MECHANISTIC EVALUATION OF MENSTRUAL BLOOD-DERIVED ENDOMETRIAL STEM CELLS IN MUTINE MODELS OF ALSOHOLIC AND NON-ALCOHOLIC FATTY LIVER DISEASE

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
×g	g-force
℃	Degree Celsius
hr	Hour
µg	Microgram
mg	Milligram
kg	Kilogram
µl	Microliter
ml	Milliliter
µm	Micrometer
mM	Millimolar
µM	Micromolar
nM	Nanomolar
min	Minutes
rpm	Revolutions per minute
%	Percentage
™	Trademark

LIST OF ABBREVIATIONS

AASLD	American association for the study of liver diseases
AC	Alcoholic cirrhosis
ADH	Alcohol dehydrogenase system
ADSCs	Adipose-derived stem cells
AFL	Alcoholic fatty liver
AH	alcoholic hepatitis
AKI	Acute kidney injury
ALC	Alcohol-associated cirrhosis
ALD	Alcohol-associated liver disease
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
AML12	alpha mouse liver 12 cells
AMPK	AMP-activated protein kinase
ASH	Alcohol steatohepatitis
ASK1	apoptosis signal-regulating kinase 1
AST	Aspartate aminotransferase
ATGL	Adipose TG lipase
AUD	Alcohol use disorder
Akt	protein kinase B
BAX	BCL2 associated x protein
BM	bone marrow
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CAM	Cell adhesion molecule
CAMKK1	calcium/calmodulin-dependent protein kinase kinase 1

CKD	Chronic kidney disease
CM	conditioned medium
COT	Gene Cluster Acyl-CoA Thio esterase gene cluster
CPT-1A	Carnitine palmitoyl transferase 1A
CVD	Cardiovascular disease
Cyp4a10/Cyp4a14	Cytochrome P450 4a10/cytochrome P450 4a14
DAMPs	Damage-associated molecular patterns
DEGs	Differentially expressed genes
DIL	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMEM-HG	High glucose Dulbecco’ s modified Eagle’ s Medium
E2F1	E2F transcription factor 1
EASL	European association for the study of the liver
ECM	Extracellular matrix
EP	Eppendorf tube
ER	Endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESPEN	The European Society for Clinical Nutrition and Metabolism
ETC	Electron transport chain
EVs	extracellular vesicles
Exo	exosome
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FDA	Food and drug administration
FFAs	Free fatty acids
FLD	Fatty liver disease
FTO	fat mass and obesity-related gene

GHT	Glucose Tolerance Test
GLP-1	Glucagon-like peptide-1
GMP	Good manufacturing practice
GO	Gene ontology
GSEA	Gene set enrichment analysis
GSH	glutathione
GTT	Glucose tolerance test
H&E	Hematoxylin & eosin
HCC	Hepatocellular carcinoma
HFD	High fat diet
HGF	Hepatocyte growth factor
HL	human liver
HNF4α	hepatocyte nuclear factor 4 alpha
HO1	heme oxygenase 1
HP	High-protein diets
HSCs	Hepatic stellate cells
HepG2 cells	human liver carcinoma cells
HepaRG cells	human liver progenitor cells
IFNβ	interferon β
IFNγ	interferon gamma
IHC	Immunohistochemical
IL-10	interleukin-10
IL-1β	interleukin-1 beta
IL-6	interleukin 6
IL17	interleukin 17
IP	Intraperitoneal
IR	Insulin resistance

IRI	Ischemia-reperfusion injury
ITT	Insulin resistance test
IUA	Intrauterine adhesions
IV	Intravenous
JNK	c-Jun n-terminal kinase
KCs	Kupffer cells
Keap1	Kelch-like ECH-associated protein 1
L-02	human normal liver cell line
LD	Lipid droplet
LDL	low-density lipoprotein
LPA	Lysophosphatidic acid
LPAR1	lysophosphatidic acid receptor 1
LPCs	Lys phosphatidylcholines
LPS	Lipopolysaccharide
LT	Liver transplantation
MAFLD	Metabolic (dysfunction) associated fatty liver disease
MCD	methionine-choline deficient diet
MD	Mediterranean-style diets
MDA	malondialdehyde
MEOS	Microsomal ethanol oxidizing system
MMPs	Matrix metalloproteinases
MPK	AMP-activated protein kinase
MPO	Hepatic myeloperoxidase
MSC	Mesenchymal stem cells
MSCs	mesenchymal stem cell
MenSCs	Menstrual blood-derived mesenchymal stem cells
NAD+	Nicotinamide adenine dinucleotide

NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCD	Normal chow diet
NCTC1469 cells	normal mouse hepatocytes
NF-κB	nuclear factor kappa B
NIAAA	According to Alcohol Abuse and Alcoholism
NK	Natural killer
NLRs	NOD-like receptors
NQO-1	NAD(P)H quinone dehydrogenase 1
Nrf2	nuclear factor erythroid 2-related factor 2
OXPHOS	oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic Acid Schiff
PI3K	phosphoinositide 3-kinase
POF	Premature ovarian failure
PPARa	Peroxisome proliferator-activated receptor alpha
PV	per vaginal
RAS	renin-angiotensin system
ROS	reactive oxygen species
S1P	Sphingosine-1-phosphate
S1PR1	sphingosine-1-phosphate receptor 1
S1PR3	sphingosine-1-phosphate receptor 3
SERCA	the sarco-/endoplasmic reticulum (ER/SR) Ca2+ ATPase
SGLT2	Sodium-glucose cotransporter-2
SHED	Stem cells from human exfoliated deciduous teeth
SHED-CM	human exfoliated deciduous teeth

SIP	sphingosine-1-phosphate
SIRT1	sirtuin 1
SREBP-1C	Sterol regulatory element-binding protein 1C
SkMSCs	skeletal muscle-derived mesenchymal stem cells
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
TGF-β	transforming growth factor β
TIMPs	Metalloproteinases
TLR4	toll-like receptor 4
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor alpha
TUNEL	TdT-mediated dUTP nick end labeling
TWEAK	tumor necrosis factor-like weak inducer of apoptosis
UC-MSC	Umbilical cord mesenchymal stem cell
UPR	Unfolded protein response
WHO	World health organization
hSkMSCs	Human skeletal muscle satellite cell-derived mesenchymal stem cells
iNOS	inducible nitric oxide synthase
mTOR	mammalian target of rapamycin
rmTSG-6	recombinant mouse tumor necrosis factor-stimulated gene-6

LIST OF APPENDICES

PENILAIAN MEKANISTIK SEL TUNJANG ENDOMETRIUM YANG DIPEROLEH DARIPADA DARAH HAID DALAM MODEL MURIN PENYAKIT HATI BERLEMAK ALKOHOLIK DAN BUKAN ALKOHOLIK

ABSTRAK

Penyakit hati berlemak (FLD), yang merangkumi penyakit hati berlemak alkoholik (ALD) dan penyakit hati berlemak bukan alkoholik (NAFLD), merupakan penyakit hati kronik yang utama. ALD adalah antara 30 penyebab utama kematian di seluruh dunia, manakala prevalens NAFLD telah meningkat kepada 38%. Kedua-dua penyakit ini boleh berkembang kepada steatohepatitis, sirosis, dan akhirnya karsinoma hepatoselular (HCC), rawatan ubat yang tersedia pada masa ini mempunyai banyak kesan sampingan. Sel induk mesenkimal yang berasal daripada sel endometri atau darah haid manusia (MenSCs) menawarkan kelebihan yang ketara berbanding dengan sumber MSC yang lain kerana cara pengumpulan yang tidak invasif, akses etika yang lebih baik, hasilan yang lebih tinggi, dan kadar proliferasi yang tinggi. Oleh demikian, MenSCs berpotensi untuk digunakan sebagai pilihan terapeutik.Walau bagaimanapun, aplikasi khusus dan mekanisme MenSCs dalam merawat ALD dan NAFLD masih dalam tahap penyelidikan. Sehubungan dengan itu, kajian ini bertujuan untuk meneroka kesan terapeutik dan mekanisme molekul MenSCs terhadap ALD dan NAFLD dalam tikus. MenSCs pada awalnya diberikan kepada model tikus ALD yang diaruh dengan alkohol dan NAFLD yang diaruh dengan diet tinggi lemak untuk menilai kesan perlindungannya. Penilaian dilakukan berdasarkan pelbagai penanda biokimia, analisis histologi, penjujukan RNA, serta analisis gen dan protein untuk menjelaskan peranan MenSCs dalam merawat ALD dan NAFLD. Hasil kajian ini menunjukkan bahawa rawatan MenSCs telah memperbaiki fungsi hati secara

signifikan, melalui pengurangan ALT dan AST. Selain itu, pengurangan steatosis hepatik dan perubahan positif dalam ekspresi mRNA dan protein Fasn, Srebp1, SCD1, CPT1, Acadn, dan PPARα menunjukkan bahawa metabolisme lipid juga bertambah baik selepas rawatan MenSCs. Perubahan signifikan pada IL-1β, IL-10, TNF-α, IL-6, dan CCL2 selepas rawatan MenScs membuktikan bahawa keradangan dan apoptosis juga berkurangan. MenSCs juga berjaya mengurangkan tekanan hepatik melalui pengawalturan proses autophagi, tekanan ER, dan penanda tekanan oksidatif seperti Beclin1, LC3, P62, ATF4, ATF6, CHOP, NRF2, HO-1, dan GCLC. Selain itu, pengurangan ekspresi Col1a1, Ctgf, α-SMA, dan TGF-β serta pemulihan morfologi tisu menunjukkan bahawa fibrosis berkurang secara signifikan selepas rawatan MenSCs. Kesimpulannya, kajian ini berjaya membina model tikus ALD dan NAFLD dan menunjukkan bahawa MenSCs dapat mengawal fungsi hati, mengurangkan pengumpulan lemak, keradangan, dan apoptosis, mengurangkan tekanan ER dan tekanan oksidatif, mengurangkan fibrosis serta meningkatkan proses autofagi. Hasil daripada kajian ini menonjolkan potensi terapeutik MenSCs yang dapat digunakan untuk penyelidikan masa depan serta aplikasi klinikal dalam ALD dan NAFLD.

MECHANISTIC EVALUATION OF MENSTRUAL BLOOD-DERIVED ENDOMETRIAL STEM CELLS IN MURINE MODELS OF ALCOHOLIC AND NON-ALCOHOLIC FATTY LIVER DISEASE

ABSTRACT

Fatty liver disease (FLD), encompassing alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), is a major chronic liver condition. ALD is among the top 30 causes of death globally, while NAFLD prevalence has risen to 38%. Both conditions can progress to steatohepatitis, cirrhosis, or ultimately hepatocellular carcinoma (HCC), current available treatment drugs have many side effects. Human endometrial or menstrual blood-derived mesenchymal stem cells (MenSCs) offer significant advantages over other mesenchymal stem cells (MSC) sources due to their non-invasive collection, ethical accessibility, higher yield, and rapid proliferation rates, thus making them a promising therapeutic option. However, the specific applications and mechanisms of MenSCs in treating ALD and NAFLD are still under investigation. This study aimed to explore the therapeutic effects and molecular mechanisms of MenSCs on ALD and NAFLD in mice. MenSCs were initially administered to mouse models of ALD induced by alcohol and NAFLD induced by a high-fat diet to assess their protective effects. The evaluation utilized a comprehensive array of biochemical markers, histological analyses, RNA sequencing, and gene and protein analyses to elucidate the roles MenSCs in ameliorating ALD and NAFLD. The results showed that MenSCs treatment significantly improved liver function, as indicated by reductions in ALT and AST. Lipid metabolism improved, with decreased hepatic steatosis and favourable changes in mRNA and protein levels of Fasn, Srebp1, SCD1, CPT1, Acadn, and PPARα. Inflammation and apoptosis were reduced, as evidenced by changes in

IL-1β, IL-10, TNF-α, IL-6, and CCL2. MenSCs also alleviated hepatic stress by modulating autophagy, ER stress, and oxidative stress markers such as Beclin1, LC3, P62, ATF4, ATF6, CHOP, NRF2, HO-1, and GCLC. Additionally, fibrosis was significantly mitigated, as shown by decreased expression of Col1a1, Ctgf, α-SMA, and TGF-β and improved tissue morphology. This study successfully established ALD and NAFLD mouse models and demonstrated that MenSCs administration effectively regulated liver function, reduced fat accumulation, inflammation, and apoptosis, mitigated ER stress and oxidative stress, alleviated fibrosis, and improved autophagy. These findings highlight the therapeutic potential of MenSCs and pave the way for future research and clinical applications in ALD and NAFLD.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Fatty liver disease (FLD) is characterized by the accumulation of fat in more than 5% of liver cells, as observed through histology. FLD is classified into alcohol-associated liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). ALD encompasses as a spectrum of liver injuries caused by alcohol consumption, from simple fatty liver to more severe forms like alcoholic hepatitis, alcohol-related cirrhosis, and acute alcoholic steatohepatitis. This condition is commonly seen in individuals who consume more than one standard drink per day for women or more than two for men (Crabb *et al.*, 2020; Staufer and Stauber, 2023). On the other hand, NAFLD is diagnosed when there is evidence of liver fat accumulation ($\geqslant$5%) via imaging or histological analysis, in the context of minimal alcohol consumption ($\leq$21 standard drinks per week for men and $\leq$14 standard drinks per week for women, where one standard drink equivalent to 14 grams of ethanol). The diagnosis of NAFLD also involves ruling out other potential causes of liver fat buildup, such as excessive alcohol use, prolonged use of fat-inducing medications, or genetic disorders. NAFLD is often linked to metabolic issues, such as obesity, diabetes, and dyslipidemia (Chalasani *et al.*, 2018; Staufer and Stauber, 2023).

1.1.1 Alcohol-associated liver diseases (ALD)

ALD and its severe complications caused by excessive alcohol consumption and can further progress into more serious issues including alcohol associated hepatitis (AH); which is severe form of alcohol steatohepatitis (ASH), alcohol-associated cirrhosis (AC), and hepatocellular carcinoma (HCC) (Wakil *et al.*, 2023). The

advancement of ALD is mainly associated with the amount and duration of alcohol usage. For example, initial stages of AH, occur when more then 50g of alcohol is consumed daily (Hoofnagle and Doo, 2024). Thus, this pathological condition is potentially reversible when alcohol consumption is reduced. However, its development is also shaped by genetic, epigenetic, and environmental influences (Higuera-de-la-Tijera *et al.,* 2022). The pathogenesis of ALD was summarized in Table 1.1.

Currently, 43% of the global population consume alcohol, and three million people die annually due to excessive alcohol consumption (Devarbhavi *et al.*, 2023). World Health Organization (WHO) reports that Europe consumes the highest amount of alcohol per capita annually and is followed by North and South America (Higuera-de-la-Tijera *et al.*, 2022). Likewise, alcohol use disorder (AUD) affects 5.1% of the worldwide population, with the European Region showing the greatest prevalence, followed by the Americas (Devarbhavi *et al.*, 2023). Around 35% of AUD individuals are likely to develop to ALD. Globally, excessive alcohol consumption is the main contributor to cirrhosis, accounting for nearly 60% of cirrhosis cases in Europe, North America, and Latin America. Excessive alcohol consumption is the main contributor to cirrhosis, accounting for nearly 60% of cirrhosis cases in North America, Europe, and Latin America (Stein *et al.*, 2016; Avila *et al.*, 2020).

1.1.2 Non-alcoholic fatty liver diseases (NAFLD)

NAFLD is a heterogeneous condition encompassing a wide range of liver disease. Recently, experts have recognized that the term NAFLD no longer aligns with the current understanding that metabolic dysfunction, rather than the mere absence of significant alcohol consumption or other known liver diseases, is the primary driver of fatty liver disease. Consequently, metabolic (dysfunction)-associated fatty liver

disease (MAFLD) has been recognized as a more accurate term, and a set of criteria has been established for diagnosing and assessing individuals with this condition (Gofton *et al.*, 2023). NAFLD or MAFLD has been identified as an emerging primary cause of fibrosis, steatosis, cirrhosis, and hepatocellular carcinoma (Sanyal *et al.*, 2021). The pathogenesis of NAFLD was summarized in Table 1.1.

Recent data suggests that the worldwide occurrence of NAFLD has grown by over 50% over the last three decades, with rates rising from 25.3% to 38.2% (Younossi *et al.*, 2023). Studies have also indicated that individuals with NAFLD face a higher risk of all-cause mortality compared to the general population, as well as an increased likelihood of developing extra-hepatic conditions, including cardiovascular disease (CVD), chronic kidney disease (CKD), and certain extra-hepatic cancers (Mantovani *et al.*, 2020b). Notably, immediate family members of those with NAFLD-related cirrhosis experience a remarkable 12-fold higher risk of developing advanced fibrosis due to genetic factors (Johnston *et al.*, 2020). In a similar light, upward trends in obesity, insulin resistance, type two diabetes, hypertension and cardiovascular disease have also been observed (Younossi *et al.*, 2019; Xie *et al.*, 2023). This comes as no surprise as the metabolic disorders mentioned above have been linked to NAFLD and this translates to increased healthcare burden globally (Golabi *et al.*, 2021).

Table 1.1 Comparison of the pathogenesis of ALD and NAFLD

Aspect	ALD	NAFLD	Ref.
Primary etiology	Excessive alcohol consumption	Metabolic dysfunction, obesity, insulin resistance	
Lipid accumulation	Alcohol promotes lipogenesis and inhibits fatty acid oxidation, leading to hepatic fat accumulation	Insulin resistance suppresses ATGL, increasing FFA influx into the liver while PPAR-α inactivation reduces fatty acid oxidation, further exacerbating steatosis	(Tanase *et al.*, 2020; Odriozola *et al.*, 2023)
Inflammation	Alcohol induces Kupffer cell activation, promoting the release of pro-inflammatory genes, which drive hepatocyte injury	Characterized by chronic inflammation, with increased levels of pro-inflammatory genes, contributing to lipotoxic liver damage	(Chen *et al.*, 2020b; Dukic *et al.*, 2023)
Oxidative stress	Alcohol metabolism generates ROS, leading to lipid peroxidation and mitochondrial damage	Mitochondrial β-oxidation dysfunction generates ROS, increased oxidative stress	(Chen *et al.*, 2020b; Zhao *et al.*, 2021)

Continued

ER stress	Ethanol metabolism disrupts ER homeostasis, leading to UPR activation	Hepatic lip toxicity and insulin resistance induces accumulation of misfolded proteins in the ER	(Chen *et al.*, 2020b; Xia *et al.*, 2020)
Fibrosis	Acetaldehyde and alcohol metabolites directly activate HSCs, leading to excessive ECM deposition and fibrosis progression	Chronic inflammation and metabolic stress activate TGF-β and HSCs, promoting fibrosis,	(Nassir, 2022; Subramaiyam, 2023)

Abbreviations: ATGL adipose triglyceride lipase, FFA increasing free fatty acid, UPR unfolded protein response, HSCs hepatic stellate cells, ECM extracellular matrix, ROS reactive oxygen species, TGF-β transforming growth factor-β.

1.2 Rationale and importance of the study

1.2.1 Rational and importance of MenSCs in ALD

Better treatment outcomes for ALD can be achieved if addressed before it progresses to the cirrhosis stage. The current treatment approaches for ALD emphasize key strategies, such as strict alcohol abstinence (Rogal et al., 2020; EASL, 2023), proper nutrition (Bischoff et al., 2020), and pharmacological interventions (Lieber et al., 2018). However, their efficacy remains limited, and patient adherence is often poor. While alcohol abstinence is the cornerstone of ALD management, the relapse rate is high, making long-term adherence challenging for many patients (Sehrawat et al., 2020; Mellinger et al., 2023). Pharmacological treatments, such as corticosteroids, are used in severe alcoholic hepatitis, yet their efficacy varies among individuals and is associated with increased risks of gastrointestinal bleeding and sepsis(Crabb et al., 2020). Other hepatoprotective agents, including N-acetylcysteine and S-adenosylmethionine, have been used as supportive therapies but lack robust clinical evidence(Liu et al., 2021). Once ALD progresses to liver fibrosis or cirrhosis, effective therapeutic options are scarce, and liver transplantation remains the only viable option for end-stage disease (Sehrawat et al., 2020). However, this approach is hindered by organ shortages, high costs, and the risk of post-transplant alcohol relapse (Terrault et al., 2023). Furthermore, chronic inflammation, oxidative stress, and gut microbiota dysbiosis, which play crucial roles in ALD pathogenesis, remain inadequately addressed by current treatments. This has prompted increasing research interest in novel therapeutic targets, such as inhibiting CYP2E1 and reducing reactive oxygen species (ROS), to develop more precise treatment approaches (Kong et al., 2019; Arab et al., 2020). Despite ongoing efforts, there are currently no officially approved

pharmacological therapies for ALD beyond risk factor management (Wong and Singal, 2019).

Mesenchymal stem cells (MSCs) are a diverse group of stem cells that originate from various tissues, including bone marrow, adipose tissue, and the umbilical cord, among others, have demonstrated significant potential in ameliorating a spectrum of liver metabolic disorders (Eom *et al.*, 2020; Watanabe *et al.*, 2021). The potential of MSCs as a treatment for liver diseases, including ALD, AH, and alcoholic cirrhosis, has been extensively studied in both experimental and clinical trials with no side effects (Suk *et al.*, 2016; Chung *et al.*, 2022). The effectiveness of MSC-based treatments in liver metabolic disorders is primarily attributed to their secretion of extracellular vesicles, various chemokines, and growth factors (Alfaifi *et al.*, 2018; Yang *et al.*, 2023). MSCs have been shown to mitigate ethanol-induced liver injury and steatosis, reduce hepatic lipid accumulation, and enhance liver function (Wan et al., 2020a). Additionally, MSCs exert anti-inflammatory effects (Chung et al., 2022) and alleviate oxidative stress in ALD (Wan *et al.*, 2020a; Wan *et al.*, 2020b). Notably, pre-conditioning human adipose-derived mesenchymal stem cells (hADMSCs) with lysophosphatidic acid (LPA) and/or sphingosine-1-phosphate (S1P) significantly attenuated lipid accumulation, inflammation, oxidative stress, and fibrosis, while simultaneously enhancing the activity of alcohol-metabolizing enzymes and improving liver function (Li et al., 2018).

MenSCs, a notable subset of MSCs, are noteworthy for their non-invasive collection method, ethical accessibility, higher concentration, and rapid proliferation rates (Liu *et al.*, 2018). These cells exhibit characteristics similar to other MSCs, such as spindle-shaped morphology, the capability to differentiate into the classical trilineage, and the presence of specific surface markers (Liu *et al.*, 2018; Chen *et al.*,

2019a; Chen *et al.*, 2019b; Chen *et al.*, 2020a). Despite comprehensive investigations into the applications of MenSCs in diverse metabolic disease models (Chen *et al.*, 2017; Du *et al.*, 2023), the understanding of their effects and potential mechanisms in the treatment of ALD remains limited.

1.2.2 Rational and importance of MenSCs in NAFLD

The management of NAFLD primarily focuses on weight management, dietary modifications, and physical activity. However, patient adherence remains a major challenge, leading to persistent disease progression (Rong *et al.*, 2022). Currently, there are no FDA approved pharmacological therapies specifically for NAFLD (Wong and Singal, 2019). Among the available options, vitamin E and pioglitazone have demonstrated some therapeutic benefits, yet their clinical applications are restricted by significant adverse effects. While vitamin E has been shown to reduce hepatic steatosis and inflammation, prolonged use may increase the risks of bleeding, prostate cancer, and heart failure (Rinella et al., 2023). Similarly, pioglitazone improves insulin resistance but is associated with weight gain, bladder cancer, and cardiovascular complications (Cusi et al., 2022). Recently, GLP-1 receptor agonists have shown promise in NAFLD management, but their long-term safety and tolerability require further investigation (Mantovani et al., 2020a; Mantovani and Dalbeni, 2021). Furthermore, once NAFLD progresses to liver fibrosis or cirrhosis, effective reversal therapies remain unavailable, and current management primarily focuses on symptom control and complication management (Nassir, 2022). Given the strong association between NAFLD and metabolic syndrome, research is increasingly directed toward targeting inflammation, oxidative stress, and metabolic dysregulation, though these approaches are still in preclinical or early clinical trial stages (Rong *et al.*, 2022).

Emerging evidence has demonstrated the therapeutic potential of MSCs in the treatment of NAFLD with no side effects. MSCs and their extracellular vesicles (MSCs-Exos) have drawn particular attention for their capability to regulate lipid metabolism (Li et al., 2019a; Cheng et al., 2021), modulate inflammation and apoptosis (Xu et al., 2020; Yang et al., 2021), and alleviate reactive oxygen species (ROS) accumulation, endoplasmic reticulum (ER) stress, and oxidative stress in NAFLD (Bi et al., 2021; Du et al., 2022a). MSC treatment has also been shown to contribute to an almost 50% reduction in collagen levels in NASH (Wang et al., 2018a; Nickel et al., 2022).

Given the advantages of MenSCs presented in the earlier sub-chapter 1.2.1, it could be a good option for NAFLD. However, their effects and underlying mechanisms in the treatment of NAFLD remain inadequately understood and require further investigation.

1.3 Hypothesis and objective of the study

1.3.1 Hypothesis

Null Hypothesis

MenSCs does not ameliorate ALD and NAFLD in mouse models.

Alternative Hypothesis (Hypothesis of this study)

MenSCs regulates lipid and glucose metabolism, inflammation, oxidative stress, ER stress, autophagy, and fibrosis in ALD/NAFLD.

1.3.2 Objective of the study

The primary aim of this study is to elucidate the therapeutic effects and molecular mechanisms of MenSCs in alleviating ALD and NAFLD. The specific objectives of this study are as outlined below:

1. To establish mouse models for ALD and NAFLD studies.

2. To study the influence of MenSCs on hepatic metabolism in the ALD and NAFLD.

3. To understand the modulatory functions of MenSCs in ALD and NAFLD associated lipid and glucose metabolism.

4. To investigate the roles of MenSCs in alleviation of ALD and NAFLD through anti-inflammatory and anti-apoptotic processes.

5. To evaluate the efficacy of MenSCs on reducing oxidative stress, ER stress, and autophagy in ALD and NAFLD.

6. To assess the effectiveness of MenSCs in reducing ALD and NAFLD associated liver fibrosis.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of alcohol-associated liver disease (ALD)

2.1.1 The prevalence of ALD

Alcohol misuse is a global health crisis, which stands as the primary etiological factor for liver disease (Hernandez-Evole et al., 2023). It increases the risk of death from liver disease by 260 times, while cardiovascular and cancer mortalities rates increase by factors of 3.2 and 5.1, respectively (Hagstrom et al., 2021; Devarbhavi et al., 2023). The overall prevalence of ALD in Asia was 4.81%, showing a significant upward trend over time. It increased from 3.82% between 2000 and 2010 to 6.62% between 2011 and 2020 (Xu et al., 2022). Additionally, alcohol is the top cause of cirrhosis worldwide, accounting for nearly half of all cirrhosis case globally. Moreover, the occurrence of alcohol-associated hepatitis has risen over the past few years (Arab *et al.*, 2019; Devarbhavi *et al.*, 2023). In Malaysia, alcohol-attributable liver disease remains a significant concern, with alcohol-related cirrhosis accounting for 9.48% of cases and alcohol-attributable hepatocellular carcinoma (HCC) comprising 5.80% of cases (Xu et al., 2022).

ALD, which is associated with excessive alcohol consumption, ranks among the top 30 causes of death globally and represents one of the most significant chronic liver conditions (Thursz *et al.*, 2019). AFLD, the initial stage of ALD, is presumably on the rise parallel to metabolic syndrome (Hamaguchi *et al.*, 2020). Recent data indicated an increase in the prevalence of ALD among male drinkers, rising from 22.3% in earlier cohorts to 36.6% in recent cohorts (Hamaguchi *et al.*, 2020). Results differed throughout the range of ALD, with the non-liver mortality rate at 43.4 per 1000 patient-

years in alcohol-related fatty liver and 22.5 per 1000 patient-years in alcoholic hepatitis. ALD patients faced a greater risk for all examined outcomes, with the relative risks of mortality linked to cardiovascular diseases (CVD), non-hepatic cancer, and infections (Theodoreson *et al.*, 2023). Furthermore, increased metabolic disturbances such as hyperglycaemia have shown both in men and women with ALD as compared to the normal population (25.4% vs 43.0% and 25.1% vs 39.1%) (Hamaguchi *et al.*, 2020).

2.1.2 ALD pathogenesis

The pathogenesis of ALD is multifactorial, involving intricate interactions among genetic, epigenetic, and environmental factors. Factors like alcohol, drinking habits, age and gender, obesity, ethnicity, smoking, and genetic predispositions are intricately associated with the onset and progression of ALD (Suresh *et al.*, 2020; Higuera-de-la-Tijera *et al.*, 2022). In the progression of ALD, various comorbidities may occur, including alcohol use disorders, cardiovascular and digestive issues, chronic infections, diabetes, and immunodeficiency (such as HIV/AIDS) (Mackowiak *et al.*, 2024). ALD includes a range of hepatic disorders, from simple steatosis, also known as alcoholic fatty liver (AFL), to more serious conditions like steatohepatitis, cirrhosis or fibrosis, and ultimately hepatocellular carcinoma (HCC) (Figure 2.2) (Zhao *et al.*, 2021).

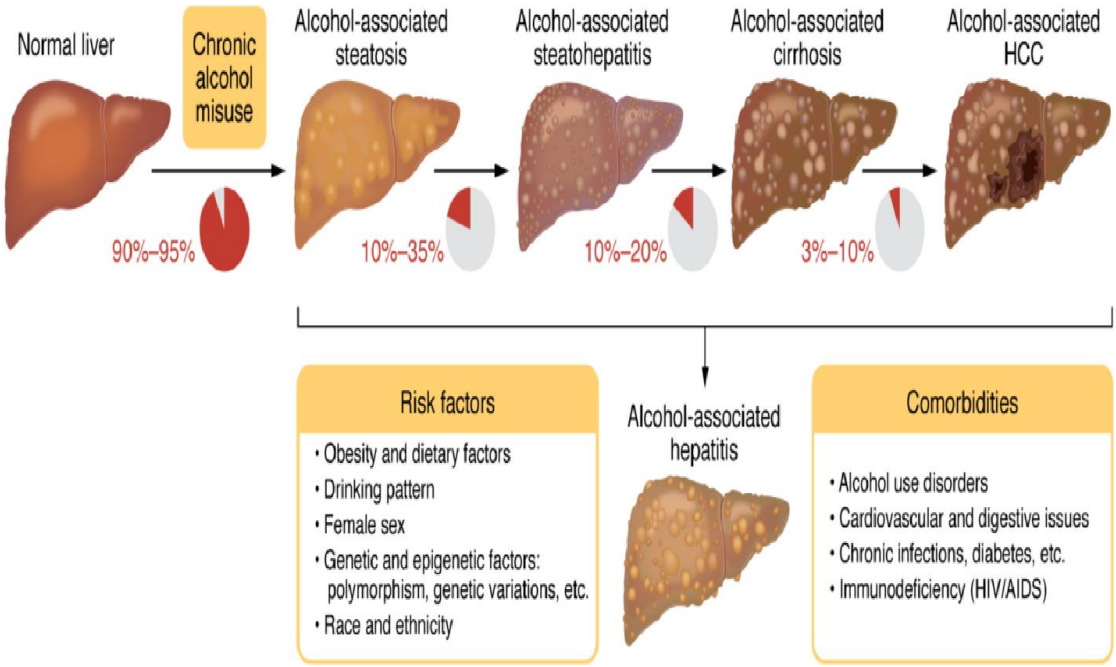

Figure 2.1 Spectrum of ALD, risk factors, and comorbidities (Mackowiak et al., 2024).

2.1.2(a) Dysregulation of alcohol metabolic pathways

About 90% of alcohol is eliminated by the liver (Jones, 2019). Alcohol is metabolized within the liver through three distinct enzymic pathways namely the cytosolic alcohol dehydrogenase system (ADH), the microsomal ethanol oxidizing system (MEOS) present in the endoplasmic reticulum, and the catalase oxidation pathway situated in peroxisomes (Figure 2.3). Predominantly, the ADH and MEOS pathways are responsible for the breakdown of alcohol, accounting for 80-90% and 10-20% of its metabolism, respectively, with less than 2% occurring through the catalase pathway (Wu *et al.*, 2023).

Alcohol metabolism is primarily facilitated by the action of cytosolic ADH, which catalyses the oxidative conversion of alcohol into acetaldehyde. The process involves the transfer from the alcohol molecule to nicotinamide adenine dinucleotide (NAD+), resulting in its reduction to NADH and the concomitant production of acetaldehyde (Buchanan and Sinclair, 2021). The excessive metabolism of alcohol

generates free radicals that compromises the stability of ADH, reducing its activity and thereby inhibiting the ADH metabolic pathway. A high NADH/NAD+ ratio affects carbohydrate metabolism and suppresses β-oxidation of fatty acids culminating in lipid accumulation in hepatic, thus precipitating early onset of fatty liver disease (Ceni *et al.*, 2014). Furthermore, this imbalance may cause an excessive flow of electrons in the mitochondrial electron transport system, with electrons accumulating and leaking at complexes I and III. This results in the generation of reactive oxygen species (ROS), triggering oxidative stress in liver (Edenberg, 2007; Kourkoumpetis and Sood, 2019; Zhao *et al.*, 2021).

MEOS is a metabolic pathway based on the cytochrome P450 enzyme system, which is predominantly located on the endoplasmic reticulum of liver cells. The various cytochrome P450 isozymes, CYP2E1 is the most crucial for catalysing the oxidation of alcohol (Buchanan and Sinclair, 2021). MEOS is an oxygen-consuming pathway that not only metabolizes alcohol but also generates a significant amount of ROS, resulting in oxidative stress in the liver (Liu *et al.*, 2021). Some free radicals can directly bind with ethanol to form hydroxyethyl free radicals. These hydroxyethyl free radicals reduce the activity of certain metabolic enzyme systems and the efficacy of functional glycoproteins in the liver. They also specifically bind with glutathione (GSH) to form stable complexes, leading to a decrease in GSH levels in the liver, which inhibits its resynthesis and thereby reduces the liver's ability to manage oxidative stress (Iain H McKillop, 2016; Teschke, 2018). The excessive accumulation of free radicals can also trigger lipid peroxidation within liver cells, thus compromising their membrane integrity, and leading to pathological changes which result in apoptosis (Teschke, 2018, 2019).

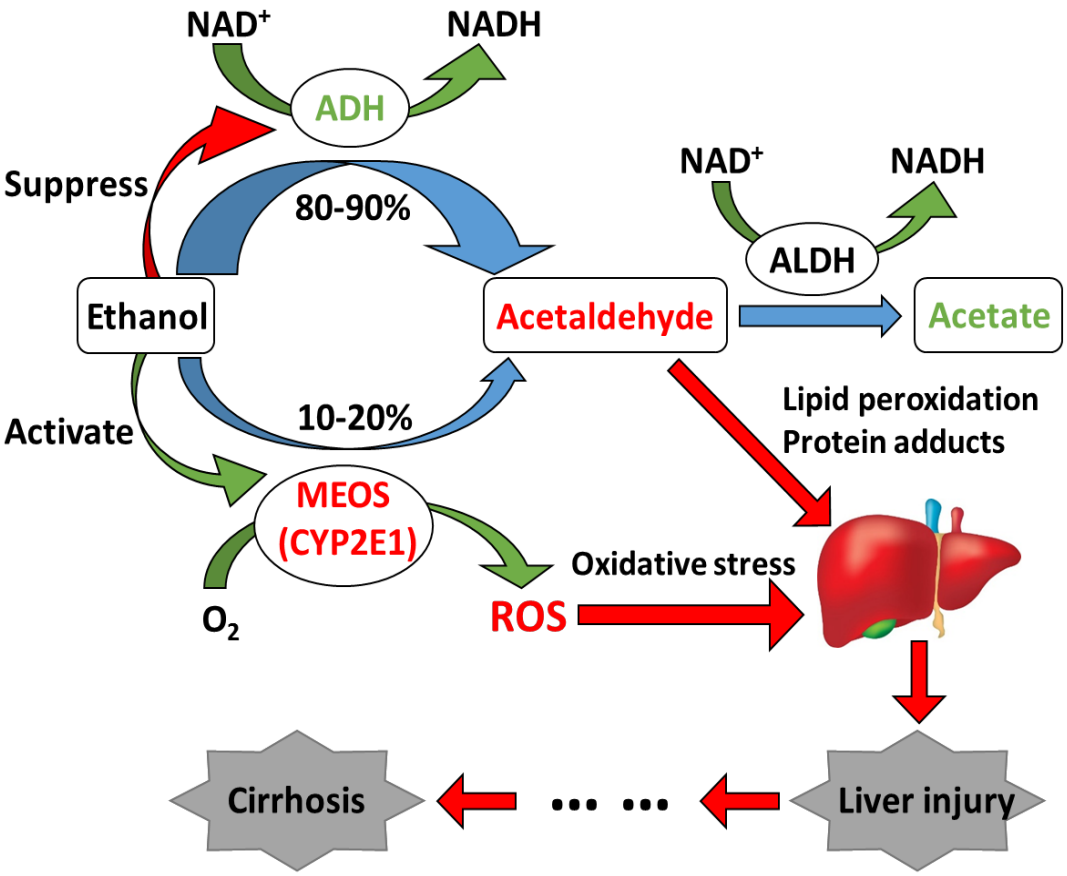

Figure 2.2 Metabolic pathways affected in the liver by excessive consumption (Xiao, 2020).

2.1.2(b) Dysregulation of metabolic pathways in the liver by excessive consumption of alcohol

Excessive alcohol consumption has been associated with the development of ALD due to the lipid accumulation such as triglycerides, cholesterol esters, and phospholipids within hepatocytes (Zhao *et al.*, 2021). This accumulation can progress through various stages of steatosis to AH, characterized by cellular ballooning and lobular inflammation (Zhao *et al.*, 2021). The activation of hepatic stellate cells plays a vital role in the development of alcohol-induced hepatic fibrosis, which potentially leads to cirrhosis (Buchanan and Sinclair, 2021). Chronic alcohol intake is also a recognized cause of HCC, with implicated mechanisms including acetaldehyde toxicity, cytochrome P4502E1 enzyme, angiogenesis induction, angiogenesis, and

oncogene mutations (Huang *et al.*, 2023). Despite extensive research, the full mechanisms behind ALD are not yet completely understood. Nevertheless, a summary of the current knowledge is presented in the subsequent sub-sections.

2.1.2(b)(i) Lipid accumulation

Alcohol has been recognized as a significant inducer of hepatic lipid accumulation through various mechanisms affecting those fat mechanisms (Ceni *et al.*, 2014). Specifically, alcohol intake raises the levels reduced NADH in hepatocytes, which in turn inhibits mitochondrial β-oxidation of fatty acids, culminating in steatosis (Herzig and Shaw, 2018). Additionally, the upregulation of SREBP1 and PPARα further promote the accumulation of hepatic fat (Yue *et al.*, 2021; Odriozola *et al.*, 2023).

Additional mechanisms also contribute significantly, notably the transformation of acetate, produced during acetaldehyde metabolism, into acetyl-CoA, a crucial precursor for synthesizing fatty acids (Odriozola et al., 2023). The consumption of alcohol also triggers lipolysis and adipocyte apoptosis, thereby increasing the concentrations of circulating fatty acids and accumulation in the liver (Odriozola et al., 2023). Furthermore, an enhanced lipid supply from the small intestine to the liver has been identified as a factor that facilitates the onset of steatosis (Herzig and Shaw, 2018).

2.1.2(b)(ii) Increase in inflammatory response

Alcohol and its metabolites, such as ROS, acetaldehyde, acetate, and lipopolysaccharide (LPS) derived from intestinal microbes, are pivotal in the development of ALD. These compounds significantly contribute to the inflammatory processes associated with ALD (Zhao *et al.*, 2021; Dukic *et al.*, 2023). Notably,

acetaldehyde and ROS are particularly detrimental to liver health, significantly contributing to inflammation (Le Dare *et al.*, 2019).

The inflammatory response in ALD involves not only immune system cells but also various resident liver cells like Kupffer cells and hepatic stellate cells. they are activated by a diverse array of exogenous and endogenous antigens (Dukic *et al.*, 2023). Specifically, hepatic inflammation is primarily triggered by gut-derived pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) released from stressed or dying hepatocytes (Seitz *et al.*, 2018). These PAMPs and DAMPs are recognized by Toll-like receptors (TLRs) and NOD-like receptors (NLRs) expressed on both immune cells and parenchymal cells in the liver. This recognition triggers the secretion of chemokines and cytokines, which further amplify the inflammatory response (Dukic et al., 2023). For example, LPS, a PAMP derived from gut bacteria, is recognized by TLR4. This binding activates NF-κB, resulting in the generation of pro-inflammatory chemokines and cytokines (Guijarro-Munoz *et al.*, 2014). Additionally, LPS stimulates Kupffer cells through the TLR4-mediated pathway, resulting in the release of macrophage inflammatory protein-2 and monocyte chemoattractant protein-1, along with key pro-inflammatory cytokines like IL-1, IL-6, and TNF-α, further intensifying the inflammatory response in ALD (Slevin *et al.*, 2020).

2.1.2(b)(iii) Accumulation of ROS and increase in oxidative stress

Exposure to alcohol results in liver injury through the modulation of various cellular processes, notably involving the generation of ROS and the resulting oxidative stress (Zhao *et al.*, 2021; Sundar and Saraswathi, 2023).

The generation of ROS related to alcohol consumption involves multiple pathways, primarily the induction of the enzyme CYP2E1 and alcohol-induced

inflammation (Leung and Nieto, 2013; Harjumaki *et al.*, 2021). The enzyme CYP2E1 plays a vital role in enhancing the activity of NADPH oxidase. This increased activity accelerates the mitochondrial transport of reduced NADH, leading to increased electron leakage from the mitochondrial respiratory chain of hepatocyte and, consequently, elevated ROS production (Harjumaki *et al.*, 2021). In the context of alcohol-induced inflammation, the generation of tumour necrosis factor (TNF) is implicated in facilitating the interaction between N-acetyl sphingosine and mitochondria, ultimately contributing to further ROS generation (Zhao *et al.*, 2021; Dukic *et al.*, 2023). Additionally, acetaldehyde, produced via both non-oxidative and oxidative metabolic pathways of alcohol, is a toxic compound that causes mitochondrial alterations. These alterations include a decrease in ATP generation through the respiratory chain and additional ROS generation in hepatocytes (Odriozola *et al.*, 2023). Moreover, alcohol itself is toxic and promotes oxidative stress, further mediated by ROS (Liu *et al.*, 2021).

Oxidative stress in the liver fundamentally stems from a disproportion between ROS and antioxidants. This condition is often triggered by the overproduction of ROS, leading to oxidative stress in liver, a key contributor in alcohol-induced liver damage (Kourkoumpetis and Sood, 2019; Zhao *et al.*, 2021).

2.1.2(b)(iv) Increase in endoplasmic reticulum (ER) stress

Alcohol consumption leads to the buildup of misfolded or unfolded proteins within the ER lumen, then triggering ER stress. The stress activates unfolded protein response (UPR) to maintain ER homeostasis. The UPR mitigates ER stress by decreasing protein synthesis, enhancing the production of enzymes involved in protein folding, and promoting protein degradation (Maiers and Malhi, 2019; Buyco *et al.*, 2021). Nonetheless, prolonged alcohol intake could exacerbate ER stress. Prolonged

ER stress may surpass the adaptive capabilities of the UPR, resulting in additional disruptions in hepatocyte function (Xia *et al.*, 2020).

Alcohol metabolism and the resulting ROS are key factors to ER stress. During alcohol metabolism via the ADH and MEOS pathways, substantial amounts of NADH or $NADP^+$ are produced, leading to ROS accumulation. An overproduction of ROS can disrupt the redox equilibrium in the ER, impairing the function of disulfide bond isomerases and leading to the buildup of unfolded proteins, which triggers ER stress (Aghara et al., 2023). Additionally, alcohol disrupts lipid metabolism, further contributing to ER stress. Dysregulation of lipid metabolism caused by alcohol can result in lipid accumulation in the ER membrane, resulting in activation of the UPR and ER stress (Xia *et al.*, 2020). Moreover, free fatty acids can disrupt protein folding and contribute to ER stress (Lepretti *et al.*, 2018). Changes in the composition and amount of lipids in the ER membrane can alter its fluidity and impair the function of membrane proteins, further leading to ER stress (Na *et al.*, 2023). In ALD, chronic ER stress and UPR activation can result in lipid metabolic disorders, hepatocyte inflammation, and apoptosis, contribute to the progression of liver inflammation and fibrosis (Liu and Green, 2019; Na *et al.*, 2023).

2.1.2(b)(v) Inhibition of autophagy

Autophagy is a dynamic process that is crucial for targeting damaged and excess cytoplasmic organelles for lysosomal degradation. This mechanism significantly counteracts the harmful effects of ROS causes oxidative stress, a key driver in the advancement of ALD (Salete-Granado *et al.*, 2023). Autophagy is essential not only for regulating hepatocyte function but also for affecting non-parenchymal cells, including macrophages, endothelial cells, and hepatic stellate cells, all of which are pivotal in the development of liver disease (Allaire et al., 2019).

In the context of ALD, acute alcohol exposure initially induces a protective and adaptive autophagy. However, chronic alcohol exposure suppresses hepatic autophagy, thereby contributing to the progression of ALD (Qian et al., 2021a). This suppression, termed “insufficient autophagy”, involved decreased lysosome number and impaired lysosomal functions in hepatocytes (Chao et al., 2018a). Experimental models in mice with chronic alcohol feeding plus acute binge (Gao-binge) demonstrate autophagy insufficiency and liver injury (Chao et al., 2018b). Concurrently, ER stress, occurring alongside oxidative stress and mitochondrial dysfunction, further complicates the autophagy landscape in ALD (Liu and Green, 2019). This complex relationship intertwines with apoptosis induced by ER stress, involving critical signalling pathways like PERK/ATF4, IRE1α, ATF6, and $Ca2^{+}$ (Song et al., 2017). Moreover, apoptosis-related proteins can hinder autophagy by breaking down vital autophagy-associated proteins (Song et al., 2017).

2.1.2(b)(vi) Acceleration of fibrosis and cirrhosis

Liver fibrosis and cirrhosis in ALD primarily arise from alcohol-induced hepatocyte injury, inflammation, and subsequent immune responses that active hepatic stellate cells (HSCs) (Nagy et al., 2016). These cells migrate, proliferate, and enhance extracellular matrix (ECM) deposition, driving the fibro genic process (Nagy et al., 2016).

The activation of HSCs plays a crucial role in the development of liver fibrosis and cirrhosis in ALD. Alcohol or acetaldehyde-induced hepatocellular damage leads to the release of hedgehog ligands (Subramaiyam, 2023). These ligands activate hedgehog-responsive genes and HSCs through a paracrine mechanism, resulting in ECM deposition in sinusoidal areas and around swollen hepatocytes, leading to pseudo lobular formation (Lackner and Tiniakos, 2019). Other crucial pathways in HSC

activation involve Kupffer cell activation and the release of growth factors and chemokines. These processes are stimulated by DAMPs from dead hepatocytes, and by PAMPs and LPS translocating from a compromised gut due to alcohol (Fujii and Kawada, 2014; Nagy et al., 2016). Moreover, the fibrogenesis process is sustained by ECM components like collagen-1 and integrin, which support the survival of activated HSCs. Conversely, alcohol consumption hinders the natural killer (NK) cell-mediated clearance of HSCs (Seitz *et al.*, 2018; Lackner and Tiniakos, 2019; Zhao *et al.*, 2022b). Activated HSCs produce tissue inhibitors of metalloproteinases (TIMPs), which inhibit the fibro lytic activity of matrix metalloproteinases (MMPs). Combined with hepatocyte loss in septal PCF regions, this contributes to the progression of fibrosis, marked by denser collagen deposition and the buildup of elastic fibres and clusters. The continuous fibrogenesis and loss of liver parenchyma ultimately disrupt the lobular architecture, leading to cirrhosis (Lackner and Tiniakos, 2019; Zhao *et al.*, 2022b).

2.1.2(b)(vii) Induction of apoptosis

Alcohol metabolism in hepatocytes triggers apoptosis through both the extrinsic (death receptor) and intrinsic (mitochondria) pathways (Subramaiyam, 2023). The extrinsic pathway involves the activation of death receptors, which is induced by alcohol-induced ER stress, ROS production, and increased gut permeability. This leads to the liver influx of LPS derived from the gut microbiome, activating hepatocytes and Kupffer cells. Consequently, pro-inflammatory cytokines are triggered. These cytokines initiate death receptor signalling, leading to apoptosis and necroptosis mediated by caspase-8 activity (Miyata and Nagy, 2020). Meanwhile, oxidative stress triggers the intrinsic pathway, leading to DNA damage and alterations in growth factors (Shojaie *et al.*, 2020). This results in mitochondrial dysfunction, causing

cytochrome c to be released into the cytoplasm. There, it combines with Apaf-1 and caspase 9 to forming the apoptosome, which subsequently triggers the activation of downstream caspases 3, 6 and 7 to carry out apoptosis (Namachivayam and Valsala Gopalakrishnan, 2021; Subramaiyam, 2023).

2.1.3 Current management and treatment strategies for ALD

The medical management of ALD emphasizes several key strategies which includes unwavering alcohol abstinence, ensuring adequate nutritional support, early detection and treatment of infections, addressing conditions such as acute kidney injury (AKI), managing the complications resulting from portal hypertension, and, in specific cases, employing corticosteroids. Furthermore, recent guidelines provide a detailed treatment framework for ALD which includes target therapies as well (Arab et al., 2023).

2.1.3(a) Alcohol abstinence: a core management strategy

Complete alcohol abstinence is crucial in improving clinical outcomes across all disease stages and for reducing mortality rates in ALD (Rogal *et al.*, 2020). For ALD patients, particularly those with AH or cirrhosis, even minimal alcohol consumption poses substantial risks. These risks include an increased likelihood of decompensating events, including variceal bleeding, ascites, and hepatic encephalopathy, a higher incidence of HCC, elevated recurrence rates of cirrhosis and mortality after transplantation, and an overall rise in liver-related mortality (Mellinger et al., 2023). In cases of severe ALD, assessment by a cessation team, which include psychiatrists and addiction specialists, is essential in addition to the recommendation of medications such as baclofen, lorazepam, and oxazepam (Cabezas, 2022).

Studies indicate that approximately 30% of patients maintain complete abstinence during follow-up (Lackner et al., 2017). However, achieving total abstinence is challenge for patients with alcohol induced liver disease as relapse rates remain high (Sehrawat *et al.*, 2020; Mellinger *et al.*, 2023). Regarding abstinence, although 27% of patients with ALD may achieve histological normalization by abstinence from alcohol, 18% still at risk progressing to cirrhosis (Lackner et al., 2017). While abstinence does not assure full recovery from the disease, it is crucial to ALD management. These findings highlight the importance of early diagnosis and intervention in ALD, emphasizing the need for both abstinence and complementary treatment strategies.

2.1.3(b) Nutritional support in ALD care

In the context of advanced ALD, malnutrition, sarcopenia, and frailty pose significant risks. These conditions often resulting from protein damage and can increase the susceptibility to bacterial infections in individuals with ALD (Kong *et al.*, 2019; Ayares *et al.*, 2022). Malnutrition, particularly in those with AH, closely correlates with disease severity, complications, and mortality (Simonetto *et al.*, 2020).

The European Society for Clinical Nutrition and Metabolism (ESPEN) recommends that individuals with AH consume 1.2-1.5 g/kg of protein and 30-35 kcal/kg daily, increasing to 1.5 g/kg protein and 40 kcal/kg for patients with decompensated cirrhosis (Bischoff *et al.*, 2020). Nutritional supplements, including vitamin B, zinc, and vitamin D, are essential for maintaining calorie intake, reducing infection, and improving liver function (Bischoff *et al.*, 2020). Nutritional supplementation, as demonstrated in multiple randomized studies, supports the maintenance of necessary caloric intake and has been demonstrated to reduce the occurrence of infections, hasten the resolution of hepatic encephalopathy, and improve