guerre-Millo M, Clément K. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes. 2006;55(6):1554-61.
45. Farhadi A, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, Kwasny MM, Keshavarzian A. Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis. Liver Int. 2008;28(7):1026-33.
46. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, Pradere JP, Schwabe RF. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. Nat Commun. 2013;4:2823.
47. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. Nat Rev Immunol. 2014;14(3):181-94.
48. Iredale JP, Benyon RC, Arthur MJ, Ferris WF, Alcolado R, Winwood PJ, Clark N, Murphy G. Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. Hepatology. 1996;24(1):176-84.
49. Angulo P, Machado MV, Diehl AM. Fibrosis in nonalcoholic Fatty liver disease: mechanisms and clinical implications. Semin Liver Dis. 2015;35(2):132-45.
50. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69-90.

51. Sanyal A, Poklepovic A, Moyneur E, Barghout V. Population-based risk factors and resource utilization for HCC: US perspective. Curr Med Res Opin. 2010;26(9):2183-91.
52. Dyson J, Jaques B, Chattopadyhay D, Lochan R, Graham J, Das D, Aslam T, Patanwala I, Gaggar S, Cole M, Sumpter K, Stewart S, Rose J, Hudson M, Manas D, Reeves HL. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. J Hepatol. 2014;60(1):110-7.
53. Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. Hepatology. 2010;51(6):1972-8.
54. Nakagawa H, Hayata Y, Kawamura S, Yamada T, Fujiwara N, Koike K. Lipid Metabolic Reprogramming in Hepatocellular Carcinoma. Cancers (Basel). 2018;10(11).
55. Kudo Y, Tanaka Y, Tateishi K, Yamamoto K, Yamamoto S, Mohri D, Isomura Y, Seto M, Nakagawa H, Asaoka Y, Tada M, Ohta M, Ijichi H, Hirata Y, Otsuka M, Ikenoue T, Maeda S, Shiina S, Yoshida H, Nakajima O, Kanai F, Omata M, Koike K. Altered composition of fatty acids exacerbates hepatotumorigenesis during activation of the phosphatidylinositol 3-kinase pathway. J Hepatol. 2011;55(6):1400-8.
56. Boege Y, Malehmir M, Healy ME, Bettermann K, Lorentzen A, Vucur M, Ahuja AK, Böhm F, Mertens JC, Shimizu Y, Frick L, Remouchamps C, Mutreja K, Kähne T, Sundaravinayagam D, Wolf MJ, Rehrauer H, Koppe C, Speicher T, Padrissa-Altés S, Maire R, Schattenberg JM, Jeong JS, Liu L, Zwirner S, Boger R, Hüser N, Davis RJ, Müllhaupt B, Moch H, Schulze-Bergkamen H, Clavien PA, Werner S, Borsig L, Luther SA, Jost PJ, Weinlich R, Unger K, Behrens A, Hillert L, Dillon C, Di Virgilio M, Wallach D, Dejardin E, Zender L, Naumann M, Walczak H, Green DR, Lopes M, Lavrik I, Luedde T, Heikenwalder M, Weber A. A Dual Role of Caspase-8 in Triggering and Sensing Proliferation-Associated DNA Damage, a Key Determinant of Liver Cancer Development. Cancer Cell. 2017;32(3):342-59.e10.
57. Daugherity EK, Balmus G, Al Saei A, Moore ES, Abi Abdallah D, Rogers AB, Weiss RS, Maurer KJ. The DNA damage checkpoint protein ATM promotes

hepatocellular apoptosis and fibrosis in a mouse model of non-alcoholic fatty liver disease. Cell Cycle. 2012;11(10):1918-28.

58. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N. Autophagy-deficient mice develop multiple liver tumors. Genes Dev. 2011;25(8):795-800.
59. Nakagawa H, Umemura A, Taniguchi K, Font-Burgada J, Dhar D, Ogata H, Zhong Z, Valasek MA, Seki E, Hidalgo J, Koike K, Kaufman RJ, Karin M. ER stress cooperates with hypernutrition to trigger TNF-dependent spontaneous HCC development. Cancer Cell. 2014;26(3):331-43.
60. Ringelhan M, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. Nat Immunol. 2018;19(3):222-32.
61. Masarone M, Rosato V, Dallio M, Gravina AG, Aglitti A, Loguercio C, Federico A, Persico M. Role of Oxidative Stress in Pathophysiology of Nonalcoholic Fatty Liver Disease. Oxid Med Cell Longev. 2018;2018:9547613.
62. Ohnishi S, Ma N, Thanan R, Pinlaor S, Hammam O, Murata M, Kawanishi S. DNA damage in inflammation-related carcinogenesis and cancer stem cells. Oxid Med Cell Longev. 2013;2013:387014.
63. Anstee QM, Reeves HL, Kotsiliti E, Govaere O, Heikenwalder M. From NASH to HCC: current concepts and future challenges. Nat Rev Gastroenterol Hepatol. 2019;16(7):411-28.
64. Jameson JL, Longo DL. Precision medicine--personalized, problematic, and promising. N Engl J Med. 2015;372(23):2229-34.
65. Collins FS, Varmus H. A new initiative on precision medicine. N Engl J Med. 2015;372(9):793-5.
66. Miller VM, Rocca WA, Faubion SS. Sex Differences Research, Precision Medicine, and the Future of Women's Health. J Womens Health (Larchmt). 2015;24(12):969-71.
67. Schumacher Dimech A, Ferretti MT, Sandset EC, Santuccione Chadha A. The role of sex and gender differences in precision medicine: the work of the Women's Brain Project. Eur Heart J. 2021;42(34):3215-7.
68. Lazo M, Hernaez R, Eberhardt MS, Bonekamp S, Kamel I, Guallar E, Koteish A, Brancati FL, Clark JM. Prevalence of nonalcoholic fatty liver disease in the United

States: the Third National Health and Nutrition Examination Survey, 1988-1994. Am J Epidemiol. 2013;178(1):38-45.

69. Ballestri S, Nascimbeni F, Baldelli E, Marrazzo A, Romagnoli D, Lonardo A. NAFLD as a Sexual Dimorphic Disease: Role of Gender and Reproductive Status in the Development and Progression of Nonalcoholic Fatty Liver Disease and Inherent Cardiovascular Risk. Adv Ther. 2017;34(6):1291-326.
70. Anderson EL, Howe LD, Jones HE, Higgins JP, Lawlor DA, Fraser A. The Prevalence of Non-Alcoholic Fatty Liver Disease in Children and Adolescents: A Systematic Review and Meta-Analysis. PLoS One. 2015;10(10):e0140908.
71. DiStefano JK. NAFLD and NASH in Postmenopausal Women: Implications for Diagnosis and Treatment. Endocrinology. 2020;161(10).
72. Lonardo A, Trande P. Are there any sex differences in fatty liver? A study of glucose metabolism and body fat distribution. J Gastroenterol Hepatol. 2000;15(7):775-82.
73. Link JC, Reue K. Genetic Basis for Sex Differences in Obesity and Lipid Metabolism. Annu Rev Nutr. 2017;37:225-45.
74. Corrigan EC, Nelson LM, Bakalov VK, Yanovski JA, Vanderhoof VH, Yanoff LB, Bondy CA. Effects of ovarian failure and X-chromosome deletion on body composition and insulin sensitivity in young women. Menopause. 2006;13(6):911-6.
75. Virani SS, Alonso A, Aparicio HJ, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, Chamberlain AM, Cheng S, Delling FN, Elkind MSV, Evenson KR, Ferguson JF, Gupta DK, Khan SS, Kissela BM, Knutson KL, Lee CD, Lewis TT, Liu J, Loop MS, Lutsey PL, Ma J, Mackey J, Martin SS, Matchar DB, Mussolino ME, Navaneethan SD, Perak AM, Roth GA, Samad Z, Satou GM, Schroeder EB, Shah SH, Shay CM, Stokes A, VanWagner LB, Wang NY, Tsao CW. Heart Disease and Stroke Statistics-2021 Update: A Report From the American Heart Association. Circulation. 2021;143(8):e254-e743.
76. Lu KT, Keen HL, Weatherford ET, Sequeira-Lopez ML, Gomez RA, Sigmund CD. Estrogen Receptor α Is Required for Maintaining Baseline Renin Expression. Hypertension. 2016;67(5):992-9.

77. Mirabito KM, Hilliard LM, Head GA, Widdop RE, Denton KM. Pressor responsiveness to angiotensin II in female mice is enhanced with age: role of the angiotensin type 2 receptor. Biol Sex Differ. 2014;5:13.
78. Kamari Y, Shaish A, Vax E, Shemesh S, Kandel-Kfir M, Arbel Y, Olteanu S, Barshack I, Dotan S, Voronov E, Dinarello CA, Apte RN, Harats D. Lack of interleukin-1α or interleukin-1β inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. J Hepatol. 2011;55(5):1086-94.
79. Cooper AJ, Gupta SR, Moustafa AF, Chao AM. Sex/Gender Differences in Obesity Prevalence, Comorbidities, and Treatment. Curr Obes Rep. 2021;10(4):458-66.
80. Tarantino G, Finelli C. Pathogenesis of hepatic steatosis: the link between hypercortisolism and non-alcoholic fatty liver disease. World J Gastroenterol. 2013;19(40):6735-43.
81. Candia R, Riquelme A, Baudrand R, Carvajal CA, Morales M, Solís N, Pizarro M, Escalona A, Carrasco G, Boza C, Pérez G, Padilla O, Cerda J, Fardella CE, Arrese M. Overexpression of 11β-hydroxysteroid dehydrogenase type 1 in visceral adipose tissue and portal hypercortisolism in non-alcoholic fatty liver disease. Liver Int. 2012;32(3):392-9.
82. Dakin RS, Walker BR, Seckl JR, Hadoke PW, Drake AJ. Estrogens protect male mice from obesity complications and influence glucocorticoid metabolism. Int J Obes (Lond). 2015;39(10):1539-47.
83. Fried SK, Lee MJ, Karastergiou K. Shaping fat distribution: New insights into the molecular determinants of depot- and sex-dependent adipose biology. Obesity (Silver Spring). 2015;23(7):1345-52.
84. Snijder MB, Dekker JM, Visser M, Yudkin JS, Stehouwer CD, Bouter LM, Heine RJ, Nijpels G, Seidell JC. Larger thigh and hip circumferences are associated with better glucose tolerance: the Hoorn study. Obes Res. 2003;11(1):104-11.
85. Hocking SL, Wu LE, Guilhaus M, Chisholm DJ, James DE. Intrinsic depot-specific differences in the secretome of adipose tissue, preadipocytes, and adipose tissue-derived microvascular endothelial cells. Diabetes. 2010;59(12):3008-16.

86. Pedersen SB, Kristensen K, Hermann PA, Katzenellenbogen JA, Richelsen B. Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution. J Clin Endocrinol Metab. 2004;89(4):1869-78.
87. Park YM, Pereira RI, Erickson CB, Swibas TA, Cox-York KA, Van Pelt RE. Estradiol-mediated improvements in adipose tissue insulin sensitivity are related to the balance of adipose tissue estrogen receptor α and β in postmenopausal women. PLoS One. 2017;12(5):e0176446.
88. Matsuo K, Gualtieri MR, Cahoon SS, Jung CE, Paulson RJ, Shoupe D, Muderspach LI, Wakatsuki A, Wright JD, Roman LD. Surgical menopause and increased risk of nonalcoholic fatty liver disease in endometrial cancer. Menopause. 2016;23(2):189-96.
89. Bambha K, Belt P, Abraham M, Wilson LA, Pabst M, Ferrell L, Unalp-Arida A, Bass N. Ethnicity and nonalcoholic fatty liver disease. Hepatology. 2012;55(3):769-80.
90. Paik JM, Henry L, De Avila L, Younossi E, Racila A, Younossi ZM. Mortality Related to Nonalcoholic Fatty Liver Disease Is Increasing in the United States. Hepatol Commun. 2019;3(11):1459-71.
91. Hamaguchi M, Kojima T, Ohbora A, Takeda N, Fukui M, Kato T. Aging is a risk factor of nonalcoholic fatty liver disease in premenopausal women. World J Gastroenterol. 2012;18(3):237-43.
92. Klair JS, Yang JD, Abdelmalek MF, Guy CD, Gill RM, Yates K, Unalp-Arida A, Lavine JE, Clark JM, Diehl AM, Suzuki A. A longer duration of estrogen deficiency increases fibrosis risk among postmenopausal women with nonalcoholic fatty liver disease. Hepatology. 2016;64(1):85-91.
93. Turola E, Petta S, Vanni E, Milosa F, Valenti L, Critelli R, Miele L, Maccio L, Calvaruso V, Fracanzani AL, Bianchini M, Raos N, Bugianesi E, Mercorella S, Di Giovanni M, Craxì A, Fargion S, Grieco A, Cammà C, Cotelli F, Villa E. Ovarian senescence increases liver fibrosis in humans and zebrafish with steatosis. Dis Model Mech. 2015;8(9):1037-46.

94. Chung GE, Yim JY, Kim D, Lim SH, Yang JI, Kim YS, Yang SY, Kwak MS, Kim JS, Cho SH. The influence of metabolic factors for nonalcoholic Fatty liver disease in women. Biomed Res Int. 2015;2015:131528.
95. McKenzie J, Fisher BM, Jaap AJ, Stanley A, Paterson K, Sattar N. Effects of HRT on liver enzyme levels in women with type 2 diabetes: a randomized placebo-controlled trial. Clin Endocrinol (Oxf). 2006;65(1):40-4.
96. Yang JD, Abdelmalek MF, Guy CD, Gill RM, Lavine JE, Yates K, Klair J, Terrault NA, Clark JM, Unalp-Arida A, Diehl AM, Suzuki A. Patient Sex, Reproductive Status, and Synthetic Hormone Use Associate With Histologic Severity of Nonalcoholic Steatohepatitis. Clin Gastroenterol Hepatol. 2017;15(1):127-31.e2.
97. Khan D, Ansar Ahmed S. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. Front Immunol. 2015;6:635.
98. Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. Annu Rev Nutr. 2006;26:229-50.
99. Fischer LM, daCosta KA, Kwock L, Stewart PW, Lu TS, Stabler SP, Allen RH, Zeisel SH. Sex and menopausal status influence human dietary requirements for the nutrient choline. Am J Clin Nutr. 2007;85(5):1275-85.
100. Clegg D, Hevener AL, Moreau KL, Morselli E, Criollo A, Van Pelt RE, Vieira-Potter VJ. Sex Hormones and Cardiometabolic Health: Role of Estrogen and Estrogen Receptors. Endocrinology. 2017;158(5):1095-105.
101. Perreault L, Bergman BC, Hunerdosse DM, Eckel RH. Altered intramuscular lipid metabolism relates to diminished insulin action in men, but not women, in progression to diabetes. Obesity (Silver Spring). 2010;18(11):2093-100.
102. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: it's not just about sex hormones. J Clin Endocrinol Metab. 2011;96(4):885-93.
103. Reaven GM, Bernstein RM. Effect of obesity on the relationship between very low density lipoprotein production rate and plasma triglyceride concentration in normal and hypertriglyceridemic subjects. Metabolism. 1978;27(9):1047-54.

104. Palmisano BT, Zhu L, Eckel RH, Stafford JM. Sex differences in lipid and lipoprotein metabolism. Mol Metab. 2018;15:45-55.
105. Ferri N, Ruscica M, Coggi D, Bonomi A, Amato M, Frigerio B, Sansaro D, Ravani A, Veglia F, Capra N, Lupo MG, Macchi C, Castelnuovo S, Savonen K, Silveira A, Kurl S, Giral P, Pirro M, Strawbridge RJ, Gigante B, Smit AJ, Tremoli E, Colombo GI, Baldassarre D. Sex-specific predictors of PCSK9 levels in a European population: The IMPROVE study. Atherosclerosis. 2020;309:39-46.
106. Levenson AE, Shah AS, Khoury PR, Kimball TR, Urbina EM, de Ferranti SD, Maahs DM, Dolan LM, Wadwa RP, Biddinger SB. Obesity and type 2 diabetes are associated with elevated PCSK9 levels in young women. Pediatr Diabetes. 2017;18(8):755-60.
107. Ghosh M, Gälman C, Rudling M, Angelin B. Influence of physiological changes in endogenous estrogen on circulating PCSK9 and LDL cholesterol. J Lipid Res. 2015;56(2):463-9.
108. Persson L, Henriksson P, Westerlund E, Hovatta O, Angelin B, Rudling M. Endogenous estrogens lower plasma PCSK9 and LDL cholesterol but not Lp(a) or bile acid synthesis in women. Arterioscler Thromb Vasc Biol. 2012;32(3):810-4.
109. Ruscica M, Ferri N, Macchi C, Meroni M, Lanti C, Ricci C, Maggioni M, Fracanzani AL, Badiali S, Fargion S, Magni P, Valenti L, Dongiovanni P. Liver fat accumulation is associated with circulating PCSK9. Ann Med. 2016;48(5):384-91.
110. Atkins DL, de Caen AR, Berger S, Samson RA, Schexnayder SM, Joyner BL, Jr., Bigham BL, Niles DE, Duff JP, Hunt EA, Meaney PA. 2017 American Heart Association Focused Update on Pediatric Basic Life Support and Cardiopulmonary Resuscitation Quality: An Update to the American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care. Circulation. 2018;137(1):e1-e6.
111. VanWagner LB, Wilcox JE, Colangelo LA, Lloyd-Jones DM, Carr JJ, Lima JA, Lewis CE, Rinella ME, Shah SJ. Association of nonalcoholic fatty liver disease with subclinical myocardial remodeling and dysfunction: A population-based study. Hepatology. 2015;62(3):773-83.

112. Salah HM, Pandey A, Soloveva A, Abdelmalek MF, Diehl AM, Moylan CA, Wegermann K, Rao VN, Hernandez AF, Tedford RJ, Parikh KS, Mentz RJ, McGarrah RW, Fudim M. Relationship of Nonalcoholic Fatty Liver Disease and Heart Failure With Preserved Ejection Fraction. JACC Basic Transl Sci. 2021;6(11):918-32.
113. Younossi ZM, Tampi R, Priyadarshini M, Nader F, Younossi IM, Racila A. Burden of Illness and Economic Model for Patients With Nonalcoholic Steatohepatitis in the United States. Hepatology. 2019;69(2):564-72.
114. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med. 2010;362(18):1675-85.
115. Kubota Y, Iso H, Date C, Kikuchi S, Watanabe Y, Wada Y, Inaba Y, Tamakoshi A. Dietary intakes of antioxidant vitamins and mortality from cardiovascular disease: the Japan Collaborative Cohort Study (JACC) study. Stroke. 2011;42(6):1665-72.
116. Klein EA, Thompson IM, Jr., Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, Gaziano JM, Karp DD, Lieber MM, Walther PJ, Klotz L, Parsons JK, Chin JL, Darke AK, Lippman SM, Goodman GE, Meyskens FL, Jr., Baker LH. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). JAMA. 2011;306(14):1549-56.
117. Tang H, Shi W, Fu S, Wang T, Zhai S, Song Y, Han J. Pioglitazone and bladder cancer risk: a systematic review and meta-analysis. Cancer Med. 2018;7(4):1070-80.
118. Vuppalanchi R, Noureddin M, Alkhouri N, Sanyal AJ. Therapeutic pipeline in nonalcoholic steatohepatitis. Nat Rev Gastroenterol Hepatol. 2021;18(6):373-92.
119. Kim CW, Addy C, Kusunoki J, Anderson NN, Deja S, Fu X, Burgess SC, Li C, Ruddy M, Chakravarthy M, Previs S, Milstein S, Fitzgerald K, Kelley DE, Horton JD. Acetyl CoA Carboxylase Inhibition Reduces Hepatic Steatosis but Elevates Plasma Triglycerides in Mice and Humans: A Bedside to Bench Investigation. Cell Metab. 2017;26(2):394-406.e6.

120. Huard K, Smith AC, Cappon G, Dow RL, Edmonds DJ, El-Kattan A, Esler WP, Fernando DP, Griffith DA, Kalgutkar AS, Ross TT, Bagley SW, Beebe D, Bi YA, Cabral S, Crowley C, Doran SD, Dowling MS, Liras S, Mascitti V, Niosi M, Pfefferkorn JA, Polivkova J, Préville C, Price DA, Shavnya A, Shirai N, Smith AH, Southers JR, Tess DA, Thuma BA, Varma MV, Yang X. Optimizing the Benefit/Risk of Acetyl-CoA Carboxylase Inhibitors through Liver Targeting. J Med Chem. 2020;63(19):10879-96.
121. Stiede K, Miao W, Blanchette HS, Beysen C, Harriman G, Harwood HJ, Jr., Kelley H, Kapeller R, Schmalbach T, Westlin WF. Acetyl-coenzyme A carboxylase inhibition reduces de novo lipogenesis in overweight male subjects: A randomized, double-blind, crossover study. Hepatology. 2017;66(2):324-34.
122. Syed-Abdul MM, Parks EJ, Gaballah AH, Bingham K, Hammoud GM, Kemble G, Buckley D, McCulloch W, Manrique-Acevedo C. Fatty Acid Synthase Inhibitor TVB-2640 Reduces Hepatic de Novo Lipogenesis in Males With Metabolic Abnormalities. Hepatology. 2020;72(1):103-18.
123. Loomba R, Mohseni R, Lucas KJ, Gutierrez JA, Perry RG, Trotter JF, Rahimi RS, Harrison SA, Ajmera V, Wayne JD, O'Farrell M, McCulloch W, Grimmer K, Rinella M, Wai-Sun Wong V, Ratziu V, Gores GJ, Neuschwander-Tetri BA, Kemble G. TVB-2640 (FASN Inhibitor) for the Treatment of Nonalcoholic Steatohepatitis: FASCINATE-1, a Randomized, Placebo-Controlled Phase 2a Trial. Gastroenterology. 2021;161(5):1475-86.
124. Beysen C, Schroeder P, Wu E, Brevard J, Ribadeneira M, Lu W, Dole K, O'Reilly T, Morrow L, Hompesch M, Hellerstein MK, Li K, Johansson L, Kelly PF. Inhibition of fatty acid synthase with FT-4101 safely reduces hepatic de novo lipogenesis and steatosis in obese subjects with non-alcoholic fatty liver disease: Results from two early-phase randomized trials. Diabetes Obes Metab. 2021;23(3):700-10.
125. Harrison SA, Fecht W, Brunt EM, Neuschwander-Tetri BA. Orlistat for overweight subjects with nonalcoholic steatohepatitis: A randomized, prospective trial. Hepatology. 2009;49(1):80-6.
126. Safadi R, Konikoff FM, Mahamid M, Zelber-Sagi S, Halpern M, Gilat T, Oren R. The fatty acid-bile acid conjugate Aramchol reduces liver fat content in patients

with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2014;12(12):2085-91.e1.

127. Ratziu V, de Guevara L, Safadi R, Poordad F, Fuster F, Flores-Figueroa J, Arrese M, Fracanzani AL, Ben Bashat D, Lackner K, Gorfine T, Kadosh S, Oren R, Halperin M, Hayardeny L, Loomba R, Friedman S, Sanyal AJ. Aramchol in patients with nonalcoholic steatohepatitis: a randomized, double-blind, placebo-controlled phase 2b trial. Nat Med. 2021;27(10):1825-35.

128. Loomba R, Morgan E, Watts L, Xia S, Hannan LA, Geary RS, Baker BF, Bhanot S. Novel antisense inhibition of diacylglycerol O-acyltransferase 2 for treatment of non-alcoholic fatty liver disease: a multicentre, double-blind, randomised, placebo-controlled phase 2 trial. Lancet Gastroenterol Hepatol. 2020;5(9):829-38.

129. Saxena A CKSVOADS. Diacylglycerol acyltransferase 2 (DGAT2) inhibitor PF-06865571 reduces liver fat by MRI-PDFF after 2 weeks in adults with NAFLD | Cochrane Library. Hepatology. 2019;70:1260A-.

130. Kimura Y, Hyogo H, Yamagishi S, Takeuchi M, Ishitobi T, Nabeshima Y, Arihiro K, Chayama K. Atorvastatin decreases serum levels of advanced glycation endproducts (AGEs) in nonalcoholic steatohepatitis (NASH) patients with dyslipidemia: clinical usefulness of AGEs as a biomarker for the attenuation of NASH. J Gastroenterol. 2010;45(7):750-7.

131. Fouda A, Abdelaziz AE, Hussien M, Ali AA, Abdelkawy KS, Elbarbry F. A randomized controlled trial comparing the effects of Vitamin E, Ursodeoxycholic acid and Pentoxifylline on Egyptian non-alcoholic steatohepatitis patients. Eur Rev Med Pharmacol Sci. 2021;25(23):7449-59.

132. Nadinskaia M, Maevskaya M, Ivashkin V, Kodzoeva K, Pirogova I, Chesnokov E, Nersesov A, Kaibullayeva J, Konysbekova A, Raissova A, Khamrabaeva F, Zueva E. Ursodeoxycholic acid as a means of preventing atherosclerosis, steatosis and liver fibrosis in patients with nonalcoholic fatty liver disease. World J Gastroenterol. 2021;27(10):959-75.

133. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarathy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-

alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet. 2015;385(9972):956-65.

134. Younossi ZM, Ratziu V, Loomba R, Rinella M, Anstee QM, Goodman Z, Bedossa P, Geier A, Beckebaum S, Newsome PN, Sheridan D, Sheikh MY, Trotter J, Knapple W, Lawitz E, Abdelmalek MF, Kowdley KV, Montano-Loza AJ, Boursier J, Mathurin P, Bugianesi E, Mazzella G, Olveira A, Cortez-Pinto H, Graupera I, Orr D, Gluud LL, Dufour JF, Shapiro D, Campagna J, Zaru L, MacConell L, Shringarpure R, Harrison S, Sanyal AJ. Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. Lancet. 2019;394(10215):2184-96.

135. Mudaliar S, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, Adorini L, Sciacca CI, Clopton P, Castelloe E, Dillon P, Pruzanski M, Shapiro D. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. Gastroenterology. 2013;145(3):574-82.e1.

136. Ratziu V, Rinella ME, Neuschwander-Tetri BA, Lawitz E, Denham D, Kayali Z, Sheikh A, Kowdley KV, Desta T, Elkhashab M, DeGrauw J, Goodwin B, Ahmad A, Adda N. EDP-305 in patients with NASH: A phase II double-blind placebo-controlled dose-ranging study. J Hepatol. 2022;76(3):506-17.

137. Patel K, Harrison SA, Elkhashab M, Trotter JF, Herring R, Rojter SE, Kayali Z, Wong VW, Greenbloom S, Jayakumar S, Shiffman ML, Freilich B, Lawitz EJ, Gane EJ, Harting E, Xu J, Billin AN, Chung C, Djedjos CS, Subramanian GM, Myers RP, Middleton MS, Rinella M, Noureddin M. Cilofexor, a Nonsteroidal FXR Agonist, in Patients With Noncirrhotic NASH: A Phase 2 Randomized Controlled Trial. Hepatology. 2020;72(1):58-71.

138. Tully DC, Rucker PV, Chianelli D, Williams J, Vidal A, Alper PB, Mutnick D, Bursulaya B, Schmeits J, Wu X, Bao D, Zoll J, Kim Y, Groessl T, McNamara P, Seidel HM, Molteni V, Liu B, Phimister A, Joseph SB, Laffitte B. Discovery of Tropifexor (LJN452), a Highly Potent Non-bile Acid FXR Agonist for the Treatment of Cholestatic Liver Diseases and Nonalcoholic Steatohepatitis (NASH). J Med Chem. 2017;60(24):9960-73.

139. Harrison SA, Bashir MR, Lee KJ, Shim-Lopez J, Lee J, Wagner B, Smith ND, Chen HC, Lawitz EJ. A structurally optimized FXR agonist, MET409, reduced liver fat content over 12 weeks in patients with non-alcoholic steatohepatitis. J Hepatol. 2021;75(1):25-33.
140. Stefan N, Ramsauer M, Jordan P, Nowotny B, Kantartzis K, Machann J, Hwang JH, Nowotny P, Kahl S, Harreiter J, Hornemann S, Sanyal AJ, Stewart PM, Pfeiffer AF, Kautzky-Willer A, Roden M, Häring HU, Fürst-Recktenwald S. Inhibition of 11β-HSD1 with RO5093151 for non-alcoholic fatty liver disease: a multicentre, randomised, double-blind, placebo-controlled trial. Lancet Diabetes Endocrinol. 2014;2(5):406-16.
141. Harrison SA, Bashir MR, Guy CD, Zhou R, Moylan CA, Frias JP, Alkhouri N, Bansal MB, Baum S, Neuschwander-Tetri BA, Taub R, Moussa SE. Resmetirom (MGL-3196) for the treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. Lancet. 2019;394(10213):2012-24.
142. Harrison SA, Bashir M, Moussa SE, McCarty K, Pablo Frias J, Taub R, Alkhouri N. Effects of Resmetirom on Noninvasive Endpoints in a 36-Week Phase 2 Active Treatment Extension Study in Patients With NASH. Hepatol Commun. 2021;5(4):573-88.
143. Loomba R NJMRBDSRDMSSMCHKTBMMMHLB. LBP-20-VK2809, a Novel Liver-Directed Thyroid Receptor Beta Agonist, Significantly Reduces Liver Fat with Both Low and High Doses in Patients with Non-Alcoholic Fatty Liver Disease: A Phase 2 Randomized, Placebo-Controlled Trial. J Hepatol. 2019;70:141-382.
144. Harrison SA, Rinella ME, Abdelmalek MF, Trotter JF, Paredes AH, Arnold HL, Kugelmas M, Bashir MR, Jaros MJ, Ling L, Rossi SJ, DePaoli AM, Loomba R. NGM282 for treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. Lancet. 2018;391(10126):1174-85.
145. Harrison SA, Neff G, Guy CD, Bashir MR, Paredes AH, Frias JP, Younes Z, Trotter JF, Gunn NT, Moussa SE, Kohli A, Nelson K, Gottwald M, Chang WCG, Yan AZ, DePaoli AM, Ling L, Lieu HD. Efficacy and Safety of Aldafermin, an

Engineered FGF19 Analog, in a Randomized, Double-Blind, Placebo-Controlled Trial of Patients With Nonalcoholic Steatohepatitis. Gastroenterology. 2021;160(1):219-31.e1.

146. Sanyal A, Charles ED, Neuschwander-Tetri BA, Loomba R, Harrison SA, Abdelmalek MF, Lawitz EJ, Halegoua-DeMarzio D, Kundu S, Noviello S, Luo Y, Christian R. Pegbelfermin (BMS-986036), a PEGylated fibroblast growth factor 21 analogue, in patients with non-alcoholic steatohepatitis: a randomised, double-blind, placebo-controlled, phase 2a trial. Lancet. 2019;392(10165):2705-17.

147. Harrison SA, Ruane PJ, Freilich BL, Neff G, Patil R, Behling CA, Hu C, Fong E, de Temple B, Tillman EJ, Rolph TP, Cheng A, Yale K. Efruxifermin in non-alcoholic steatohepatitis: a randomized, double-blind, placebo-controlled, phase 2a trial. Nat Med. 2021;27(7):1262-71.

148. Nakajima A, Eguchi Y, Yoneda M, Imajo K, Tamaki N, Suganami H, Nojima T, Tanigawa R, Iizuka M, Iida Y, Loomba R. Randomised clinical trial: Pemafibrate, a novel selective peroxisome proliferator-activated receptor α modulator (SPPARMα), versus placebo in patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 2021;54(10):1263-77.

149. Olson EJ, Pearce GL, Jones NP, Sprecher DL. Lipid effects of peroxisome proliferator-activated receptor-δ agonist GW501516 in subjects with low high-density lipoprotein cholesterol: characteristics of metabolic syndrome. Arterioscler Thromb Vasc Biol. 2012;32(9):2289-94.

150. Risérus U, Sprecher D, Johnson T, Olson E, Hirschberg S, Liu A, Fang Z, Hegde P, Richards D, Sarov-Blat L, Strum JC, Basu S, Cheeseman J, Fielding BA, Humphreys SM, Danoff T, Moore NR, Murgatroyd P, O'Rahilly S, Sutton P, Willson T, Hassall D, Frayn KN, Karpe F. Activation of peroxisome proliferator-activated receptor (PPAR)delta promotes reversal of multiple metabolic abnormalities, reduces oxidative stress, and increases fatty acid oxidation in moderately obese men. Diabetes. 2008;57(2):332-9.

151. Cariou B, Hanf R, Lambert-Porcheron S, Zaïr Y, Sauvinet V, Noël B, Flet L, Vidal H, Staels B, Laville M. Dual peroxisome proliferator-activated receptor α/δ

agonist GFT505 improves hepatic and peripheral insulin sensitivity in abdominally obese subjects. Diabetes Care. 2013;36(10):2923-30.

152. Ratziu V, Harrison SA, Francque S, Bedossa P, Lehert P, Serfaty L, Romero-Gomez M, Boursier J, Abdelmalek M, Caldwell S, Drenth J, Anstee QM, Hum D, Hanf R, Roudot A, Megnien S, Staels B, Sanyal A. Elafibranor, an Agonist of the Peroxisome Proliferator-Activated Receptor-α and -δ, Induces Resolution of Nonalcoholic Steatohepatitis Without Fibrosis Worsening. Gastroenterology. 2016;150(5):1147-59.e5.

153. Gawrieh S, Noureddin M, Loo N, Mohseni R, Awasty V, Cusi K, Kowdley KV, Lai M, Schiff E, Parmar D, Patel P, Chalasani N. Saroglitazar, a PPAR-α/γ Agonist, for Treatment of NAFLD: A Randomized Controlled Double-Blind Phase 2 Trial. Hepatology. 2021;74(4):1809-24.

154. Kaul U, Parmar D, Manjunath K, Shah M, Parmar K, Patil KP, Jaiswal A. New dual peroxisome proliferator activated receptor agonist-Saroglitazar in diabetic dyslipidemia and non-alcoholic fatty liver disease: integrated analysis of the real world evidence. Cardiovasc Diabetol. 2019;18(1):80.

155. Cusi K, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, Tio F, Hardies J, Darland C, Musi N, Webb A, Portillo-Sanchez P. Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. Ann Intern Med. 2016;165(5):305-15.

156. Bril F, Kalavalapalli S, Clark VC, Lomonaco R, Soldevila-Pico C, Liu IC, Orsak B, Tio F, Cusi K. Response to Pioglitazone in Patients With Nonalcoholic Steatohepatitis With vs Without Type 2 Diabetes. Clin Gastroenterol Hepatol. 2018;16(4):558-66.e2.

157. Francque SM, Bedossa P, Ratziu V, Anstee QM, Bugianesi E, Sanyal AJ, Loomba R, Harrison SA, Balabanska R, Mateva L, Lanthier N, Alkhouri N, Moreno C, Schattenberg JM, Stefanova-Petrova D, Vonghia L, Rouzier R, Guillaume M, Hodge A, Romero-Gómez M, Huot-Marchand P, Baudin M, Richard MP, Abitbol JL, Broqua P, Junien JL, Abdelmalek MF. A Randomized, Controlled Trial of the Pan-PPAR Agonist Lanifibranor in NASH. N Engl J Med. 2021;385(17):1547-58.

158. Buse JB, Klonoff DC, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, Maggs DG, Wintle ME. 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. 2007;29(1):139-53.
159. Shao N, Kuang HY, Hao M, Gao XY, Lin WJ, Zou W. Benefits of exenatide on obesity and non-alcoholic fatty liver disease with elevated liver enzymes in patients with type 2 diabetes. Diabetes Metab Res Rev. 2014;30(6):521-9.
160. Dutour A, Abdesselam I, Ancel P, Kober F, Mrad G, Darmon P, Ronsin O, Pradel V, Lesavre N, Martin JC, Jacquier A, Lefur Y, Bernard M, Gaborit B. Exenatide decreases liver fat content and epicardial adipose tissue in patients with obesity and type 2 diabetes: a prospective randomized clinical trial using magnetic resonance imaging and spectroscopy. Diabetes Obes Metab. 2016;18(9):882-91.
161. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K, Abouda G, Aldersley MA, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hübscher SG, Newsome PN. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet. 2016;387(10019):679-90.
162. Petit JM, Cercueil JP, Loffroy R, Denimal D, Bouillet B, Fourmont C, Chevallier O, Duvillard L, Vergès B. Effect of Liraglutide Therapy on Liver Fat Content in Patients With Inadequately Controlled Type 2 Diabetes: The Lira-NAFLD Study. J Clin Endocrinol Metab. 2017;102(2):407-15.
163. Khoo J, Hsiang JC, Taneja R, Koo SH, Soon GH, Kam CJ, Law NM, Ang TL. Randomized trial comparing effects of weight loss by liraglutide with lifestyle modification in non-alcoholic fatty liver disease. Liver Int. 2019;39(5):941-9.
164. Newsome PN, Buchholtz K, Cusi K, Linder M, Okanoue T, Ratziu V, Sanyal AJ, Sejling AS, Harrison SA. A Placebo-Controlled Trial of Subcutaneous Semaglutide in Nonalcoholic Steatohepatitis. N Engl J Med. 2021;384(12):1113-24.
165. Harrison SA, Calanna S, Cusi K, Linder M, Okanoue T, Ratziu V, Sanyal A, Sejling AS, Newsome PN. Semaglutide for the treatment of non-alcoholic

steatohepatitis: Trial design and comparison of non-invasive biomarkers. Contemp Clin Trials. 2020;97:106174.

166. Newsome P, Francque S, Harrison S, Ratziu V, Van Gaal L, Calanna S, Hansen M, Linder M, Sanyal A. Effect of semaglutide on liver enzymes and markers of inflammation in subjects with type 2 diabetes and/or obesity. Aliment Pharmacol Ther. 2019;50(2):193-203.

167. O'Neil PM, Birkenfeld AL, McGowan B, Mosenzon O, Pedersen SD, Wharton S, Carson CG, Jepsen CH, Kabisch M, Wilding JPH. Efficacy and safety of semaglutide compared with liraglutide and placebo for weight loss in patients with obesity: a randomised, double-blind, placebo and active controlled, dose-ranging, phase 2 trial. Lancet. 2018;392(10148):637-49.

168. Hartman ML, Sanyal AJ, Loomba R, Wilson JM, Nikooienejad A, Bray R, Karanikas CA, Duffin KL, Robins DA, Haupt A. Effects of Novel Dual GIP and GLP-1 Receptor Agonist Tirzepatide on Biomarkers of Nonalcoholic Steatohepatitis in Patients With Type 2 Diabetes. Diabetes Care. 2020;43(6):1352-5.

169. Pirro V, Roth KD, Lin Y, Willency JA, Milligan PL, Wilson JM, Ruotolo G, Haupt A, Newgard CB, Duffin KL. Effects of Tirzepatide, a Dual GIP and GLP-1 RA, on Lipid and Metabolite Profiles in Subjects With Type 2 Diabetes. J Clin Endocrinol Metab. 2022;107(2):363-78.

170. Nahra R, Wang T, Gadde KM, Oscarsson J, Stumvoll M, Jermutus L, Hirshberg B, Ambery P. Effects of Cotadutide on Metabolic and Hepatic Parameters in Adults With Overweight or Obesity and Type 2 Diabetes: A 54-Week Randomized Phase 2b Study. Diabetes Care. 2021;44(6):1433-42.

171. Cui J, Philo L, Nguyen P, Hofflich H, Hernandez C, Bettencourt R, Richards L, Salotti J, Bhatt A, Hooker J, Haufe W, Hooker C, Brenner DA, Sirlin CB, Loomba R. Sitagliptin vs. placebo for non-alcoholic fatty liver disease: A randomized controlled trial. J Hepatol. 2016;65(2):369-76.

172. Joy TR, McKenzie CA, Tirona RG, Summers K, Seney S, Chakrabarti S, Malhotra N, Beaton MD. Sitagliptin in patients with non-alcoholic steatohepatitis: A randomized, placebo-controlled trial. World J Gastroenterol. 2017;23(1):141-50.

173. Alam S, Ghosh J, Mustafa G, Kamal M, Ahmad N. Effect of sitagliptin on hepatic histological activity and fibrosis of nonalcoholic steatohepatitis patients: a 1-year randomized control trial. Hepat Med. 2018;10:23-31.
174. Kahl S, Gancheva S, Straßburger K, Herder C, Machann J, Katsuyama H, Kabisch S, Henkel E, Kopf S, Lagerpusch M, Kantartzis K, Kupriyanova Y, Markgraf D, van Gemert T, Knebel B, Wolkersdorfer MF, Kuss O, Hwang JH, Bornstein SR, Kasperk C, Stefan N, Pfeiffer A, Birkenfeld AL, Roden M. Empagliflozin Effectively Lowers Liver Fat Content in Well-Controlled Type 2 Diabetes: A Randomized, Double-Blind, Phase 4, Placebo-Controlled Trial. Diabetes Care. 2020;43(2):298-305.
175. Kuchay MS, Krishan S, Mishra SK, Farooqui KJ, Singh MK, Wasir JS, Bansal B, Kaur P, Jevalikar G, Gill HK, Choudhary NS, Mithal A. Effect of Empagliflozin on Liver Fat in Patients With Type 2 Diabetes and Nonalcoholic Fatty Liver Disease: A Randomized Controlled Trial (E-LIFT Trial). Diabetes Care. 2018;41(8):1801-8.
176. Pokharel A, Kc S, Thapa P, Karki N, Shrestha R, Jaishi B, Paudel MS. The Effect of Empagliflozin on Liver Fat in Type 2 Diabetes Mellitus Patients With Non-Alcoholic Fatty Liver Disease. Cureus. 2021;13(7):e16687.
177. Chehrehgosha H, Sohrabi MR, Ismail-Beigi F, Malek M, Reza Babaei M, Zamani F, Ajdarkosh H, Khoonsari M, Fallah AE, Khamseh ME. Empagliflozin Improves Liver Steatosis and Fibrosis in Patients with Non-Alcoholic Fatty Liver Disease and Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial. Diabetes Ther. 2021;12(3):843-61.
178. Lai LL, Vethakkan SR, Nik Mustapha NR, Mahadeva S, Chan WK. Empagliflozin for the Treatment of Nonalcoholic Steatohepatitis in Patients with Type 2 Diabetes Mellitus. Dig Dis Sci. 2020;65(2):623-31.
179. Latva-Rasku A, Honka MJ, Kullberg J, Mononen N, Lehtimäki T, Saltevo J, Kirjavainen AK, Saunavaara V, Iozzo P, Johansson L, Oscarsson J, Hannukainen JC, Nuutila P. The SGLT2 Inhibitor Dapagliflozin Reduces Liver Fat but Does Not Affect Tissue Insulin Sensitivity: A Randomized, Double-Blind, Placebo-Controlled Study With 8-Week Treatment in Type 2 Diabetes Patients. Diabetes Care. 2019;42(5):931-7.

180. Aso Y, Kato K, Sakurai S, Kishi H, Shimizu M, Jojima T, Iijima T, Maejima Y, Shimomura K, Usui I. Impact of dapagliflozin, an SGLT2 inhibitor, on serum levels of soluble dipeptidyl peptidase-4 in patients with type 2 diabetes and non-alcoholic fatty liver disease. Int J Clin Pract. 2019;73(5):e13335.
181. Tobita H, Sato S, Miyake T, Ishihara S, Kinoshita Y. Effects of Dapagliflozin on Body Composition and Liver Tests in Patients with Nonalcoholic Steatohepatitis Associated with Type 2 Diabetes Mellitus: A Prospective, Open-label, Uncontrolled Study. Curr Ther Res Clin Exp. 2017;87:13-9.
182. Cusi K, Bril F, Barb D, Polidori D, Sha S, Ghosh A, Farrell K, Sunny NE, Kalavalapalli S, Pettus J, Ciaraldi TP, Mudaliar S, Henry RR. Effect of canagliflozin treatment on hepatic triglyceride content and glucose metabolism in patients with type 2 diabetes. Diabetes Obes Metab. 2019;21(4):812-21.
183. Seko Y, Nishikawa T, Umemura A, Yamaguchi K, Moriguchi M, Yasui K, Kimura M, Iijima H, Hashimoto T, Sumida Y, Okanoue T, Itoh Y. Efficacy and safety of canagliflozin in type 2 diabetes mellitus patients with biopsy-proven nonalcoholic steatohepatitis classified as stage 1-3 fibrosis. Diabetes Metab Syndr Obes. 2018;11:835-43.
184. Inoue M, Hayashi A, Taguchi T, Arai R, Sasaki S, Takano K, Inoue Y, Shichiri M. Effects of canagliflozin on body composition and hepatic fat content in type 2 diabetes patients with non-alcoholic fatty liver disease. J Diabetes Investig. 2019;10(4):1004-11.
185. Harrison SA, Alkhouri N, Davison BA, Sanyal A, Edwards C, Colca JR, Lee BH, Loomba R, Cusi K, Kolterman O, Cotter G, Dittrich HC. Insulin sensitizer MSDC-0602K in non-alcoholic steatohepatitis: A randomized, double-blind, placebo-controlled phase IIb study. J Hepatol. 2020;72(4):613-26.
186. Calle R BA, Somayaji V, Chidsey K, Kazierad D. PS-110-Ketohexokinase inhibitor PF-06835919 administered for 6 weeks reduces whole liver fat as measured by magnetic resonance imaging-proton density fat fraction in subjects with non-alcoholic fatty liver disease. J Hepatol. 2019;70:69-70.
187. Frenette CT, Morelli G, Shiffman ML, Frederick RT, Rubin RA, Fallon MB, Cheng JT, Cave M, Khaderi SA, Massoud O, Pyrsopoulos N, Park JS, Robinson JM, Yamashita M, Spada AP, Chan JL, Hagerty DT. Emricasan Improves Liver

Function in Patients With Cirrhosis and High Model for End-Stage Liver Disease Scores Compared With Placebo. Clin Gastroenterol Hepatol. 2019;17(4):774-83.e4.

188. Garcia-Tsao G, Bosch J, Kayali Z, Harrison SA, Abdelmalek MF, Lawitz E, Satapathy SK, Ghabril M, Shiffman ML, Younes ZH, Thuluvath PJ, Berzigotti A, Albillos A, Robinson JM, Hagerty DT, Chan JL, Sanyal AJ. Randomized placebo-controlled trial of emricasan for non-alcoholic steatohepatitis-related cirrhosis with severe portal hypertension. J Hepatol. 2020;72(5):885-95.

189. Harrison SA, Goodman Z, Jabbar A, Vemulapalli R, Younes ZH, Freilich B, Sheikh MY, Schattenberg JM, Kayali Z, Zivony A, Sheikh A, Garcia-Samaniego J, Satapathy SK, Therapondos G, Mena E, Schuppan D, Robinson J, Chan JL, Hagerty DT, Sanyal AJ. A randomized, placebo-controlled trial of emricasan in patients with NASH and F1-F3 fibrosis. J Hepatol. 2020;72(5):816-27.

190. Mehta G, Rousell S, Burgess G, Morris M, Wright G, McPherson S, Frenette C, Cave M, Hagerty DT, Spada A, Jalan R. A Placebo-Controlled, Multicenter, Double-Blind, Phase 2 Randomized Trial of the Pan-Caspase Inhibitor Emricasan in Patients with Acutely Decompensated Cirrhosis. J Clin Exp Hepatol. 2018;8(3):224-34.

191. Shiffman M, Freilich B, Vuppalanchi R, Watt K, Chan JL, Spada A, Hagerty DT, Schiff E. Randomised clinical trial: emricasan versus placebo significantly decreases ALT and caspase 3/7 activation in subjects with non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 2019;49(1):64-73.

192. Chalasani N, Abdelmalek MF, Garcia-Tsao G, Vuppalanchi R, Alkhouri N, Rinella M, Noureddin M, Pyko M, Shiffman M, Sanyal A, Allgood A, Shlevin H, Horton R, Zomer E, Irish W, Goodman Z, Harrison SA, Traber PG. Effects of Belapectin, an Inhibitor of Galectin-3, in Patients With Nonalcoholic Steatohepatitis With Cirrhosis and Portal Hypertension. Gastroenterology. 2020;158(5):1334-45.e5.

193. Harrison SA, Marri SR, Chalasani N, Kohli R, Aronstein W, Thompson GA, Irish W, Miles MV, Xanthakos SA, Lawitz E, Noureddin M, Schiano TD, Siddiqui M, Sanyal A, Neuschwander-Tetri BA, Traber PG. Randomised clinical study: GR-MD-02, a galectin-3 inhibitor, vs. placebo in patients having non-alcoholic

steatohepatitis with advanced fibrosis. Aliment Pharmacol Ther. 2016;44(11-12):1183-98.

194. Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, Francque S, Farrell G, Kowdley KV, Craxi A, Simon K, Fischer L, Melchor-Khan L, Vest J, Wiens BL, Vig P, Seyedkazemi S, Goodman Z, Wong VW, Loomba R, Tacke F, Sanyal A, Lefebvre E. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. Hepatology. 2018;67(5):1754-67.

195. Ratziu V, Sanyal A, Harrison SA, Wong VW, Francque S, Goodman Z, Aithal GP, Kowdley KV, Seyedkazemi S, Fischer L, Loomba R, Abdelmalek MF, Tacke F. Cenicriviroc Treatment for Adults With Nonalcoholic Steatohepatitis and Fibrosis: Final Analysis of the Phase 2b CENTAUR Study. Hepatology. 2020;72(3):892-905.

196. Harrison SA, Wong VW, Okanoue T, Bzowej N, Vuppalanchi R, Younes Z, Kohli A, Sarin S, Caldwell SH, Alkhouri N, Shiffman ML, Camargo M, Li G, Kersey K, Jia C, Zhu Y, Djedjos CS, Subramanian GM, Myers RP, Gunn N, Sheikh A, Anstee QM, Romero-Gomez M, Trauner M, Goodman Z, Lawitz EJ, Younossi Z. Selonsertib for patients with bridging fibrosis or compensated cirrhosis due to NASH: Results from randomized phase III STELLAR trials. J Hepatol. 2020;73(1):26-39.

197. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, Diehl AM, Djedjos CS, Han L, Myers RP, Subramanian GM, McHutchison JG, Goodman ZD, Afdhal NH, Charlton MR. The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: A randomized, phase 2 trial. Hepatology. 2018;67(2):549-59.

198. Harrison SA, Abdelmalek MF, Caldwell S, Shiffman ML, Diehl AM, Ghalib R, Lawitz EJ, Rockey DC, Schall RA, Jia C, McColgan BJ, McHutchison JG, Subramanian GM, Myers RP, Younossi Z, Ratziu V, Muir AJ, Afdhal NH, Goodman Z, Bosch J, Sanyal AJ. Simtuzumab Is Ineffective for Patients With Bridging Fibrosis or Compensated Cirrhosis Caused by Nonalcoholic Steatohepatitis. Gastroenterology. 2018;155(4):1140-53.

199. Van Wagner LB, Koppe SW, Brunt EM, Gottstein J, Gardikiotes K, Green RM, Rinella ME. Pentoxifylline for the treatment of non-alcoholic steatohepatitis: a randomized controlled trial. Ann Hepatol. 2011;10(3):277-86.
200. Zein CO, Yerian LM, Gogate P, Lopez R, Kirwan JP, Feldstein AE, McCullough AJ. Pentoxifylline improves nonalcoholic steatohepatitis: a randomized placebo-controlled trial. Hepatology. 2011;54(5):1610-9.
201. Kane AE, Bisset ES, Heinze-Milne S, Keller KM, Grandy SA, Howlett SE. Maladaptive Changes Associated With Cardiac Aging Are Sex-Specific and Graded by Frailty and Inflammation in C57BL/6 Mice. J Gerontol A Biol Sci Med Sci. 2021;76(2):233-43.
202. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, Verstegen NJM, Ciampricotti M, Hawinkels L, Jonkers J, de Visser KE. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;522(7556):345-8.
203. Kucsera D, Tóth VE, Sayour NV, Kovács T, Gergely TG, Ruppert M, Radovits T, Fábián A, Kovács A, Merkely B, Ferdinandy P, Varga ZV. IL-1β neutralization prevents diastolic dysfunction development, but lacks hepatoprotective effect in an aged mouse model of NASH. Sci Rep. 2023;13(1):356.
204. Kucsera D, Tóth VE, Gergő D, Vörös I, Onódi Z, Görbe A, Ferdinandy P, Varga ZV. Characterization of the CDAA Diet-Induced Non-alcoholic Steatohepatitis Model: Sex-Specific Differences in Inflammation, Fibrosis, and Cholesterol Metabolism in Middle-Aged Mice. Front Physiol. 2021;12:609465.
205. Diaz Brinton R. Minireview: translational animal models of human menopause: challenges and emerging opportunities. Endocrinology. 2012;153(8):3571-8.
206. Huby T, Gautier EL. Immune cell-mediated features of non-alcoholic steatohepatitis. Nat Rev Immunol. 2021:1-15.
207. Wu H, Ballantyne CM. Metabolic Inflammation and Insulin Resistance in Obesity. Circ Res. 2020;126(11):1549-64.
208. Tilg H, Moschen AR, Szabo G. Interleukin-1 and inflammasomes in alcoholic liver disease/acute alcoholic hepatitis and nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. Hepatology. 2016;64(3):955-65.

209. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017;377(12):1119-31.
210. Schnelle M, Catibog N, Zhang M, Nabeebaccus AA, Anderson G, Richards DA, Sawyer G, Zhang X, Toischer K, Hasenfuss G, Monaghan MJ, Shah AM. Echocardiographic evaluation of diastolic function in mouse models of heart disease. J Mol Cell Cardiol. 2018;114:20-8.
211. McTiernan CF, Lemster BH, Frye C, Brooks S, Combes A, Feldman AM. Interleukin-1 beta inhibits phospholamban gene expression in cultured cardiomyocytes. Circ Res. 1997;81(4):493-503.
212. Chung MK, Gulick TS, Rotondo RE, Schreiner GF, Lange LG. Mechanism of cytokine inhibition of beta-adrenergic agonist stimulation of cyclic AMP in rat cardiac myocytes. Impairment of signal transduction. Circ Res. 1990;67(3):753-63.
213. Liu SJ, Zhou W, Kennedy RH. Suppression of beta-adrenergic responsiveness of L-type Ca2+ current by IL-1beta in rat ventricular myocytes. Am J Physiol. 1999;276(1):H141-8.
214. Campana L, Esser H, Huch M, Forbes S. Liver regeneration and inflammation: from fundamental science to clinical applications. Nat Rev Mol Cell Biol. 2021;22(9):608-24.
215. Torres S, Brol MJ, Magdaleno F, Schierwagen R, Uschner FE, Klein S, Ortiz C, Tyc O, Bachtler N, Stunden J, Bertheloot D, Kitanovic A, Sanchez B, Schrum J, Roush WR, Franchi L, Byth K, Latz E, Trebicka J. The Specific NLRP3 Antagonist IFM-514 Decreases Fibrosis and Inflammation in Experimental Murine Non-Alcoholic Steatohepatitis. Front Mol Biosci. 2021;8:715765.
216. Morrison MC, Mulder P, Salic K, Verheij J, Liang W, van Duyvenvoorde W, Menke A, Kooistra T, Kleemann R, Wielinga PY. Intervention with a caspase-1 inhibitor reduces obesity-associated hyperinsulinemia, non-alcoholic

steatohepatitis and hepatic fibrosis in LDLR-/-.Leiden mice. Int J Obes (Lond). 2016;40(9):1416-23.

217. Mridha AR, Wree A, Robertson AAB, Yeh MM, Johnson CD, Van Rooyen DM, Haczeyni F, Teoh NC, Savard C, Ioannou GN, Masters SL, Schroder K, Cooper MA, Feldstein AE, Farrell GC. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. J Hepatol. 2017;66(5):1037-46.

218. Leung BP, Culshaw S, Gracie JA, Hunter D, Canetti CA, Campbell C, Cunha F, Liew FY, McInnes IB. A role for IL-18 in neutrophil activation. J Immunol. 2001;167(5):2879-86.

219. Zhu C, Tabas I, Schwabe RF, Pajvani UB. Maladaptive regeneration - the reawakening of developmental pathways in NASH and fibrosis. Nat Rev Gastroenterol Hepatol. 2021;18(2):131-42.

220. Feldstein AE, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology. 2003;125(2):437-43.

221. Shojaie L, Iorga A, Dara L. Cell Death in Liver Diseases: A Review. Int J Mol Sci. 2020;21(24).

222. Hirsova P, Bohm F, Dohnalkova E, Nozickova B, Heikenwalder M, Gores GJ, Weber A. Hepatocyte apoptosis is tumor promoting in murine nonalcoholic steatohepatitis. Cell Death Dis. 2020;11(2):80.

223. Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, Lencioni R, Koike K, Zucman-Rossi J, Finn RS. Hepatocellular carcinoma. Nat Rev Dis Primers. 2021;7(1):6.

224. Lohitesh K, Chowdhury R, Mukherjee S. Resistance a major hindrance to chemotherapy in hepatocellular carcinoma: an insight. Cancer Cell Int. 2018;18:44.

225. Sangro B, Sarobe P, Hervás-Stubbs S, Melero I. Advances in immunotherapy for hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol. 2021;18(8):525-43.

226. Pfister D, Núñez NG, Pinyol R, Govaere O, Pinter M, Szydlowska M, Gupta R, Qiu M, Deczkowska A, Weiner A, Müller F, Sinha A, Friebel E, Engleitner T, Lenggenhager D, Moncsek A, Heide D, Stirm K, Kosla J, Kotsiliti E, Leone V,

Dudek M, Yousuf S, Inverso D, Singh I, Teijeiro A, Castet F, Montironi C, Haber PK, Tiniakos D, Bedossa P, Cockell S, Younes R, Vacca M, Marra F, Schattenberg JM, Allison M, Bugianesi E, Ratziu V, Pressiani T, D'Alessio A, Personeni N, Rimassa L, Daly AK, Scheiner B, Pomej K, Kirstein MM, Vogel A, Peck-Radosavljevic M, Hucke F, Finkelmeier F, Waidmann O, Trojan J, Schulze K, Wege H, Koch S, Weinmann A, Bueter M, Rössler F, Siebenhüner A, De Dosso S, Mallm JP, Umansky V, Jugold M, Luedde T, Schietinger A, Schirmacher P, Emu B, Augustin HG, Billeter A, Müller-Stich B, Kikuchi H, Duda DG, Kütting F, Waldschmidt DT, Ebert MP, Rahbari N, Mei HE, Schulz AR, Ringelhan M, Malek N, Spahn S, Bitzer M, Ruiz de Galarreta M, Lujambio A, Dufour JF, Marron TU, Kaseb A, Kudo M, Huang YH, Djouder N, Wolter K, Zender L, Marche PN, Decaens T, Pinato DJ, Rad R, Mertens JC, Weber A, Unger K, Meissner F, Roth S, Jilkova ZM, Claassen M, Anstee QM, Amit I, Knolle P, Becher B, Llovet JM, Heikenwalder M. NASH limits anti-tumour surveillance in immunotherapy-treated HCC. Nature. 2021;592(7854):450-6.

227. Zong Z, Zou J, Mao R, Ma C, Li N, Wang J, Wang X, Zhou H, Zhang L, Shi Y. M1 Macrophages Induce PD-L1 Expression in Hepatocellular Carcinoma Cells Through IL-1β Signaling. Front Immunol. 2019;10:1643.

228. Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A. 2003;100(5):2645-50.

229. Dmitrieva-Posocco O, Dzutsev A, Posocco DF, Hou V, Yuan W, Thovarai V, Mufazalov IA, Gunzer M, Shilovskiy IP, Khaitov MR, Trinchieri G, Waisman A, Grivennikov SI. Cell-Type-Specific Responses to Interleukin-1 Control Microbial Invasion and Tumor-Elicited Inflammation in Colorectal Cancer. Immunity. 2019;50(1):166-80.e7.

230. Wu T, Hong Y, Jia L, Wu J, Xia J, Wang J, Hu Q, Cheng B. Modulation of IL-1β reprogrammes the tumor microenvironment to interrupt oral carcinogenesis. Sci Rep. 2016;6:20208.

231. Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, Kammula US, Chen Y, Qin LX, Tang ZY, Wang XW. Prediction of venous metastases, recurrence, and

prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell. 2006;10(2):99-111.

232. Maina V, Sutti S, Locatelli I, Vidali M, Mombello C, Bozzola C, Albano E. Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. Clin Sci (Lond). 2012;122(11):545-53.

233. Zhang Y, Li JQ, Jiang ZZ, Li L, Wu Y, Zheng L. CD169 identifies an anti-tumour macrophage subpopulation in human hepatocellular carcinoma. J Pathol. 2016;239(2):231-41.

234. Rébé C, Ghiringhelli F. Interleukin-1β and Cancer. Cancers (Basel). 2020;12(7).

235. Purwar R, Schlapbach C, Xiao S, Kang HS, Elyaman W, Jiang X, Jetten AM, Khoury SJ, Fuhlbrigge RC, Kuchroo VK, Clark RA, Kupper TS. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. Nat Med. 2012;18(8):1248-53.

236. Chen LC, Wang LJ, Tsang NM, Ojcius DM, Chen CC, Ouyang CN, Hsueh C, Liang Y, Chang KP, Chen CC, Chang YS. Tumour inflammasome-derived IL-1β recruits neutrophils and improves local recurrence-free survival in EBV-induced nasopharyngeal carcinoma. EMBO Mol Med. 2012;4(12):1276-93.

237. Della Torre S, Benedusi V, Fontana R, Maggi A. Energy metabolism and fertility: a balance preserved for female health. Nat Rev Endocrinol. 2014;10(1):13-23.

238. Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. FEMS Immunol Med Microbiol. 2003;38(1):13-22.

239. Theocharidou E, Papademetriou M, Reklou A, Sachinidis A, Boutari C, Giouleme O. The Role of PCSK9 in the Pathogenesis of Non-alcoholic Fatty Liver Disease and the Effect of PCSK9 Inhibitors. Curr Pharm Des. 2018;24(31):3654-7.

240. Lai Q, Giralt A, Le May C, Zhang L, Cariou B, Denechaud PD, Fajas L. E2F1 inhibits circulating cholesterol clearance by regulating Pcsk9 expression in the liver. JCI Insight. 2017;2(10).

241. Lebeau PF, Byun JH, Platko K, Al-Hashimi AA, Lhoták Š, MacDonald ME, Mejia-Benitez A, Prat A, Igdoura SA, Trigatti B, Maclean KN, Seidah NG, Austin RC. Pcsk9 knockout exacerbates diet-induced non-alcoholic steatohepatitis, fibrosis and liver injury in mice. JHEP Rep. 2019;1(6):418-29.

242. Panta R, Dahal K, Kunwar S. Efficacy and safety of mipomersen in treatment of dyslipidemia: a meta-analysis of randomized controlled trials. J Clin Lipidol. 2015;9(2):217-25.
243. Hooper AJ, Burnett JR, Watts GF. Contemporary aspects of the biology and therapeutic regulation of the microsomal triglyceride transfer protein. Circ Res. 2015;116(1):193-205.

9. LIST OF OWN PUBLICATIONS

9.1. Publications related to the candidate's PhD dissertation

I. Dániel Kucsera, Viktória E. Tóth, Nabil V. Sayour, Tamás Kovács, Tamás G. Gergely, Mihály Ruppert, Tamás Radovits, Alexandra Fábián, Attila Kovács, Béla Merkely, Péter Ferdinandy, Zoltán V. Varga (2023). "IL-1β neutralization prevents diastolic dysfunction development, but lacks hepatoprotective effect in an aged mouse model of NASH" *Scientific Reports.* **IF: 4.997**

II. Dániel Kucsera, Viktória E. Tóth, Dorottya Gergő, Imre Vörös, Zsófia Onódi, Anikó Görbe, Péter Ferdinandy, Zoltán V. Varga (2021). "Characterization of the CDAA Diet-Induced Non-alcoholic Steatohepatitis Model: Sex-Specific Differences in Inflammation, Fibrosis, and Cholesterol Metabolism in Middle-Aged Mice" *Frontiers in Physiology*. **IF: 4.755**

Sum of impact factors of dissertation-related publications: 9.752

9.2. Publications not related to the candidate' PhD dissertation

III. Tamás G. Gergely, Dániel Kucsera, Viktória E. Tóth, Tamás Kovács, Nabil V. Sayour, Zsófia D. Drobni, Mihály Ruppert, Balázs Petrovich, Bence Ágg, Zsófia Onódi, Nóra Fekete, Éva Pállinger, Edit I. Buzás, Laura I. Yousif, Wouter C. Meijers, Tamás Radovits, Béla Merkely, Péter Ferdinandy, Zoltán V. Varga (2022). "Characterization of immune checkpoint inhibitor-induced cardiotoxicity reveals interleukin-17A as a driver of cardiac dysfunction after anti-PD-1 treatment" *British Journal of Pharmacology*. **IF: 9.473**

IV. Zsófia Onódi, Mihály Ruppert, Dániel Kucsera, Alex Ali Sayour, Viktória E. Tóth, Gábor Koncsos, Julianna Novák, Gábor B. Brenner, András Makkos, Tamás Baranyai, Zoltán Giricz, Anikó Görbe, Przemyslaw Leszek, Mariann Gyöngyösi, Iván G. Horváth, Rainer Schulz, Béla Merkely, Péter Ferdinandy, Tamás Radovits, Zoltán V. Varga (2021). "AIM2-driven inflammasome activation in heart failure" *Cardiovascular research.* **IF: 14,239**

V. Judit Szepesy, Gabriella Miklós, János Farkas, Dániel Kucsera, Zoltán Giricz, Anita Gáborján, Gábor Polony, Ágnes Szirma, László Tamás, László Köles, Zoltán V. Varga, Tibor Zelles (2020). "Anti-PD-1 Therapy Does Not Influence Hearing Ability

in the Most Sensitive Frequency Range, but Mitigates Outer Hair Cell Loss in the Basal Cochlear Region" *International Journal of Molecular Sciences.* **IF: 5.924**

Sum of all impact factor: 39.388

10. ACKNOWLEDGEMENT

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NYILATKOZAT EREDETISÉGRŐL ÉS SZERZŐI JOGRÓL

a PhD disszertáció elkészítésére vonatkozó szabályok betartásáról

Alulírott Kucsera Dániel jelen nyilatkozat aláírásával kijelentem, hogy a **Targeting IL-1β and assessing sex specific molecular differences in mouse models of non-alcoholic steatohepatitis** című PhD értekezésem önálló munkám, a dolgozat készítése során betartottam a szerzői jogról szóló 1999. évi LXXVI tv. vonatkozó rendelkezéseit, a már megjelent vagy közlés alatt álló közlemény(ek)ből felhasznált ábra/szöveg nem sérti a kiadó vagy más jogi vagy természetes személy jogait.

Jelen nyilatkozat aláírásával tudomásul veszem, hogy amennyiben igazolható, hogy a dolgozatban nem saját eredményeimet használtam fel vagy a dolgozattal kapcsolatban szerzői jog megsértése merül fel, a Semmelweis Egyetem megtagadja PhD dolgozatom befogadását, velem szemben fegyelmi eljárást indít, illetve visszavonja a már odaítélt PhD fokozatot.

A dolgozat befogadásának megtagadása és a fegyelmi eljárás indítása nem érinti a szerzői jogsértés miatti egyéb (polgári jogi, szabálysértési jogi, büntetőjogi) jogkövetkezményeket.

Tudomásul veszem, hogy a PhD értekezés nyilvánosan elérhető formában feltöltésre kerül az Országos Doktori Tanács honlapjára.

Budapest, 2023.03.21.

Kucsera Dániel