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

PhD thesis

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LIST OF ABBREVIATIONS

11 β -HSD1	11 β -hydroxysteroid dehydrogenase type 1
ACC	acetyl-CoA carboxylase
AGE	advanced glycation endproducts
AFP	α -fetoprotein
ALT	alanine aminotransferase
Anti-IL-1 β	anti-interleukin-1 β monoclonal antibody
ASK	apoptosis signal-regulating kinase
AST	aspartate aminotransferase
AT ₂ R	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- κ B	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/kexin 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	sterol-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.

Hepatosteatosi 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).

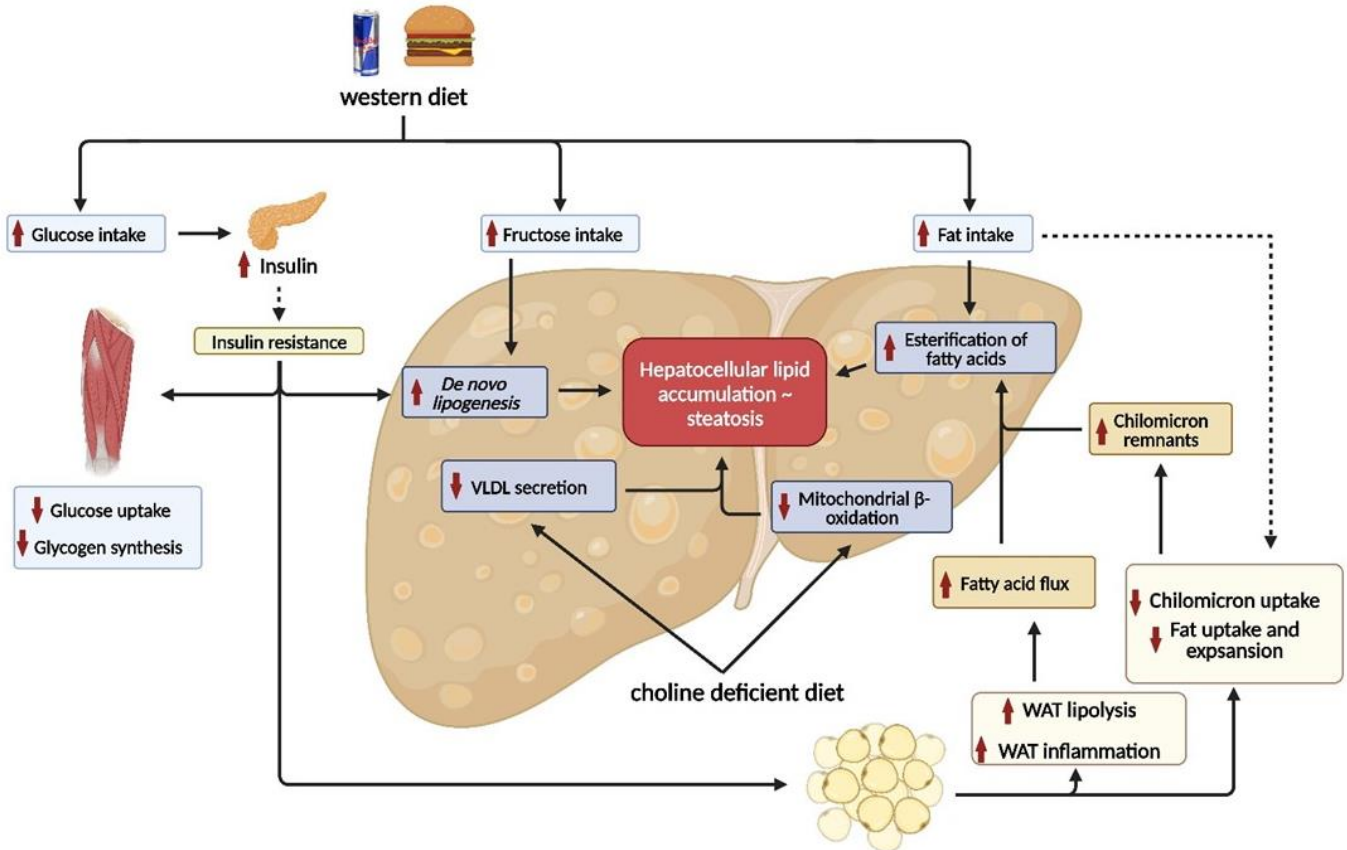


Figure 1 – Contributing factors of hepatic lipid accumulation – steatosis.

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 hepatosteatosi. (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-1 β , IL-6, IL-18, TNF α , 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).

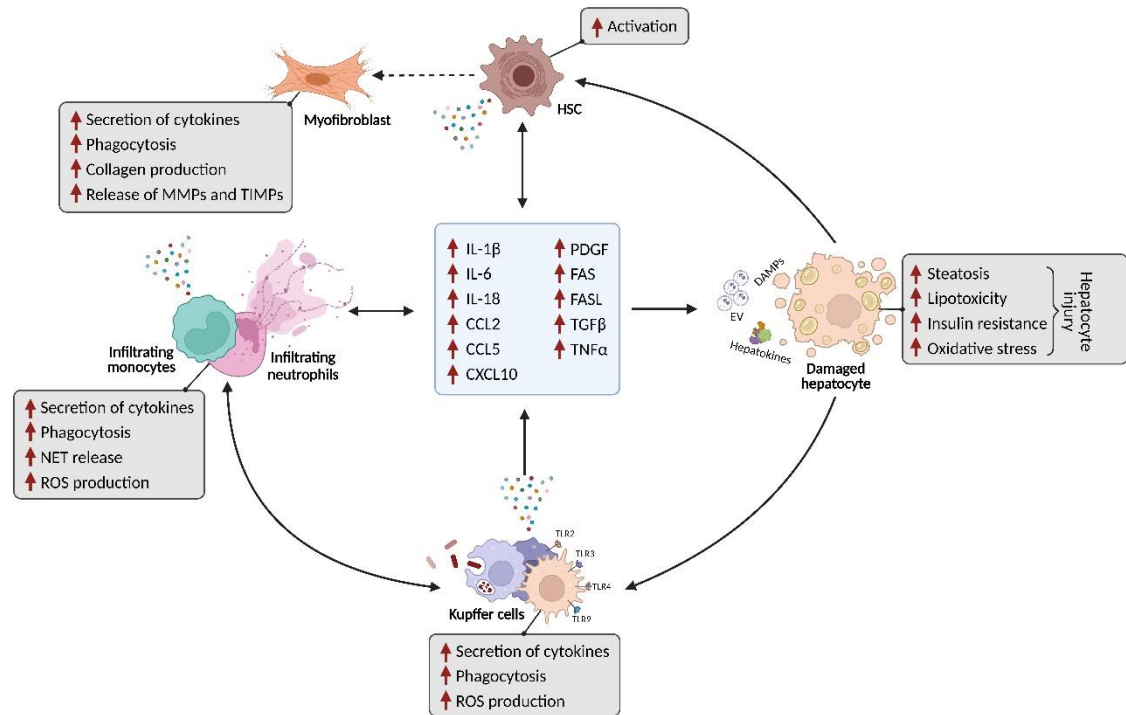


Figure 2 – Cellular contributors of NASH progression.

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 steatohepatic 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).

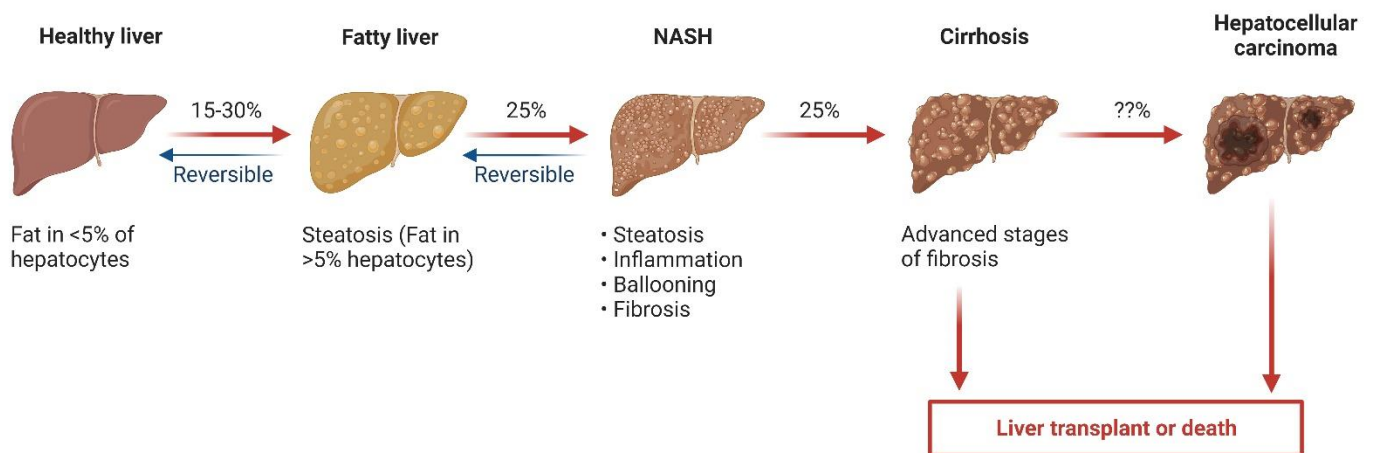


Figure 3 – Stages of non-alcoholic fatty liver disease.

NAFLD is a progressive disease, starting with benign and reversible steatosis. If this state is not alleviated within a reasonable 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 AT₂R 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-rich VLDLs (102), while men produce apoB-rich 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.




Factors	Male 	Young female 	Elder female 
Metabolic syndrome	—	+	—
Hypertension	—	+	—
Obesity	—	+	—
Fat distribution	"apple shape"	┌ "pear shape" ─┐	
NAFLD/NASH	—	+	—
Lobular inflammation	+	—	+
Advanced fibrosis	—	+	—
Dietary choline requirement	NA	lower	higher
PCSK9 serum level	+	—	—

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 in 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**.

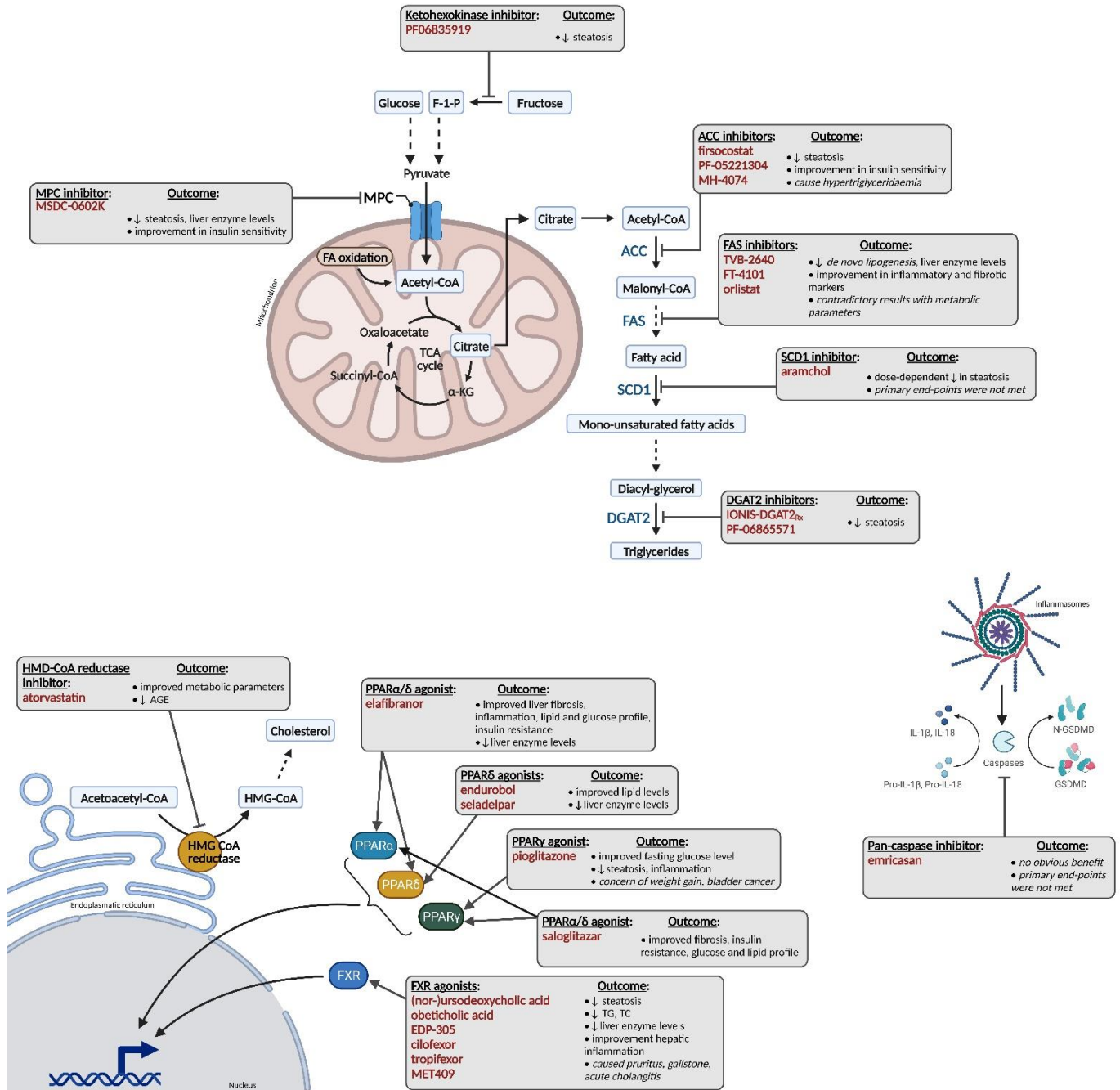


Figure 5 – Summary of major drug candidates for NASH.

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, <i>but hypertriglyceridemia occurred</i>	(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, <i>but glucose parameters did not change</i> 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, <i>but LDL increased</i>	(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 <i>Concern of weight gain, bladder cancer</i>	(155, 156)
PPARα/δ/γ agonist	lanifibranor	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) <i>Did not meet the primary endpoint</i>	(185)
Ketohexokinase inhibitor	PF-06835919	Reduced steatosis <i>No effect on insulin sensitivity</i>	(186)

Table 2 – Inflammation and fibrosis targeting clinical trials for NASH

Drug classes	Drug candidates	Main results	Ref
Caspase inhibitor	emricasan	<i>No obvious benefit was observed, and may even worsened fibrosis</i>	(187-191)
Galectin 3 inhibitor	belapectin	<i>No improvement, did not meet the endpoints</i>	(192, 193)
CCR2/CCR5 inhibitor	cenicriviroc	Improvement in fibrosis Decreased level of inflammatory biomarkers <i>No improvement in key NASH features, program terminated</i>	(194, 195)
ASK1 inhibitor	selonsertib	<i>Primary end-points were not met, program terminated</i>	(196, 197)
LOXL2 inhibitor	simtuzumab	Improvement in fibrosis <i>Trial terminated due to lack of efficacy</i>	(198)
TNFα inhibitor	pentoxifylline	<i>Contradictory effects in NASH</i>	(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-1 β 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-1 β 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-1 β 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 μ g/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 $6.3\times$ 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) Substrate, 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 $2^{-\Delta\Delta C_p}$ 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-1 β (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 \pm 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-1 β is a major pro-inflammatory cytokine with key importance in the pathophysiology of NASH. Both hepatic and immune cells are capable to secrete IL-1 β , 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-1 β 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-1 β 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-1 β 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**).

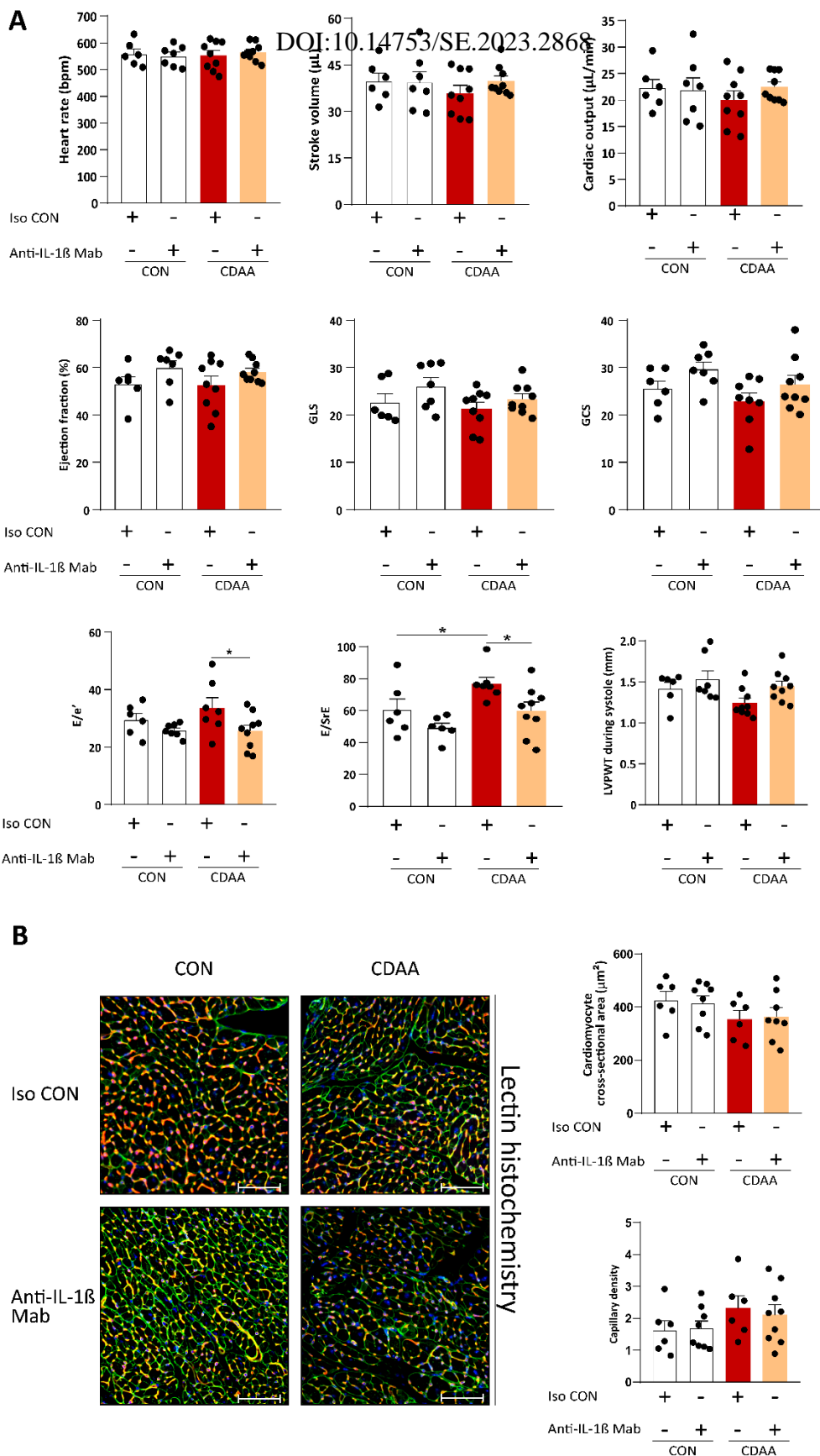


Figure 6. Cardiac function and remodeling in an aged NASH mouse model.

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

4.1.2. Interleukin-1 β 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-1 β neutralization decreased the expression of *Col1a1* and *Col3a1*. However, the overall quantification of fibrosis did not change macroscopically (**FIGURE 7**).

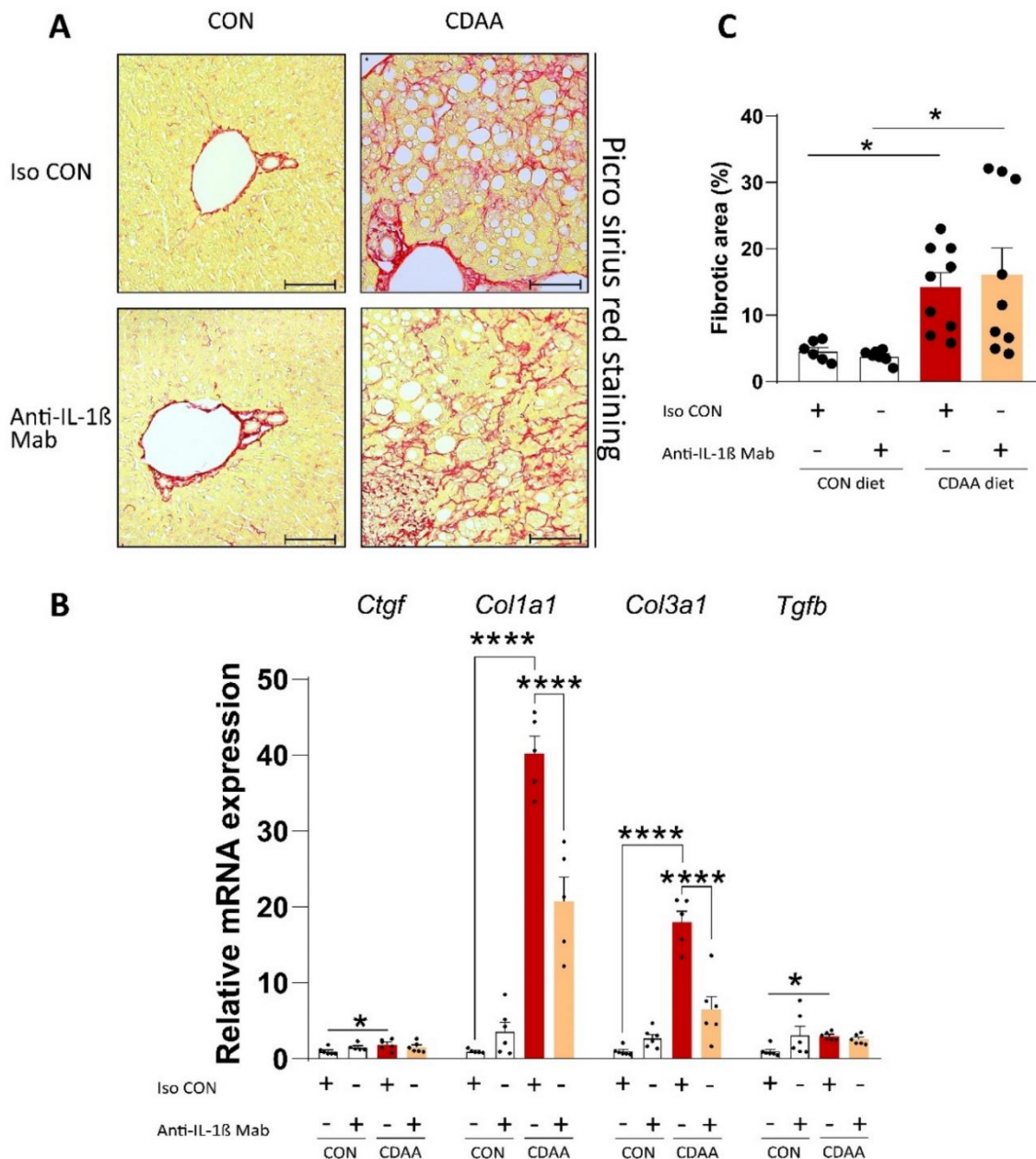


Figure 7 – Histological and molecular analysis of hepatic fibrosis.

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-1 β 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-1 β 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).

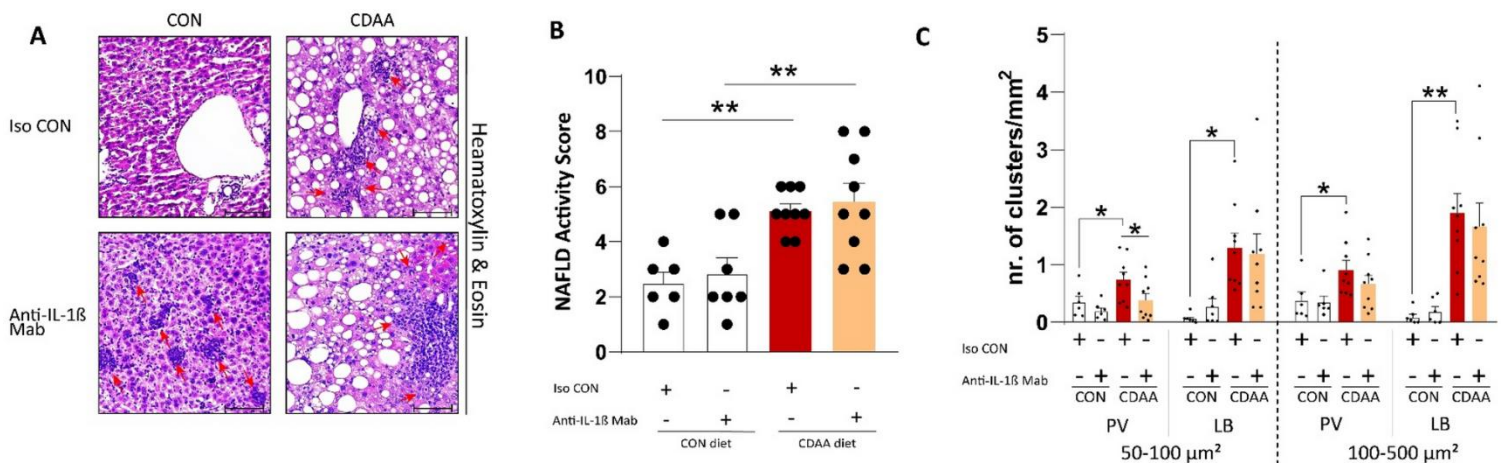


Figure 8 – Investigation of hepatic inflammatory cell infiltrations and NAS.

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-1 β 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-1 β 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-1 β 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-1 β inhibitor showed a compensatory increase of *Il1b* in hepatocytes (**Figure 9C**). The pro-tumorigenic CD163⁺ macrophages were depleted in CDAA-fed and treated animals.

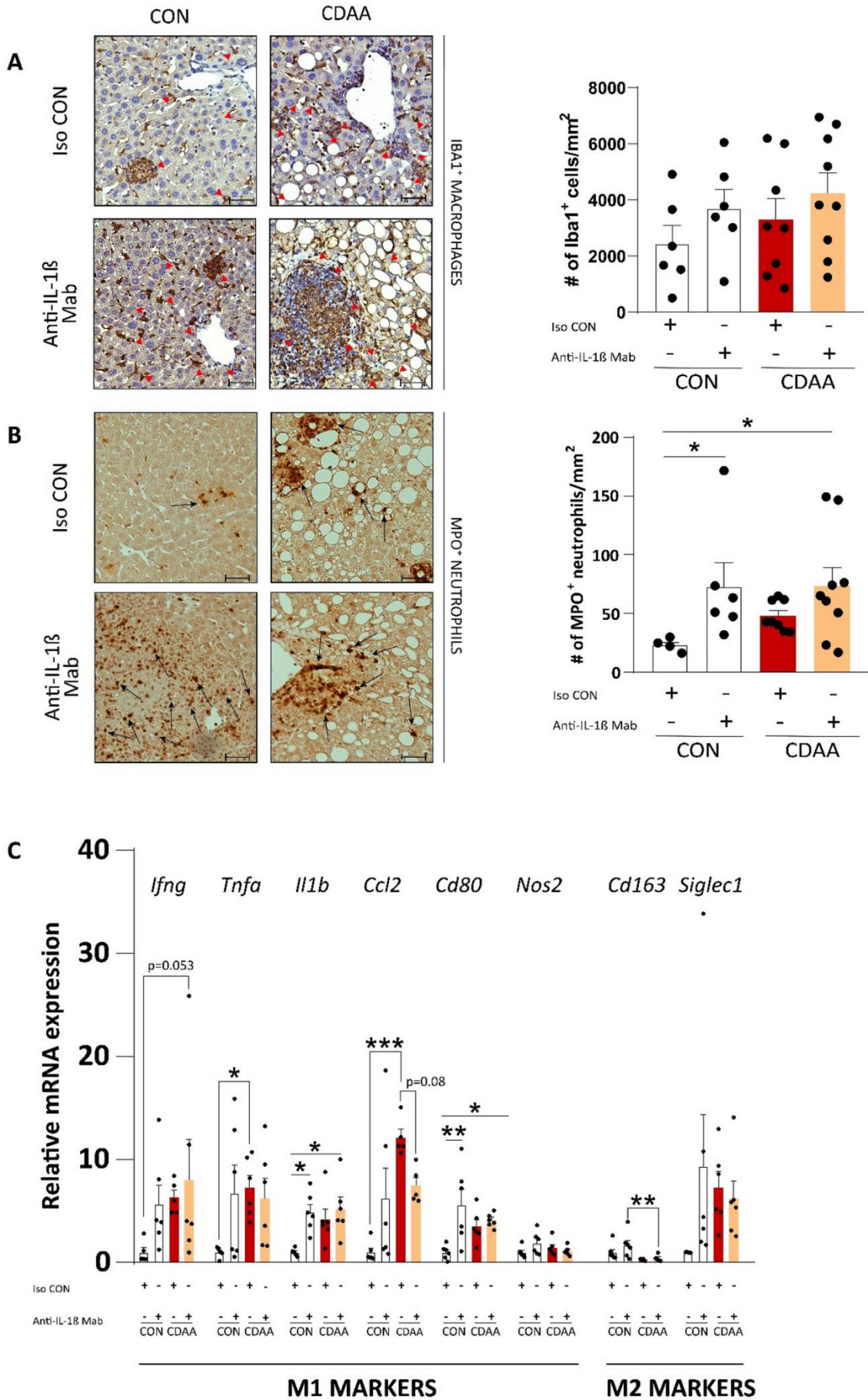


Figure 9 – Assessment of pro-inflammatory cells and mediators.

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 μ m. 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-1 β 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-1 β 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-11* was increased by the diet, while the treatment induced the up-regulation of *Pd-1* and *Ctla4* (FIGURE 10).

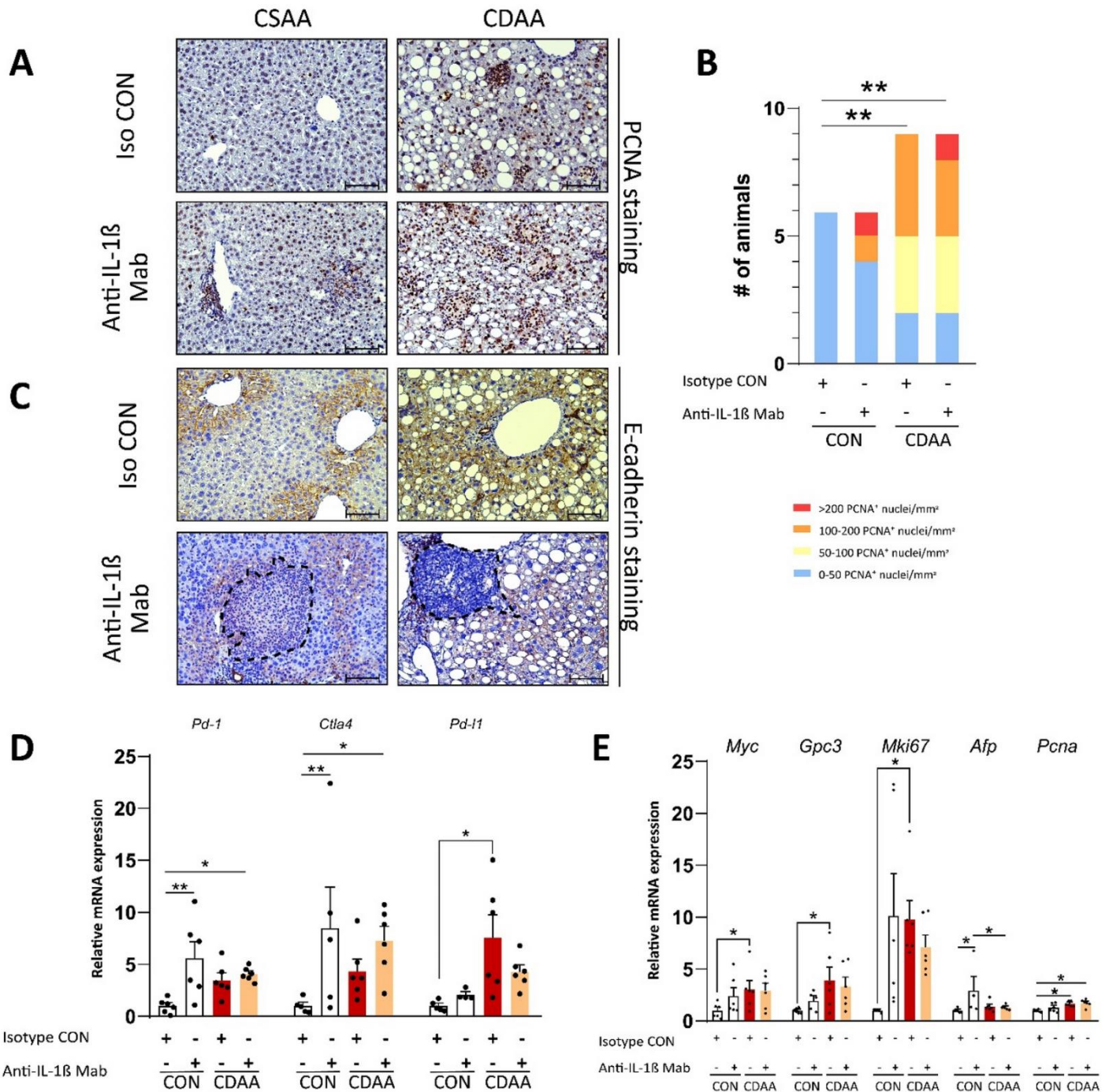


Figure 10 – Histological and molecular analysis of hepatic microenvironment.

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 μ m. 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**).

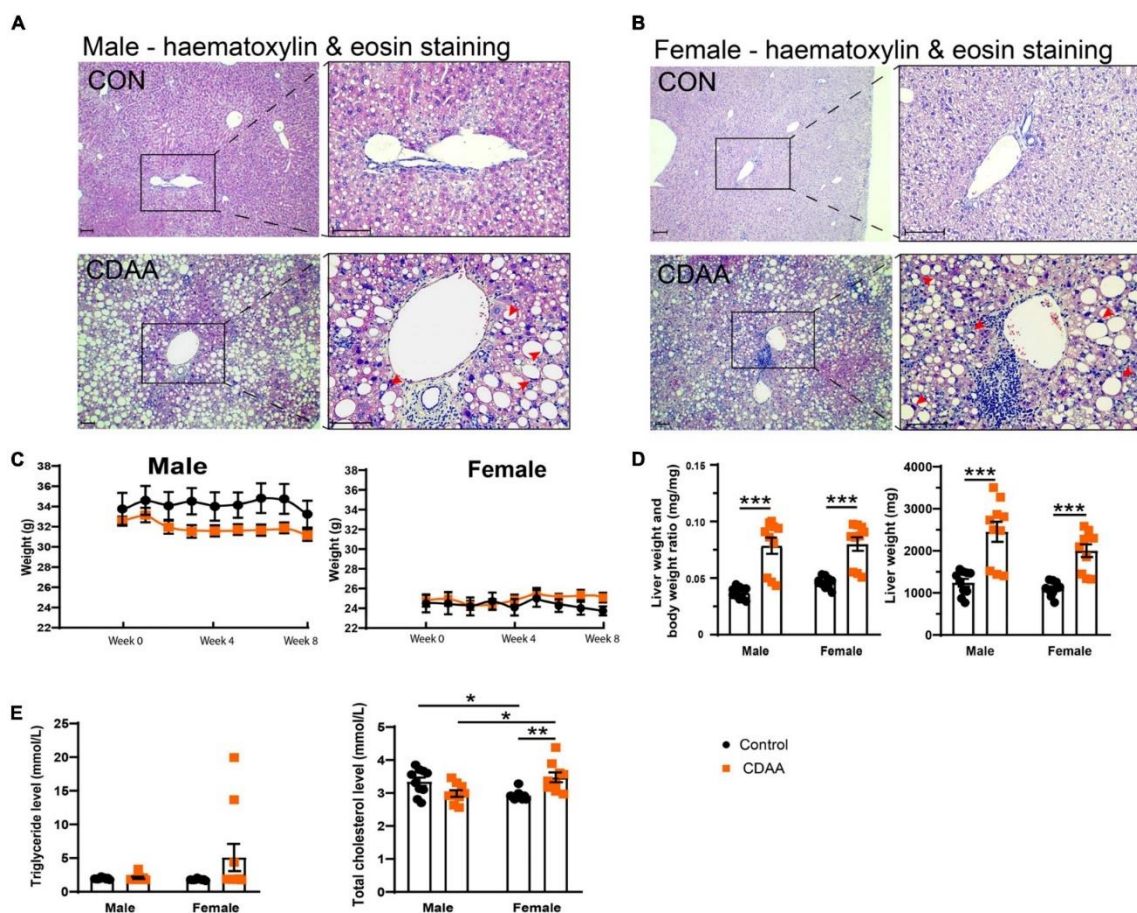


Figure 11 – Sex-specific differences in hepatic architecture and serum lipid levels.

Depiction of steatosis in males (A) and females (B) on hematoxylin-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**).

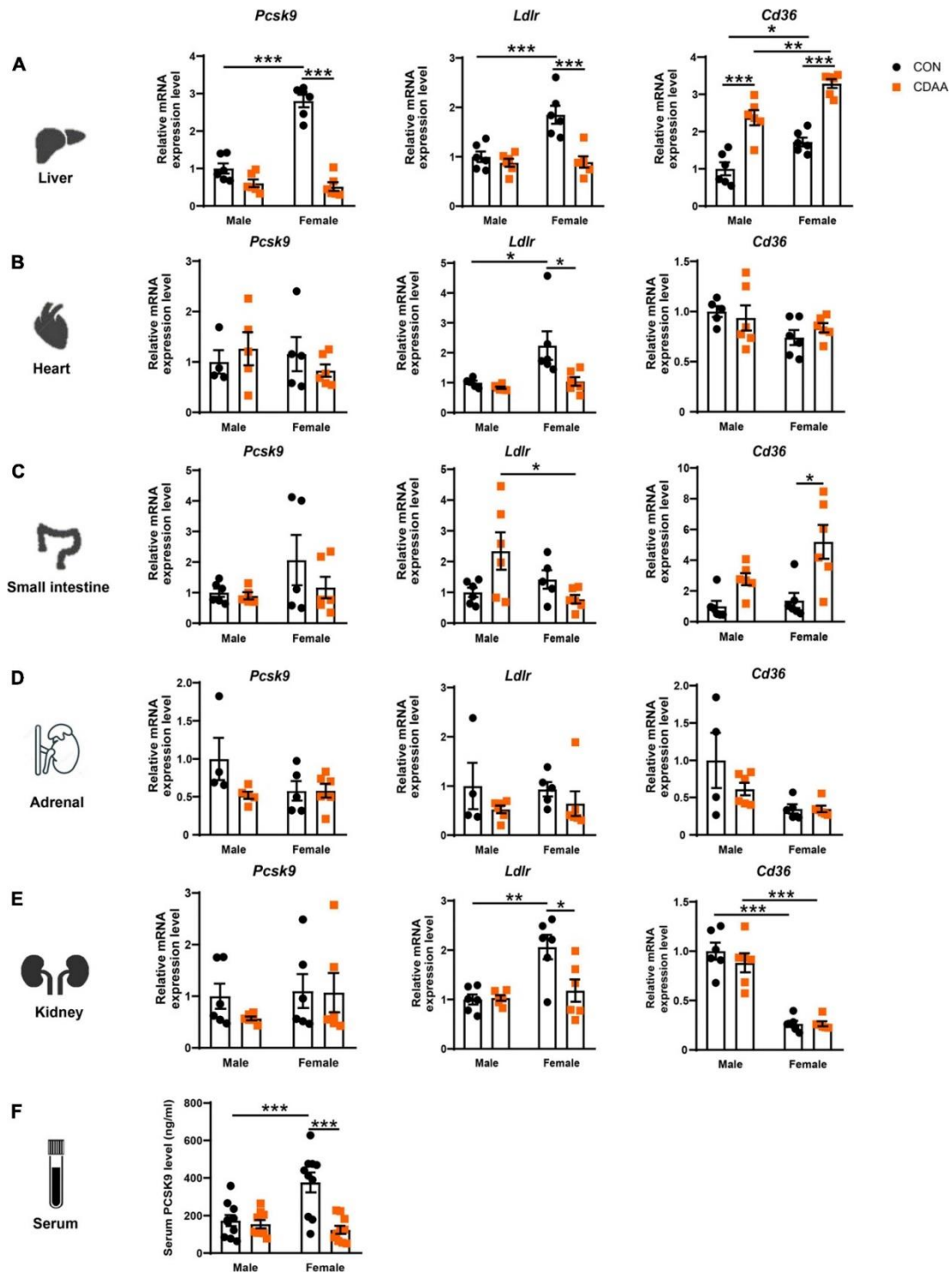


Figure 12 – Sex-dependent gene expression differences of *Pcsk9* and its major targets.

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**).

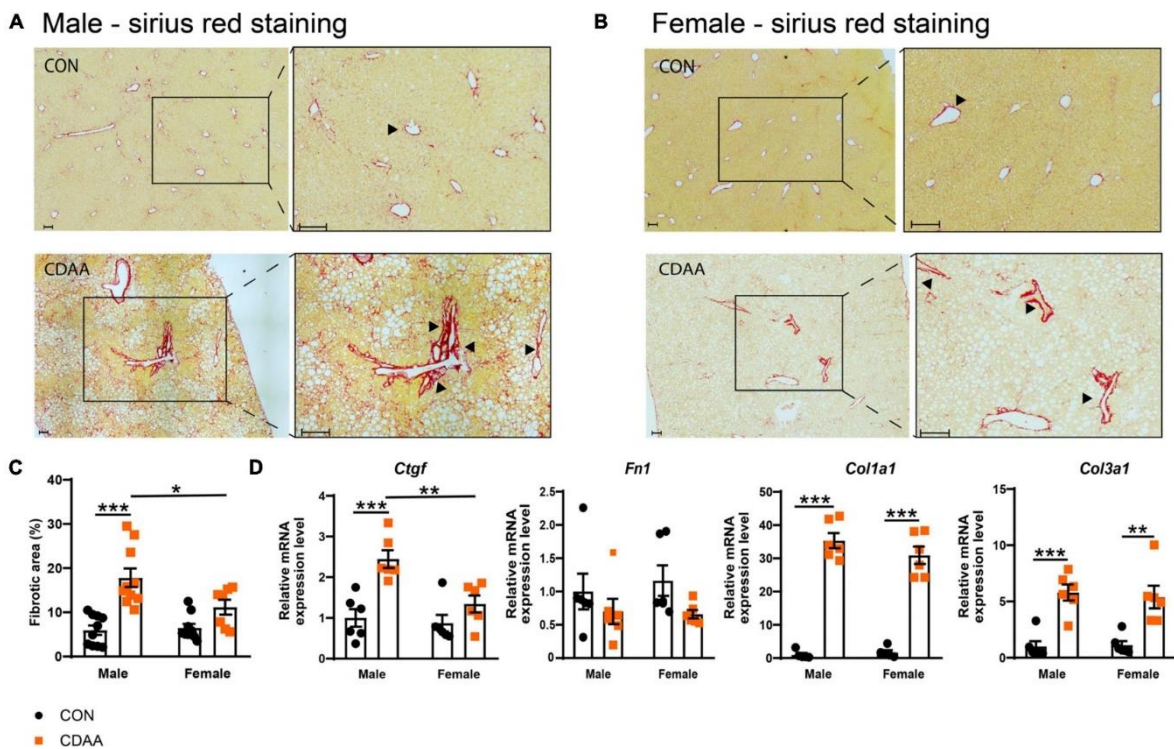
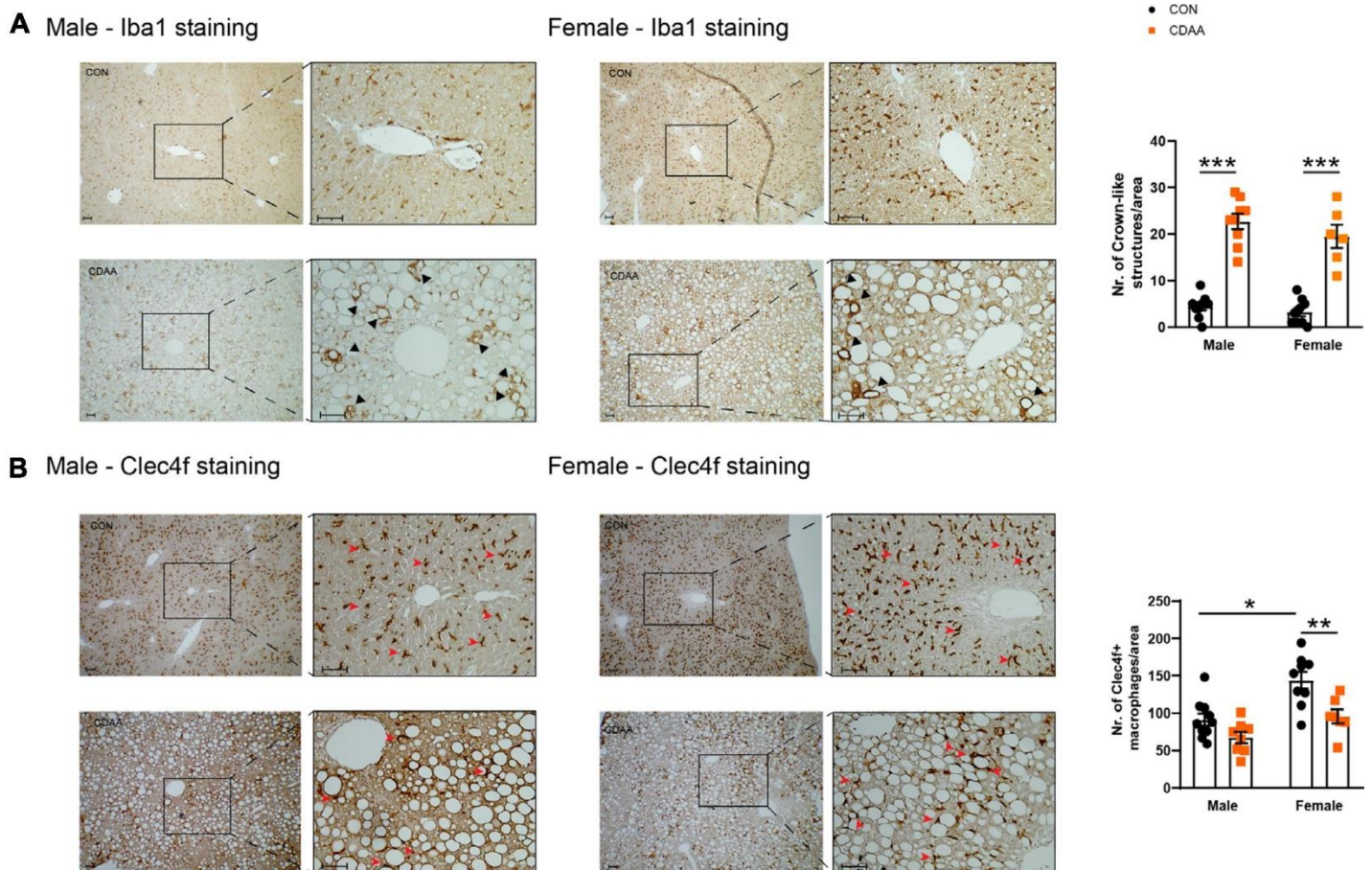


Figure 13 – Sex differences in hepatic fibrosis.

Depiction of hepatic fibrosis on picosirius-red stained sections in males (**A**) and (**B**). Quantification of overall fibrosis, $n = 10/\text{group}$ (**C**). Transcriptomic analysis of major pro-fibrotic genes, $n = 6/\text{group}$ (**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**).



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**).

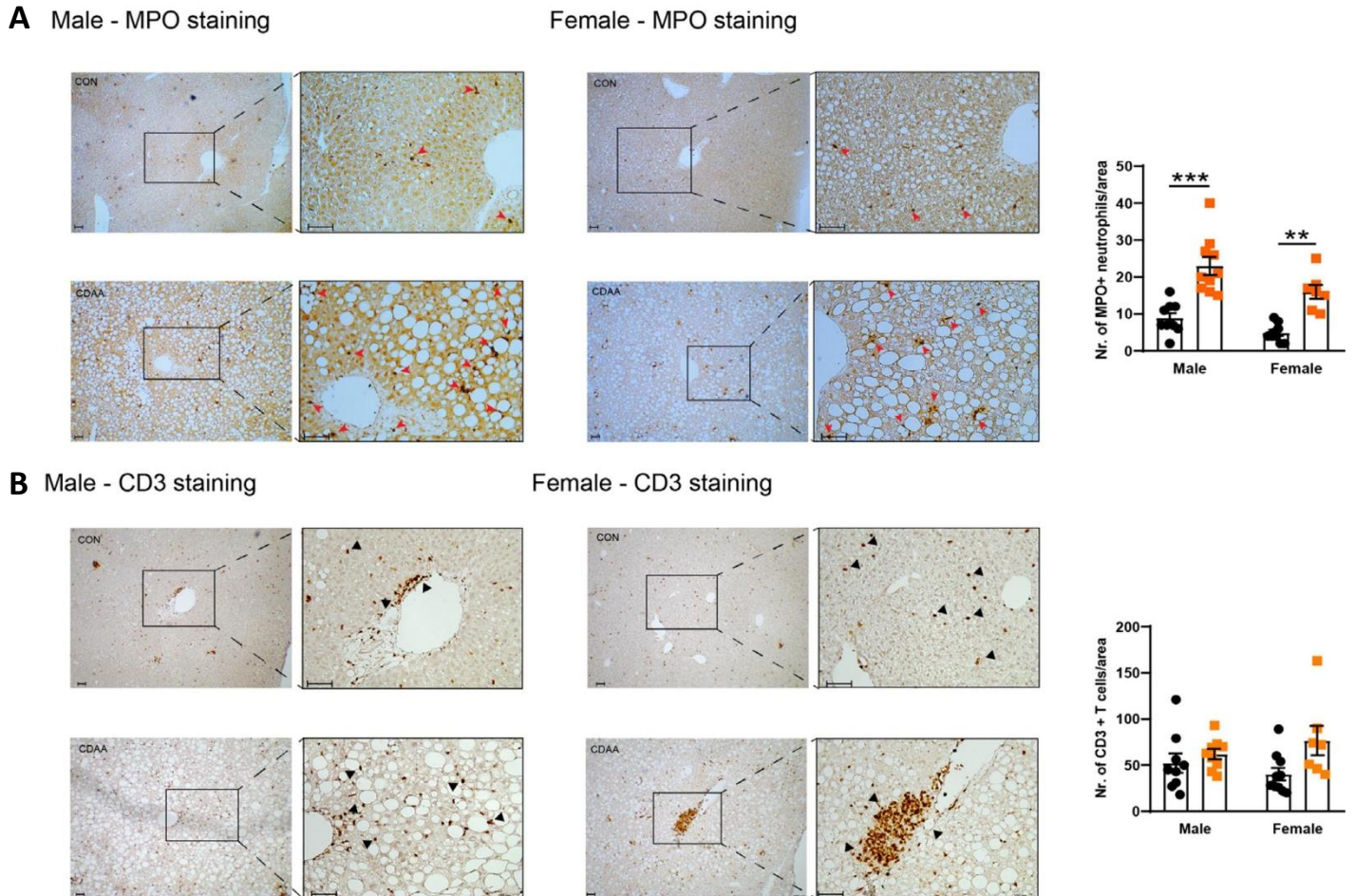


Figure 15 – Immunostaining of neutrophils and T cells

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-1 β 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-1 β in females with NASH. The responsible inflammasome for the enzymatic cleavage of IL-1 β 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).

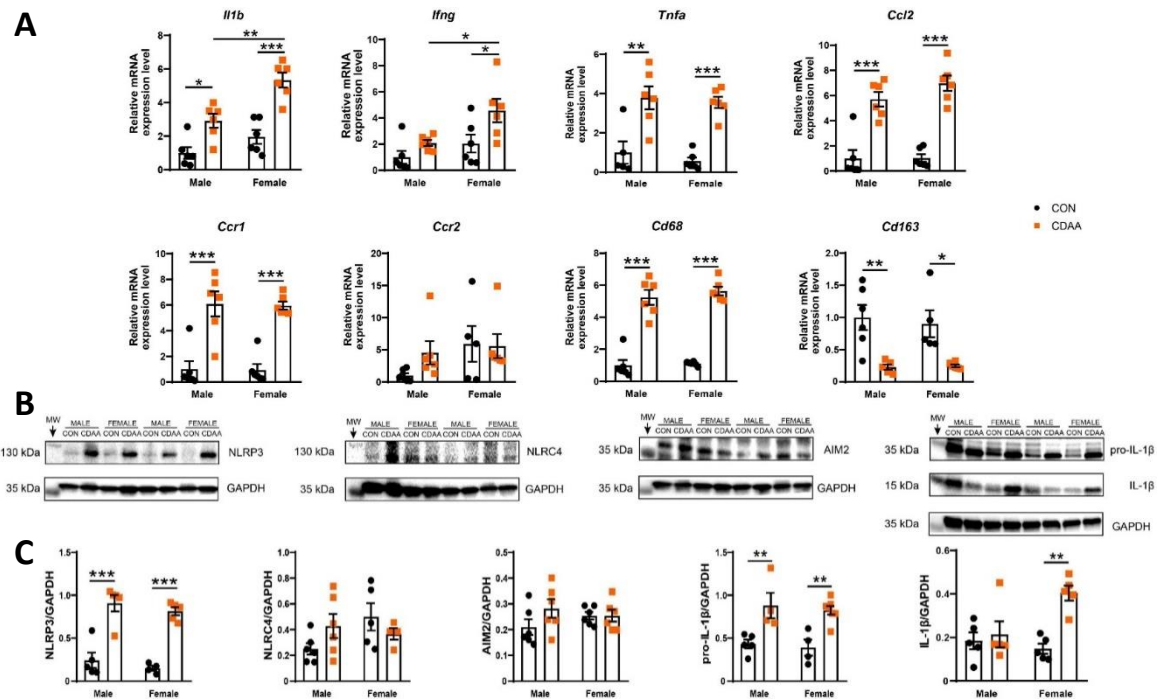


Figure 16 – Sex-dependent differences in hepatic inflammation.

Gene expression analysis of major pro-inflammatory cytokines (A), n = 6/group. Western blot analysis of major inflammasomes and IL-1 β , 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-1 β 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-1 β , because of its extensive role in NASH pathomechanism (208). Additionally, canakinumab - a human monoclonal antibody against circulating IL-1 β – 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-1 β inhibition, which was observable only with speckle tracking echocardiography. This finding is in line with studies, where it was shown that IL-1 β signaling may interfere with the transduction of β -adrenergic receptors, causing impairment in cardiac function (211-213).

Unfortunately, IL-1 β 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-1 β and hepatocellular debris (47). Interestingly, IL-1 β blockade decreased the transcription of *Coll1a1* and *Col3a1*; however, macroscopic quantification of fibrosis showed no overall change in anti-IL-1 β monoclonal antibody treated mice. We may assume that IL-1 β 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-1 β by inhibition of NLRP3 inflammasome or caspase-1 reduced hepatic fibrosis, proving that targeting mechanism that subsequently decreases IL-1 β 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-1 β 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-1 β blocker, while transcription of *Ccl2* gene was marginally diminished by the treatment. NF- κ B, the downstream transcription factor of IL-1 β , 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-1 β 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-1 β 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-1 β 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-1 β 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-1 β , 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-1 β 's vicious cycle, then we might be able to halt further loss of liver cells. However, anti-IL-1 β 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-1 β blockade affects the transcription of immune checkpoints. We report that microenvironment of NASH is characterized by increased expression of *Pd-*

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

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

associated neutrophils resulting a tumorlytic effect, thus IL-1 β blockade might prevent this beneficial effect of neutrophils (236).

It is clear that IL-1 β has a wide range of effects in different cancers and cardiometabolic diseases as well, thus future treatments with IL-1 β 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-1 β 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-1 β 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-1 β 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-1 β 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 *I1b* and higher rate of IL-1 β maturation level.

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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-1 β 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-1 β 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