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EPIGENETIC REGULATION OF INFLAMMATORY PATHWAYS IN ALCOHOL RELATED HEPATITIS

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EPIGENETIC REGULATION OF INFLAMMATORY PATHWAYS IN ALCOHOL RELATED HEPATITIS

by

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Author's Declaration

At no time during the registration of the degree of Doctor of Philosophy has the author been registered for any other university award without prior agreement of the Doctoral College Quality Sub-Committee.

This thesis has been proofread by both supervisors, no factual changes or additions or amendments to the argument were made because of this process.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Abstract

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Background

Alcohol-related liver disease (ArLD) contributes to approximately 1% of deaths worldwide and impacts on both economy and healthcare system. Alcohol related hepatitis (AH) - phenomenon of ArLD which is characterised by overactive inflammation is associated with 30% mortality rate at 90 days. Glucocorticoids (GC) are commonly used to treat AH. However, more than 1 in 3 patients fail to respond to GC therapy. Within the GC responders, the efficacy is variable due to individual glucocorticoid resistance. Improving our comprehension of GC non-responsiveness/ resistance is critical for early stratification of these patients and development of alternative therapies. This piece of research aimed to interrogate the role of oxidative stress and compare healthy volunteers (HVs) with AH survivors and non-survivors. These data aimed to assess if oxidative stress measurement can be used as a candidate prognostic biomarker in AH. The effects of oxidative stress on the activities of histone (a protein complex that provides structural support for a chromosome), histone gene expression and phosphoinositide 3-kinase delta (PI3Kδ) signalling were also examined, and followed by a trial of potential therapies to re-sensitize cellular response to GC.

Methods

Patients admitted to University Hospitals Plymouth NHS Trust who were diagnosed with AH were recruited. Their peripheral bloods were obtained and segregated into T cells and monocytes. Both cell subtypes were defined by their distinctive surface markers CD3+ and CD14+. 2'-7'dichlorofluorescin diacetate (DCFH-DA) staining which fluoresces on oxidation, was used to directly measure level of oxidative stress. Simultaneously, *ex vivo* exposure to tert-butylhydroperoxide (TBH) to stimulate maximal intracellular oxidative stress acts as positive control. Histone deacetylases (HDAC), histone acetyltransferases (HAT) and antioxidants expression were assessed by qPCR whereas functional activities of class I HDAC, HAT, and PI3Kδ were assessed using ELISA.

Results

In the flow cytometry experiments, percentage maximal oxidative stress in AH patients was significantly higher than HV in CD14+ monocytes. Among the AH patients, percentage maximal oxidative stress of non-survivors were higher

than survivors and close to maximal level. Within the HDAC genes, HDAC 2 and 8 gene expression were significantly upregulated in the survivor group. Further, the functional activities of class 1 HDACs, HATs and PI3Kδ were significantly downregulated in patients with AH in contrast to HVs; similarly in AH non-survivors when compared to survivors. Lastly, GC responsiveness were assessed with BrdU Lymphocyte Incorporation Steroid Sensitivity (BLISS) assay in cell cultures which were treated with Zinc, N-acetylcysteine, sodium butyrate and pan-HDAC inhibitor (Vorinostat). There was no statistically significant change in the *in vitro* GC responsiveness with these treatments.

Conclusion

Overall, this thesis presents novel data and highlights that both T cell and monocyte subsets of patients who did not survive AH, have consistently expressed near to maximal levels of oxidative stress. Therefore, oxidative stress can be used as a supplementary prognostic marker for AH. The significantly different expressions of HDAC and HAT genes and the functional activities of class 1 HDACs, HATs and PI3Kδ in AH patients suggest inflammation driven epigenetic modifications in AH. The work presented in this thesis represents a preliminary step forward in understanding the presence and implications of oxidative stress on epigenetic regulation of inflammatory pathways in AH. Further validation with large in-vitro human tissue models and clinical trials are warranted to investigate how these influence immune dysfunction in AH.

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List of Abbreviations

7 AAD- 7-aminoactinomycin D

A- Alive

ADH- Alcohol dehydrogenase

AH- Alcohol related hepatitis

AIH- Autoimmune hepatitis

AKT- Protein kinase B

ALDH- Aldehyde dehydrogenase

ALP- Alkaline phosphatase

ALT- Alanine transaminase

AP1- Activator protein 1

ArLD- Alcohol related liver disease

ASK- Apoptosis signal-regulating kinase 1

AST- Aspartate transaminase

ASH- Alcoholic steatohepatitis

ATAC-Seq- Assay for Transposase-Accessible Chromatin with high-throughput sequencing

AUC- Area under curve

BAX- BCL2- associated X protein

BCL2- B-cell CLL/lymphoma 2

BLISS- BrdU incorporation in lymphocyte steroid sensitivity assay

BSA- Bovine serum albumin

CAT- Catalase

CBG- Corticosteroid-binding albumin

CBP- cAMP response element binding protein

CCL20- Chemokine ligand 20

CCR2- C-C chemokine receptor type 2

ChIP- Chromatin Immunoprecipitation

ChIP-Seq- ChIP followed by high throughput sequencing

COPD- Chronic obstructive pulmonary disease

CREB- cAMP response element binding protein

CRH- Corticotrophin releasing hormone

CRP- C reactive protein

Cu- Copper

CXCL1- CXC motif chemokine 1

CXCL1/2- CXC receptor types 1/2

CXCL5- CXC motif chemokine 5

CXCL6- CXC motif chemokine 6

CYP2E1- Cytochrome p450 2E1 family member

D- Dead

DAMP- Damage associated molecular pattern

DC- Dendritic cell

DCF- 2,7-dichlorofluorescein

DCFH-DA- 2,7-dichlorofluorescein diacetate

Dex- Dexamethasone

DF- Maddrey's discriminant function

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

ECM- Extracellular matrix

EDTA- Ethylenediaminetetraacetic

EIF2A- Eukaryotic translation initiation factor 2A

EIF2AK3- Eukaryotic translation initiation factor 2 alpha kinase 3

ELISA- Enzyme-linked immunosorbent assay

ETC- Electron transport chain

FACS- Fluorescence activated cell sorting

FAS- FAS ligand

FoxO3- Forkhead box O3

FOXP3- Forkhead box P3

GAHS- Glasgow alcoholic hepatitis score

GC- Glucocorticoid

GCK- Glucokinase

GI- Gastrointestinal

GJA1- Gap junction protein 1

GM-CSF- Granulocyte-macrophage colony stimulating factor

GPX-1- Glutathione peroxidase 1

GR- Glucocorticoid receptor

GRE- Glucocorticoid response element

GRIP- GR interacting protein 1

GSH- Glutathione

GWAS- Genome wide association study

H^+- Hydron

H_2O_2- Hydrogen peroxide

H_2O- Water

HAT- Histone acetylase

HDAC- Histone deacetylase

HDM- Histone demethylases

Hep- Hepatocytes

HMT- Histone methyltransferases

HNE- 4-hyroxy-2-nonenal

HPA- Hypothalamic pituitary adrenal axis

HSC- Hepatic stellate cell

Hsp- Heat shock proteins

HSPA4- Heat shock protein 70kDa protein 4

HSPA5- Heat shock protein 70kDa protein 5

HV- Healthy volunteer

IFN- Interferon

IL-Interleukin

IL1 β- Interleukin beta

IL6- Interleukin 6

IL8- Interleukin 8

IL12- Interleukin 12

IL15- Interleukin 15

IL17- Interleukin 17

IMAX- Maximum inhibition with Dex

INR- International normalised ratio

IRF3- Interferon regulatory factor 3

JAK- Janus kinase

JNK- c-Jun N-terminal kinase

KC- Kupffer cells

KMT- lysine methyltransferases

LOOH- lipid hydroperoxides

LPS- Lipopolysaccharide

MAA- Malondialdehyde-acetaldehyde

MACS- Magnetic-activated cell sorting

MAPK- Mitogen-activated protein kinase

MDA- Malondialdehyde

MDF- Maddrey's discriminant function

MELD- Model for end-stage liver disease

MEOS- Microsomal ethanol oxidizing system

MiRNA- microRNA

MKK4- MAPK kinase 4

MKP1- Mitogen-activated protein kinase phosphatase 1

Mn- Manganese

MtDNA- Mitochondria DNA

MUP- Minimum unit pricing

MyD88- Myeloid differentiation primary response

NaBu- Sodium Butyrate

NAC- N-acetylcysteine

NAD^+- Nicotinamide adenine dinucleotide

NADPH- Reduced nicotinamide adenine dinucleotide phosphate

$NADP^+$- Nicotinamide adenine dinucleotide phosphate

Neut- Neutrophils

NF-κB - Nuclear factor kappa B

NF-κB1- Nuclear factor kappa light polypeptide gene enhancer in beta cells 1

NHS- National health service

NLR- Neutrophil to lymphocyte ratio

NK- Natural killer cells

NO- Nitric oxide

NOX- NADPH oxidase

O_2- Oxygen

OH^- -Hydroxide

P- Phosphorylation

PAMP- Pattern associated molecular pattern

PBMC- Peripheral blood mononuclear cell

PBS- Phosphate buffered saline

PCK1- Proprotein convertase subtilisin/kexin type 1

PCSK2- Proprotein convertase subtilisin/kexin type 2

PCR- Polymerase chain reaction

PD1- Programmed death 1 molecule

PHA- Phaetohaemagluttinin

PI3Kδ- Phosphatidylinositol 3 kinase delta

PRMT- Arginine methyltransferases

PT- Prothrombin time

QFM- Quantiferon monitor assay

R^2- Correlation coefficient

RCT- Randomised controlled trial

RNA- Ribonucleic acid

RPMI- Roswell Park Memorial Institute

ROC- Receiver operating characteristic curve

ROS- Reactive oxygen species

SAB- SH3 domain-binding protein

SIRS- Systemic inflammatory response syndrome

SOD1- Superoxide dismutase 1

SOD2- Superoxide dismutase 2

SREBP-1c- Sterol-element-binding-protein 1c

STAT- Signal transducer and activator of transcription

STAT5- Signal transducer and activator of transcription response 5

T_{eff}- Effector T cells

TBARS- Thiobarbituric acid reactive substances

TBH- Tert-butylhydroperoxide

Th17- T helper 17 cells

TF- Transcription factor

TIM3- T-cell immunoglobulin and mucin domain containing-3

TLR- Toll like receptor

TLR 2- Toll like receptor 2

TLR 4- Toll like receptor 4

TNF- Tumour necrosis factor

Ub- Ubiquitination

UC- Ulcerative colitis

UCP- Uncoupling protein

UHP- University Hospitals Plymouth NHS Trust

WCC- White blood cells

Zn- Zinc

$ZnSO_4$- Zinc Sulphate

Chapter 1: Introduction

Alcohol is the most universally received and misused substance in the world. Globally, the average alcohol consumption is 6.4L per person per year [1]. Alcohol attributable mortalities are responsible for roughly 3 million of all deaths annually [1]. This represents 5% of worldwide deaths and incidences are on the rise with each passing year [1]. This is no doubt a worrying public health issue [1].

In UK particularly, the average alcohol consumption is a staggering 9.7L per person per year [2]. There are over 500,000 alcohol dependent adults in England [2]. Each year, alcohol contributes to approximately 1.2 million hospital admissions, 23,500 deaths and 12,800 cases of cancer [3-5]. Alcohol continues to be one of the leading causes of premature deaths with an unprecedented increase in alcohol attributable deaths from 11.8 (per 100,000 populations) in 2019 to 14.0 in 2020 [3-5].

Among the alcohol attributable deaths, 77.8% were due to ArLD [5]. In 2020 alone, over five thousand ArLD deaths were recorded, a rise of almost 21% compared to 3% pre-COVID [3-6]. The spike in ArLD deaths has outnumbered cardiovascular disease and cancer for which mortality rate has remained stagnant or reduced [5, 6].

Unsurprisingly, ArLD is causing significant socioeconomic losses to individuals and society [7]. The commonest age of death secondary to ArLD is between 40 to 50 years old [7,8], and therefore the disease is costing £7.3 billion in lost productivity per year [7,8]. It is also inflicting the National Health Service (NHS) with disproportionate demand of resources and healthcare cost of over £3.5 billion per year [7,8].

Attempts to reduce premature mortality caused by the lifestyle issue of excess alcohol consumption and to treat ArLD effectively have been underway for years. As a result of advanced medical research, many stages and aspects of the pathogenesis of ArLD have been discovered. Of these, AH stands out as one which deserves further attention from clinical and research perspective. AH is a form of ArLD which is distinguished by florid inflammation and patients with severe AH has an unacceptably high mortality rate of 30% within 90 days [9].

To date, there are no efficacious therapies with impactful long term benefit for AH. The only therapy for AH is GC, which is only recommended for a small number of patients [9]. In addition, GC treatment has proven to have very limited long-term prognostic benefit [9]. This is why there is a need for major advances in understanding AH and new therapeutic directions.

1.1: Alcohol metabolism

The liver sustains greatest degree of tissue injury by hazardous drinking because the liver is the primary site of alcohol metabolism [10].

The body eliminates alcohol via different processes and there are three major pathways currently known to us (Figure 1) [10-12]. The chief pathway is via a route which mandates a NAD^+ requiring enzyme alcohol dehydrogenase (ADH) which metabolizes alcohol to acetaldehyde [10-12]. In a healthy liver, acetaldehyde then goes into the mitochondria and gets quickly processed into acetate by another enzyme named aldehyde dehydrogenase (ALDH) (Figure 1) [10-12]. Then, acetate is further broken down to end products which consist of carbon dioxide and water [10-12]. These end products then get eliminated out of the system.

For individuals who have drunk alcohol excessively over a sustained period of time (chronic users), their ADH/ALDH pathways are saturated [10-13]. When this happens, alcohol gets metabolized via the overflow pathway, which is known as the microsomal ethanol oxidizing system (MEOS) (Figure 1) [10-13]. This pathway oxidizes alcohol into acetaldehyde with help of hydrogen from NADPH and enzyme cytochrome CYP2E1 (Figure 1) [10-13]. NADPH then gets further reduced into NADP+ and H_2O [10-13]. This oxidized process prompts the generation of reactive oxygen species (ROS) [13-15].

ROS is usually neutralized by our inbuilt antioxidants [13-15]. However in the chronic alcohol users, the antioxidant defence system is often dysfunctional [13-15] due to depletion of mitochondrial glutathione (GSH) and impaired hepatocyte tolerance to inflammatory cytokines like tumour necrosis factor alpha (TNF-α) [16].

In these individuals, excessive unhindered ROS proceeds to activate c-Jun N-terminal kinase (JNK) and activator protein 1 (AP-1) transcription factor which stimulates lipid peroxidation [17-19]. Among the well-known end products of lipid peroxidation are 4-hydroxy-2-nonenal (HNE), lipid peroxides and malondialdehyde-acetaldehyde (MAA) [17-19]. In excess, these end products will tip the intracellular homeostasis into oxidative stress. They also bind to adenosine and cytosine to form protein adducts which subsequently adversely activate the innate immune system [17-19].

The third accessory pathway in alcohol metabolism utilizes peroxisomal enzyme catalase in the presence of H_2O_2 (Figure 1) [10-13]. Catalase converts ethanol to acetaldehyde while simultaneously breaking down H_2O_2 into H_2O [10-13]. This is the least used pathway due to low innate production of H_2O_2 [10-13]. In a healthy liver, up to 80% of the ingested ethanol is metabolized through the ADH pathway; the remainder 10-20% through MEOS pathway and only approximately 2% through the catalase pathway [10-13].

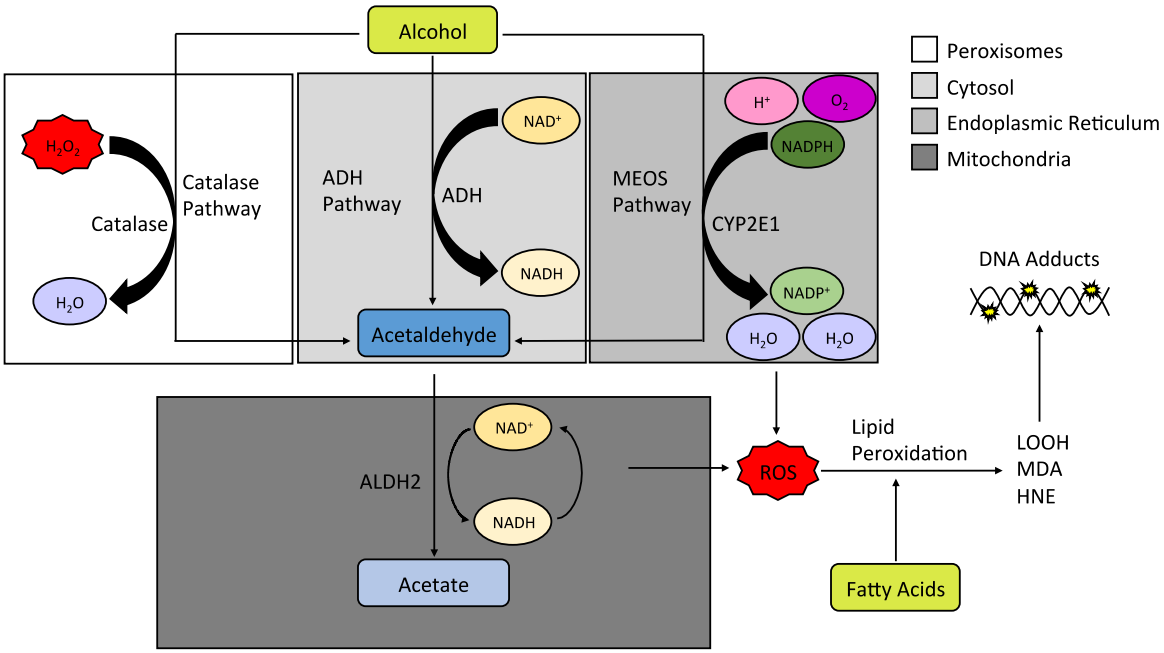


Figure 1 [11]: The three major pathways of alcohol metabolism. ADH-alcohol dehydrogenase; ALDH2-aldehyde dehydrogenase; CYP2E1-cytochrome P450 enzyme 2E1; DNA adducts- Deoxyribonucleic acid adducts; HNE- 4-hydroxy-2-nonenal; H_2O_2 – hydrogen peroxide; H_2O- water; H^+- hydron; O_2 -oxygen; NAD^+-Nicotinamide adenine dinucleotide; NADH- reduced Nicotinamide adenine dinucleotide; $NADP^+$- Nicotinamide adenine dinucleotide phosphate; NADPH-reduced Nicotinamide adenine dinucleotide phosphate; LOOH-lipid hydroperoxides; MDA- malondialdehyde; MEOS-microsomal ethanol oxidizing system; ROS-reactive oxygen species.

1.2: Development of ArLD and AH

Among the individuals who drink excessive amount of alcohol, about 35% of them will develop ArLD [20]. The rate and prevalence of progression is dependent on variables like age, concomitant substance misuse and health comorbidities [21-23].

ArLD consists of a range of stages, with fatty liver (medically known as steatosis) being the earliest transformation within the liver in response to heavy drinking [21-23]. It is distinguished by a surplus of fat formation in the liver which is then capable of progressing into inflammation within the liver cells (hepatocytes) [21-23].

If inflammation persists, the hepatocytes will slowly be replaced with permanent scar tissues comprised of extracellular matrix (ECM) and fibrillary collagen [21-24]. This process is called fibrosis [24]. If the insult towards the liver persists, advanced liver fibrosis precipitates further hepatic architecture distortion and vascular alteration [21-24]. This process consequently causes haemodynamic instability and development of portal hypertension which constitute the end stage of liver damage named cirrhosis [21-24].

Patients with a diagnosis of liver cirrhosis are predisposed to developing life-threatening complications for instance, liver cancer (hepatocellular carcinoma), bleeding from enlarged veins in the oesophagus or stomach (variceal bleeding) and disturbance to brain functionality (encephalopathy) [21-23].

AH is a distinctive clinical syndrome characterized by acute, over-activation of cellular inflammation driven liver injury [21,22,25]. AH can superimpose on any stage of ArLD described above. The occurrence of AH signifies deterioration of a patient's liver disease trajectory and prognosis [21,22,25]. The quantity and pattern of alcohol consumption can affect AH severity, whereas mortality from AH is associated with baseline physiological reserve, co-existent health comorbidity and infection propensity [21-25].

The precise incidence of AH remains unclear due to challenges in identifying patients from hospital attendance coding and lack of registry for this specific condition [25,26]. A study based on liver biopsy data demonstrated that in 1604 patients who drank alcohol excessively, 20% developed AH clinically [25,26]. From the prognosis perspective, 90 days mortality rate for patients with severe AH is as high as 30%, whilst for non-severe AH it's 7% [25,26].

To further our understanding of AH, we first need to dive into the effects of alcohol on the liver, gut, and immune systems.

Alcohol has undeviating impact on hepatocytes by producing acetaldehyde adducts and ROS in the process of alcohol metabolism [18, 19]. In a person who

drinks alcohol excessively, ROS is overproduced and accumulate in cells and tissues [27, 28]. The system's inability to detoxify these ROS generates a phenomenon called oxidative stress [19, 27, 28]. Studies have shown that oxidative stress can render both innate and adaptive immune systems dysfunctional [27-29]. This phenomenon is one of the hallmarks of AH.

This process involves the interferon regulatory factor 3 (IRF3) and TIR domain containing adapter inducing interferon-β (TRIF) [30, 31]. IRF3-TRIF signalling further induces production of ROS by NADPH oxidation [31, 32]. This triggers activation of NF-kappa B inflammatory pathway and histone acetylation [31, 32]. Further on, pathogen-associated molecular patterns (PAMPs), which are products from the gut microbiome, may signal via IRF3-TRIF which then triggers infiltrating macrophages and pro-inflammatory cytokines for instance, TNF-α, lL-1β, lL-17 and IL-8 into the portal circulation, leading to aberrant penetration and activation of immune cells into the tissues [33,34,35].

TNF-α produced by activated Kupffer cells invigorates sterol-element-binding-protein 1c (SREBP-1c), which further upregulates expression of pro-inflammatory cytokines spurring cell death [35-37]. When the system fails to get rid of these circulating pro-inflammatory cytokines, hepatocytes cascade into unabated inflammatory process and progress into AH [35-37].

In addition, alcohol has direct impact on the LPS-TLR system which upregulates the levels of negative inhibitory molecules programmed death 1 (PD-1) and T cell immunoglobulin and mucin domain 3 (TIM-3) on effector T cells [35-37]. The dysfunctional TLR system then opens the floodgate of pro-inflammatory chemokines and cytokines infiltration into the neutrophils, monocytes and Th17 cells [35-37]. This pathway stimulates fibrogenesis via the hepatic stellate cells; and drives hepatocytes into apoptosis/ necrosis [35-37]. Figure 2 shows the immune dysfunction processes of AH [11].

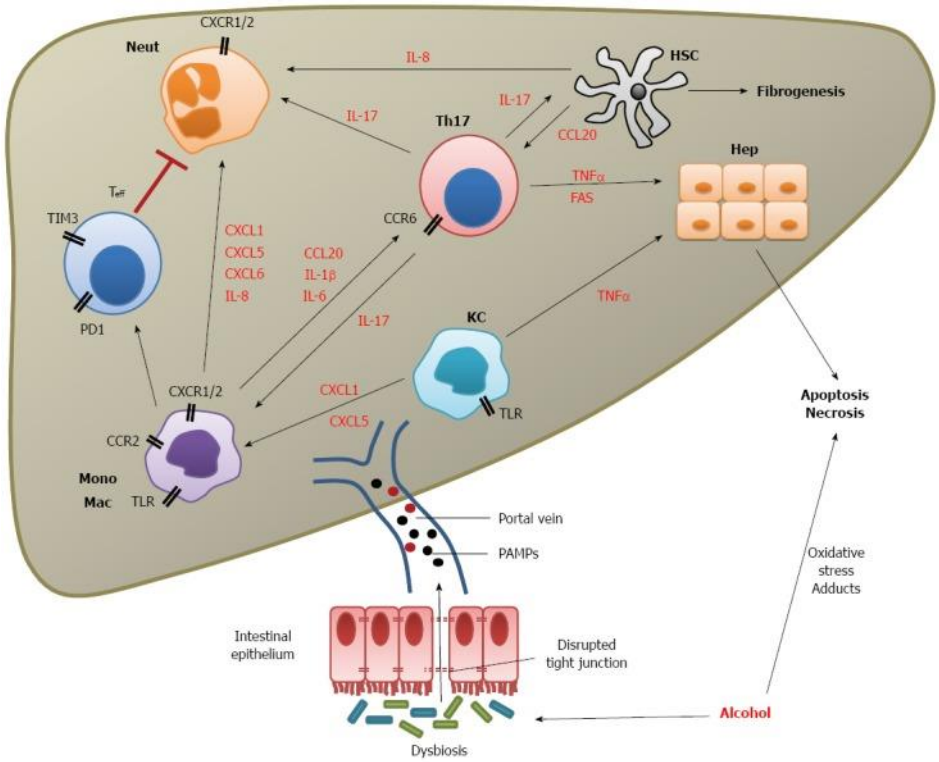


Figure 2 [29]: The immune dysfunction processes of AH. PAMPs- pathogen associated molecular pattern; CXCL5- CXC motif chemokine 5; CXCL1- CXC motif chemokine 1; CXCR 1/2- C-X-C receptor types 1/2; CCR2- C-C chemokine receptor type 2; TLR- Toll like receptor; PD1- Programmed death 1; TIM3- T cell immunoglobulin and mucin domain 3; T_{eff} – effector T cells; CXCL6- CXC motif chemokine 6; IL-8- Interleukin 8; IL-17- Interleukin 17; IL-6- Interleukin 6; IL-1β- Interleukin 1 beta; CCL 20- chemokine ligand 20; TNFα- TNF alpha; FAS- FAS ligand; HSC- Hepatic stellate cells; Hep- Hepatocytes; KC-Kupffer cells; Mono-Monocytes; Mac- Macrophages; Neut-Neutrophils.

On the other hand, the gastrointestinal (GI) tract as the initial touchpoint to any food or drinks ingested is at risk of direct injury from alcohol [38-40]. Alcohol disrupts the intestinal barrier and increases intestinal permeability [38-40]. Increased intestinal permeability induces accelerated translocation of PAMPs and lipopolysaccharides (LPS) to the systemic circulation [38-40]. When these products are exposed to Kupffer cells (liver resident macrophages), the Toll-like receptors (TLRs) and myeloid differentiation primary response (MyD88) inflammatory signalling pathways are activated [38-40], triggering systemic inflammatory response.

In addition, alcohol disrupts the bacterial homeostasis in the gut and causes dysbiosis [41, 42]. Animal studies demonstrated that alcohol reduces the gut bacteria diversity and composition whereby beneficial bacterias such as Lactobacillus and Bifidobacterium are reduced and proportion of pathogenic bacterias namely the Proteobacteria and Bacilli [41-42] are increased. Both animal and human research studies have proposed that the alterations in gut

microbiome could be a direct effect from alcohol, but it could also be indirect effect from alcohol causing abnormal intestinal motility [43-47]. Alcohol is able to modify the bile -acid metabolism causing a reduction of bile acid, further precipitating bacterial overgrowth [43-47]. Consequently, these changes would cause weakening of the intestinal barrier and subsequently breakdown of intestinal immune homeostasis.

Preliminary trials to replenish or stimulate growth of beneficial bacteria like Lactobacillus to prevent the shift in intestinal microbiota composition has attenuated liver injury in mice [45-47] and similar short term benefits in patients with liver cirrhosis [45-47]. This phenomenon of gut dysbiosis results in production and release of endotoxins, and accumulation of acetaldehyde in the gut which promote inflammation [45-47].

1.3: The involvement of immune cells in AH

AH is distinguished by over-activated systemic inflammation and dysfunctional immune responses. So, when looking for therapies to the disease, attempts to reverse the inflammation and/or correct the dysfunctional immune system are theoretically promising approach. However, which immune cells and which inflammatory pathway is dominant in driving the disease, remain under research.

In humans, there are two basic immune systems which are known as innate and adaptive immune systems [48]. These two systems mediate protective functions in the liver [48]. Major cell components of the innate immune system comprise of the phagocytic cells (neutrophils, basophils, eosinophils and mast cells), macrophages, dendritic cells, natural killer cells, and natural killer T cells [48].

Of which, monocytes are the undifferentiated predecessors of macrophages and dendritic cells [48-50]. They are leucocytes derived from bone marrow, and they circulate in the peripheral blood system and spleen [48-50]. Monocytes are the first line of cellular defence as they have innate abilities to detect and recognize PAMPs and damage associated molecular patterns (DAMPs) [48-50]. When presented with pathogens, monocytes can engulf pathogenic cells, multiply and secrete chemokines. Once enlisted into the tissues, monocytes differentiate into macrophages and dendritic cells [48-50]. There are different subset of monocytes and they are categorized into three distinct subsets based on their proclamation of surface markers [48-50].

AH is characteristic of its non-resolving inflammation driven by both PAMPs and DAMPs [51, 52]. Previous research have shown that both liver tissue-resident macrophages and peripheral monocytes are dynamically responsive to the bacterial liposaccharides (LPS) translocated from the gut after alcohol use [51-55]. These macrophages and monocytes respond by secreting pro-inflammatory cytokine and chemokine, and allowing unopposed infiltration of immune cells into the liver causing liver injury [51-55]. A common finding in both animal and human models of AH is increased circulating peripheral monocytes, especially that of intermediate monocytes (a subset of monocytes) [51-55]. This suggests that the monocytes are important contributors in the systemic inflammation cascade and form my reasons for assessing monocytes in my research.

On the other hand, the adaptive immune system is classified into T cell mediated cellular immunity and B cell mediated humoral immunity [48]. Cells of the adaptive immune system include CD4+ T helper cells, cytotoxic CD8+ T cells, and B cells [48, 56- 58]. T cells eliminate intracellular microbe, whilst B cells give rise to antibodies to get rid of extracellular pathogens [48, 56-58].

Previous animal and human model studies have demonstrated that alcohol in excess modified T cells in both quantities and activities [56- 58]. Studies on inflammatory uveitis and inflammatory bowel disease have further demonstrated that a subpopulation of $CD4^+$ T cells continued to thrive and multiply despite subjected to high amount of dexamethasone (a form of glucocorticoids) [59, 60].

On the other hand, a distinctive kind of effector memory T-helper cells called Th17 cells were found to be pro-inflammatory in patients with inflammatory arthritis [61, 62]. These Th17 cells were frequently found in abundance in patients with autoimmune diseases who are refractory to GC therapy, suggesting Th17 cells' potential contributions to GC resistance [61, 62].

Although these concepts of glucocorticoid resistant phenotype may not manifest uniformly in all autoimmune and inflammatory diseases, but there is clear potential for assessing T cell function and phenotype while testing glucocorticoid resistance in AH.

1.4: Regulation of inflammatory responses by histones and epigenetics in AH

To fully understand the underlying inflammatory mechanism that drives AH, we need to identify the key players accountable for the inflammatory pathways [63, 64]. This involves investigating the epigenetic influences behind the activation and repression of pro-inflammatory transcription factors [63, 64].

Epigenetic represents gene activities (expressions) controlled by the cells without alterations to the DNA sequence [65]. Modifications at the epigenetic level is a dynamic process and can vary according to cell types, exposure to oxidative stress and various environmental factors [65-68]. These modifications include histone acetylation, deacetylation, methylation, phosphorylation, and ubiquitination [65-68]. Amongst the various modifications, histone acetylation and deacetylation are most investigated [65-68].

Histones are highly conserved proteins, which wrap around two rounds of DNA to configure the nucleosome, which forms the core structure of chromatin [65-68]. There are two crucial archetype of histone proteins- the linker histone (H1), and core histones (H2A, H2B, H3, H4) [65-67]. They attach to the gateway site where DNA gets in and out of nucleosome [65-67].

Histone acetylation and deacetylation

Histone acetylation is mediated by two opposing group of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) [69-74]. Histone deacetylases (HDACs) are enzymes that detaches acetyl groups from N-acetyl lysine amino acid on a histone, conceding the histones to encase the DNA firmly, forming a condensed chromatin [69-74]. This action works as repressors of gene expression [69-74].

The opposing action of HDACs is that of histone acetyltransferases (HATs). HATs moves the acetyl groups away from acetyl-CoA and attaches to the lysine amino acid on a histone [69-74]. The process of histone acetylation involves histone wrapping the DNA loosely, resulting in a relaxed chromatin [69-74]. This allows the chromatin it to be more accessible to the transcriptional factors [69-74].

Figure 3 is a schematic representation of histone acetylation and deacetylation processes.

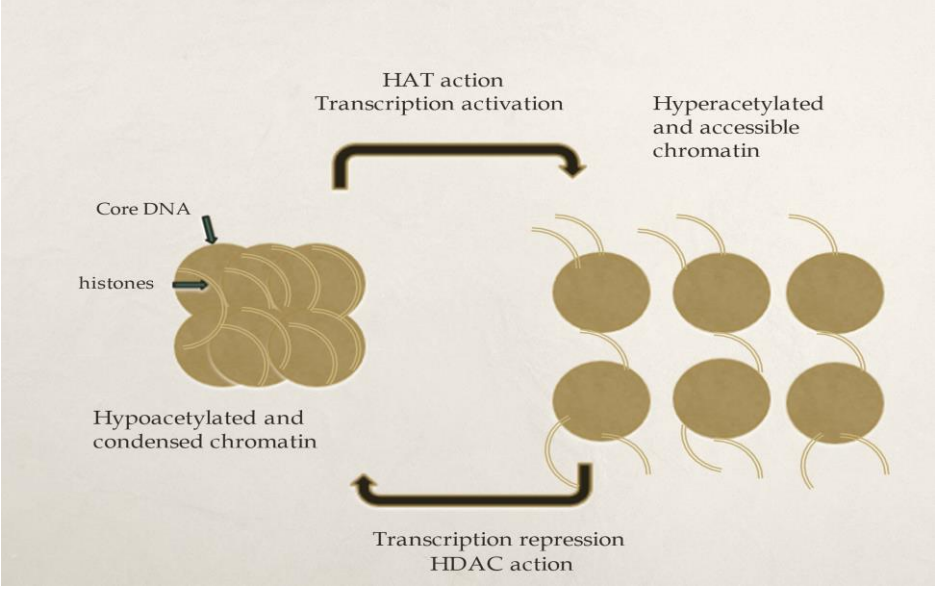


Figure 3 (own creation) shows histones wrapping around DNA to form nucleosome with their respective acetylation and deacetylation processes. HAT- histone acetyltransferases; HDAC- histone deacetylases.

HATs are grouped into five main classes [69-74]. This includes the GCN5-related N-acetyltransferase (GNAT), MYST, p300/CBP, general transcriptional factor-related HATs and steroid receptor co-activators family [69-74]. Animal studies have shown elevated HAT activity level in mice fed with alcohol [75-77]. They have also demonstrated that alcohol increases the histone 3 acetylation at lysine 9 by altering the expression of HATs in mice hepatocytes [75-77].

Among the HAT families, p300/CBP family is the most widely studied. p300 binds with CREB binding protein (CBP) and serve as a co-transactivation protein in numerous gene transcription [69-74]. In vitro animal ethanol study has demonstrated that the level of p300 is increased corresponding to the upregulation in histone 3 acetylation at lysine 9 [77, 78]. Acetylation of histone H3 at lysine 9 is one of the surrogate markers of active alcohol dehydrogenase 1 (ADH 1) gene which is responsible for alcohol metabolism [77, 78]. Previous studies have further reaffirmed that alcohol and alcohol ROS by-products increase the activity of ADH 1 gene through modulating the activity of HATs in a positive feedback loop [77, 78].

To my knowledge, the role of HAT activity has only been investigated in chronic alcohol-related liver disease models. Therefore, my research on the HAT expression levels in patients with AH is novel.

On the other hand, HDACs are grouped into four main classes (Class I, II, III, IV) [72-74]. Class III HDACs are commonly known as Sirtuins (SIRT) [72-74]. All the HDAC enzymes, except Sirtuins, are zinc and NAD^+ dependent [72-74]. NAD^+ is disintegrated into nicotinamide and binds to the acetate moiety, O-acetyl-ribose [72-74]. The end-product, nicotinamide remains a potent inhibitor of HDAC activity

[72-74]. Thus, the HDACs activity is managed by NAD^+ metabolism and is inhibited by nicotinamide [72-74].

In this context, it is not surprising that the primary pathway of alcohol metabolism i.e., the NAD^+/NADH pathway is a key mediator in these histone modifications [79-81]. In animal models, alcohol indirectly suppresses class I HDAC activities and gene expressions due to exhaustion of NAD^+ in the course of alcohol metabolism [79-81].

The role of HDACs is important in many aspects including glucocorticoid resistance. Studies on COPD suggest that oxidative stress mediated the reduction in total HDAC activity and downregulation of a particular HDAC2 [82-85]. Reduction in HDAC activity then translated to the intensity of inflammation as driven by overwhelming expression of pro-inflammatory cytokines in small airways [82-85]. Additionally, research works on COPD indicated that when HDAC activity was downregulated, HAT activity was not increased [82-85]. This indicates that the overall gene transcription in inflammatory diseases may be dominantly driven by either histone acetylation or deacetylation [82-85]. From these findings, it is not difficult to understand why a large proportion of AH patients will have reduced HDAC activities and gene expression and are therefore resistant to GC.

Theophylline (a drug which selectively inhibits the activity of PI3Kδ was found to possess the ability to restore HDAC2 activity and subsequently sensitivity to glucocorticoid [85]. As a result, theophylline and other anti-PI3Kδ drugs are currently being trialled in other inflammatory diseases [85].

Histone methylation

Histone methylation represents a process where methyl groups are catalysed and shifted from cofactor S-adenosyl-L-methionine (SAM) to either lysine or arginine surplus of H3 and H4 histones [86]. This process is mediated by three groups of histone methyltransferases (HMTs). These HMTs are named respectively, the histone demethylases (HDMs), and the lysine and arginine methyltransferases (KMTs and PRMTs)[86].

Histone methylation affects the enrolment and attachment of regulatory proteins to chromatin, resulting in the weakening or strengthening of the chemical bonds between histone tails and DNA [86-88]. This process causes either upregulation or downregulation of gene transcription and consequentially, occluding or granting DNA access to transcription factors [86-88]. Of the histone methyltransferases, KMTs are the most widely studied [86-88]. Several animal studies have shown that KMTs are responsible for the transcriptional state of ADH1 gene and KMTs are more distinctive than HATs as they earmark certain lysine residue [88-90]. These studies have demonstrated that methylation at H3meLys4 is associated with upregulation of ADH1 [88-90].

Whether histone methylation serves as a dominant epigenetic imprint in just chronic ArLD or also in acute situation like AH is still unknown.

Histone phosphorylation

Histone phosphorylation is a process where negative charge is being conferred to a histone [67, 68, 91]. It is liaised by two groups of enzymes - the kinases and phosphatases [67, 68, 91]. Kinases work by adding phosphate group to the histone whereas phosphatases remove the phosphate group [67, 68, 91]. Histone phosphorylation is associated with chromosomal condensation which affects the transcriptional activity similarly to acetylation and methylation [67, 68, 91].

Research using rat models have shown that alcohol triggers the H3 phosphorylation at location serine 10 and serine 28 in the hepatocytes [92]. This process of phosphorylation is highly dependent on p38 mitogen activated protein kinase (MAPK) activity, which is an inflammatory signalling pathway well known in ArLD [92-94].

Further, histone phosphorylation has been found to be happening in conjunction with other histone modifications like acetylation and methylation [92-94]. For instance, in an in-vivo mice hepatocyte study, alcohol induces H3 phosphorylation, but when activity of phosphorylation wanes; H3K9 acetylation activity takes over at the same histone [92-94]. Whether histone phosphorylation and acetylation were working synergistically or antagonistically remains to be investigated [92-94]. The relationship between phosphorylation and other histone modifications and whether this crosstalk represents a bigger bearing factor for the pathogenesis of AH is uncertain.

Histone ubiquitination

Amongst the histone modifications, histone ubiquitination has been known for the longest time, but ironically less well researched when compared to methylation or acetylation due to the technicalities around measuring ubiquitination activities [95-97]. Histone ubiquitination involves a process that transport ubiquitin on to the H2A and H2B which are histone core proteins [95-97]. The process of ubiquitination is mediated by histone ubiquitin ligases which is strongly associated with gene repression, whereas deubiquitinating enzymes act in opposition by removing ubiquitin and lead to gene expression [95-99].

Many in vivo models have shown that monoubiquitination where only one ubiquitin molecule is being added to one protein, plays a vital role in regulating damages within DNA [95-99]. Similarly, histone ubiquitination works in association with other histone modification [95-99]. In tumour cell studies, there are evidence to show that monoubiquitination and acetylation take place simultaneously at the same histone H3 which translate into transcription activation [95-99].

Micro-RNA

Micro-RNA, also known as miRNA is a smaller type of non-coding RNAs which can bind to the messenger RNA [100, 101]. Through its interaction with messenger RNA, miRNA could modulate post transcriptional gene expression [100, 101].

The role or miRNA in ARLD and non-alcohol related fatty liver disease (NAFLD) has been assessed in previous researches [100-102]. miRNAs have been observed to play vital regulatory function on the interaction between inflammatory signals via the expression of cytokines and enzymes directly linked to alcohol metabolism [100-102]. Further, miRNAs also play a role in regulating oxidative stress involved in ARLD pathogenesis through different pathways like the Nuclear factor erythroid 2 related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap) pathways [100-103].

Utilization of miRNAs to diagnose and prognosticate ArLD patients have been proposed, but not been well studied [104, 105]. For example, it has been suggested that miR-27a necessitates the distinction of monocytes and therefore is found abundantly in extracellular plasmatic vesicles of AH patients [104, 105]. Also, an upregulated amount of miR-182 has been associated with biopsy proven severe form of liver injury and AH [102, 106]. These findings make miRNAs a potential diagnostic tool. However, there are currently no clinical studies to progress these findings further.

In the field of oncology, miRNAs have been extensively studied due to their profound connections with histone modifications [101, 103, 107]. Recent evidence demonstrated a link between dysregulated miRNA expression, promotion of oncogenic transcription factor- MYC and epigenetic gene silencing [101, 103, 107]. However, it is unclear which miRNA is responsible for these pathological processes. Researches on miRNA and epigenetics are underway but have proven challenging due to scarcity of effective methodology and difficulties in obtaining clinical samples.

In conclusion, epigenetics and histone modifications represent a vast and intriguing research area for many allergic and inflammatory diseases. Modifications in the composition and accessibility of chromatin can exert influence on gene expression and proceed to regulate inflammatory transcription activity [92, 102, 103]. This is highly relevant and applicable to AH.

1.5: The role of oxidative stress in AH

Oxidative stress happens when there is a disparity between formation and aggregation of reactive oxygen species (ROS) and the capacity to counteract ROS with antioxidants [108].

Excessive alcohol causes breakdown of gut integrity junction and thereby making gut junction more permeable [109-111]. This breakdown triggers excessive translocation of lipopolysaccharide from gut to liver which results in ROS production through activation of toll-like receptor 4 and NADPH oxidase activation [109-111]. ROS then mediates epigenetic alterations through altering the genetic expression and activity levels of HDACs [109-111].

As previously alluded to, the alcohol metabolism pathway which utilizes cytochrome p450 2E1 contributes to production of ROS in the hepatocytes [109-112]. ROS precipitates the production of reactive nitrogen species (RNS) and thereby reduces the counteracting antioxidant cofactors like the Manganese (Mn) and Zinc (Zn) [109-112]. On the other hand, ROS also activates triggers the production of pro-inflammatory IFN-γ, interleukins, transcription factors activator protein 1 and nuclear factor κB (NF-κB), causing uncontrolled inflammation within the hepatocytes [109-112].

In a healthy liver, further functional and structural mitochondrial damage are defended by uncoupling proteins and SAMe expression [113, 114]. However, this defence mechanism is inhibited by ROS [113,114]. Figure 4 [11] below depicts the ROS mediated pathways which causes oxidative stress and damage through promoting cell death, sustaining the expression and production of pro-inflammatory mediators, and also precipitating epigenetic alterations [11].

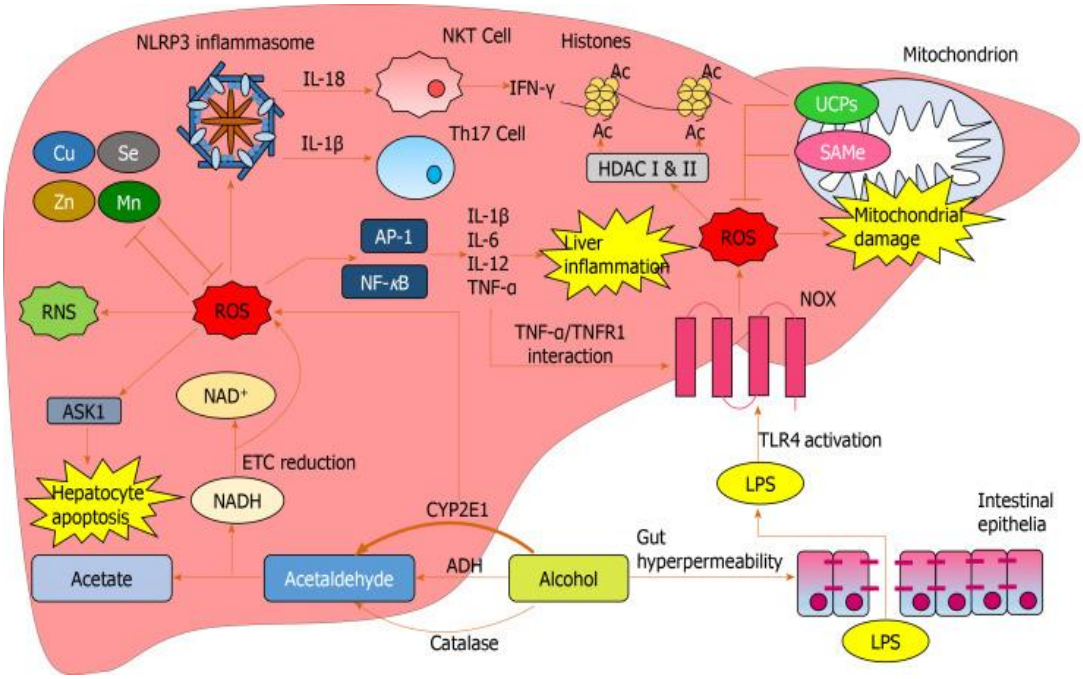


Figure 4 [11]: ROS mediated oxidative damage pathways within the liver. Ac- Acetylation; ADH- Alcohol dehydrogenase; AP-1- Activator protein 1; ASK1- Apoptosis signal-regulating kinase 1; Cu- Copper; CYP2E1- Cytochrome p450 2E1; ETC- Electron transport chain; HDAC- Histone deacetylases; IFN- Interferon; IL- Interleukin; LPS- Lipopolysaccharide; Mn- Manganese; NAD^+-Nicotinamide adenine dinucleotide; NADH- reduced form of nicotinamide adenine dinucleotide; NF-*κ*B- Nuclear factor kappa light chain enhancer of activated B cells; NKT- Natural killer T-cell; NOX- NADPH oxidase; RNS- Reactive nitrogen species; ROS- Reactive oxygen species; SAMe-S-adenosylmethionine; Se- Selenium; Th17- T-helper 17 cells; TLR4- Toll-like receptor 4; TNF-α- Tumour necrosis factor alpha; TNFR1- Tumour necrosis factor alpha receptor 1; UCP- Uncoupling protein; Zn- Zinc.

If we look closer into the mitochondria of hepatocytes, ROS downregulates the ATP and GSH levels [103,104,105]. On the other hand, when thioredoxin-interacting protein is activated by ROS, they then translocate to mitochondria and bind with thioredoxin 2. This triggers more ROS production and downstream histone changes [103,104,105].

ROS overproduction impacts on the respiratory chain in the mitochondria by producing excessive levels of NADH, and ADH [114-118]. This facilitates superoxide and hydroxyl radicals' formation and accelerates cellular DNA damage [114-118]. The other nitrogen containing oxidative species, which is known as reactive nitrogen species (RNS) acts by inhibiting mitochondrial enzyme, like ATP synthase, NADH dehydrogenase and succinate dehydrogenase which means it inhibits the functionality of mitochondria [114-118].

Excess levels of ROS induces activation of apoptosis signal-regulating kinase 1 (ASK1) [117-120]. This then triggers the cellular apoptosis pathway via cleavage of pro-caspase-3 to active caspase-3 [117-120]. ASK1 is also essential in the sustained activation of cytosolic MAPK kinase 4 (MKK4) and c-Jun N-terminal kinase (JNK), which promotes mitochondrial permeability and dysfunction [117-120]. Sustained JNK activation further interacts with Sab protein, which is an outer mitochondrial membrane protein to subsequently inhibit mitochondrial respiration and eventually cell apoptosis [117-120].

In summary, alcohol consumption causes mitochondria dysfunction, through excessive ROS production, which can trigger oxidative stress within the hepatic mitochondria. Figure 5 [11] below illustrates the ROS mediated pathways in the mitochondria which is described above [11].

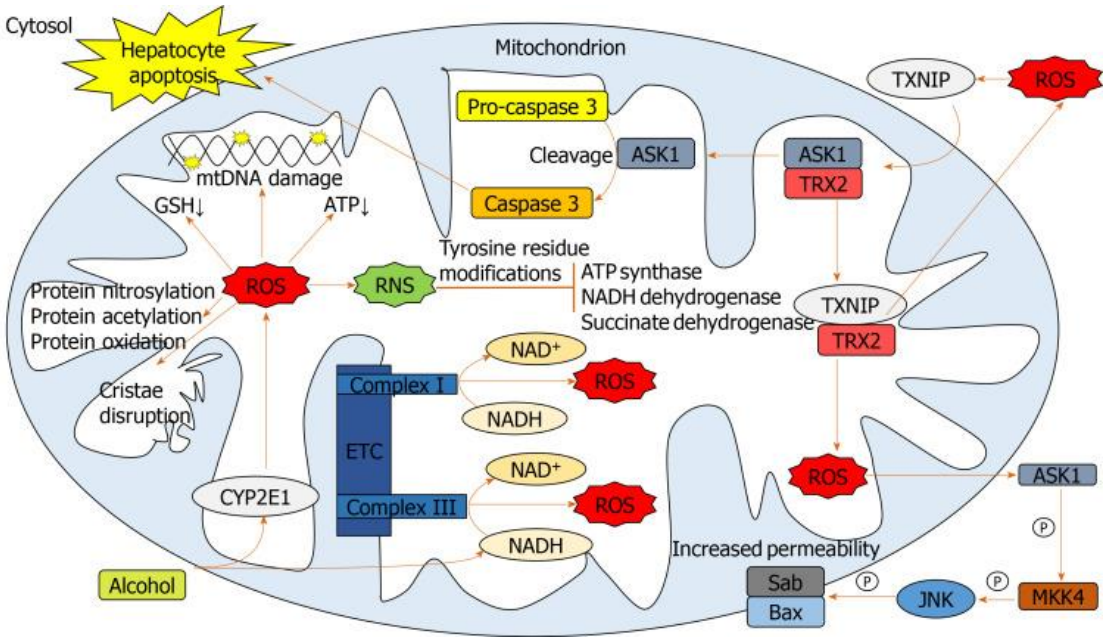


Figure 5 [11] shows the ROS mediated pathways in the mitochondria. ASK1- Apoptosis signal-regulating kinase 1; BAX- Bcl-2-associated X protein; CYP2E1- Cytochrome p450 2E1; ETC- Electron transport chain; GSH- Glutathione; JNK- C-Jun N-terminal kinase; MKK4- Mitogen-activated protein kinase kinase 4; mtDNA- Mitochondrial DNA; NAD^+-Nicotinamide adenine dinucleotide; NADH- reduced form of nicotinamide adenine dinucleotide; ROS- Reactive oxygen species; RNS- Reactive nitrogen species; Sab- SH3 domain-binding protein; TRX2- Thioredoxin 2; TXNIP- Thioredoxin-interacting protein.

In normal circumstances, the hepatic mitochondria respond to oxidative damage by uncoupling mitochondrial oxidative phosphorylation [117-120]. This diminishes the production of ROS, and as a consequence eliminates oxidative

stress [117-120]. In both animal and human models, chronic alcohol use induces structural, biological and functional change in hepatic mitochondria [120-122]. This is a result of alcohol regulating the electron transport chain, cellular energy metabolism and mitochondrial respiration which then enhances generation of ROS [120-122]. Excessive ROS subsequently causes hepatocyte apoptosis via release of cytochrome C and promotion of caspases activation [120-122]. In addition, alcohol also reduces cellular defence by modulating antioxidant defence enzymes and in turn leaving hepatic mitochondria on an unopposed cellular death pathway during cellular insult [120-122].

Increased ROS production due to alcohol metabolism prompts an increase in lipid peroxidation [13-15]. Products of lipid peroxidation include malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) that can bind to proteins to form adducts [13-15]. Acetaldehyde, which is an output of alcohol metabolism, together with MDA then bind with intracellular protein to form malondialdehyde-acetaldehyde (MAA) adducts which stimulates mass production of cytokine and chemokine [13-15, 123]. This process is seen in in vitro models of AH and ArLD with cirrhosis [13-15, 123].

Furthermore, it is observed that in ArLD, there is downregulation of mitochondrial GSH level, possibly due to ROS and lipid peroxidation [123, 124]. This then increased the lysosomal membrane permeability, allowing fat and inflammatory cell infiltration into the hepatocytes [123, 124]. More recently, there is evidence to show that in acute alcohol related liver injury like that of AH, there is decreased autophagy, which is when cell degrades its own cytosolic components as a defence mechanism [125]. The reduction in autophagy is thought to be related to the dysfunctional innate and adaptive immune system [125]. A defective autophagy system allows disinhibited inflammatory responses within the hepatocytes [125]. The compelling mechanism of this remains debatable.

The role of oxidative stress in ArLD has been highlighted here. Understanding these processes will allow us to identify which ROS mediated inflammatory pathway is likely to be culprit of the onset or progression of AH.

In vivo measurement of ROS or oxidative stress in human cells is particularly challenging due to the volatility of oxidative species with surrounding molecules [126, 127]. The most used approach is surrogate measures of ROS for instance, end products of lipid peroxidation including MDA/ HNE as described in the alcohol metabolism chapter or products of oxidatively damaged DNA which include 8-hydroxy-2-deoxyguanosine (8-OHDG) [126, 127]. These methodologies were adopted in this research project to assess oxidative stress and will be further discussed in the methodology chapter.

1.6: Diagnosis of AH

AH is a separate clinical entity which is predominantly diagnosed on the basis of clinical presentation and assessments [128, 129]. There is no single diagnostic laboratory marker for AH, but several classical biochemical disruptions are commonly seen in this cohort of patients [128, 129].

AH is characterized by recent development or worsening of jaundice with elevated serum bilirubin level of 3mg/dL [128-129] in less than 60 days of abstinence from alcohol before onset of jaundice. Patients with AH often present with elevated liver enzymes for instance, the alanine aminotransferase (ALT) and aspartate transaminase (AST) of more than 50 IU/L [128, 129]. The individual AST and ALT values typically do not exceed 400 IU/mL and AST/ALT ratio is expected to be >1.5 in AH [128, 129]. Levels out of this range raise suspicion of other differential diagnoses like drug induced liver injury (DILI) and ischaemic hepatitis [128, 129]. Additional laboratory parameters to look out for include raised total white cell counts with elevated subsets (leukocytes and neutrophils), coagulopathy (prothrombin time>12seconds or INR>1.2) [128, 129].

The duration and amount of alcohol consumption causing AH is unknown, but patients who are diagnosed with AH have typically been misusing alcohol for the preceding six months and less than 60 days without alcohol prior to onset of jaundice [128-130]. The definition of heavy alcohol use is >60g/7.5units of alcohol per day for men; and >40g/4 units of alcohol per day for women [128-130].

Obtaining an accurate alcohol consumption history can be challenging as some patients present with confusion (known as encephalopathy) and in some cases, public stigmatization made them unable to disclose information in a transparent way. In addition, patients can present with non-specific symptoms like fatigue, abdominal pain and can present with signs of liver decompensation including ascites (fluid accumulation in the abdomen), variceal bleeding (bleeding from dilated veins at the upper GI tract which is a known feature of portal hypertension) without prior knowledge of liver cirrhosis [128, 129].

Patients with AH frequently mount systemic inflammatory response syndrome (SIRS) which is the body's defense mechanism to localize and eliminate the source of insult [128, 129, 131]. In AH, this can be explained by the profuse inflammation which is a hallmark of the disease or can indicate an active infection [128, 129, 131]. Clinical parameters of SIRS criteria include tachycardia (>90bpm), pyrexia (<36'C or >38'C), tachypnoea (respiratory rate >20 breathes per minute) [128, 129, 131].

It is often onerous to differentiate AH from other liver related diagnoses due to similar presentation of symptoms and biochemical derangements. To name a

few, these include acute decompensation of liver cirrhosis, viral hepatitis A, B, C, D and E, drug induced liver injury, autoimmune hepatitis and primary sclerosing cholangitis [128, 129, 131, 132]. Therefore, AH patients must undergo non-invasive liver screen including blood tests for viral hepatitis and autoimmune liver screen, and ultrasound of abdomen [128, 129, 131, 132].

Liver biopsy with histological assessment is sometimes imperative to confirm diagnosis of AH in patients with confounding factors and to differentiate diagnoses [133]. From a histopathological perspective, macrovesicular steatosis with one of the following features: neutrophil infiltration, hepatocyte ballooning, Mallory-Denk bodies and 'chicken wire' fence fibrotic pattern is suggestive of AH [133-136]. The presence of satellite neutrophils, cholestasis and megamitochondria are indicators of poor prognosis in AH [133-136].

However, the utility of liver biopsy in AH patients is limited by the procedural risks, staff technical abilities to perform procedure, cost, operator, and sampling variations [133-136]. Among the risks associated with liver biopsy, there is a 10% bleeding risk with major bleeding occurring in less than 2% [133-136]. These quoted risks vary in accordance to AH patients' coagulopathy and thrombocytopenia [133-136]. Patient's ascites can also make the procedure technically challenging because the distance between the abdominal wall and the liver is larger, making it more difficult to obtain an adequate sample [133-136]. Unlike the conventional percutaneous liver biopsy which access the liver through the skin and liver capsule, trans-jugular approach which accesses the liver through the superior vena cava (neck vein) has a lower bleeding risk [133-136]. However, this technique is not widely available due to differences in training and technical availabilities across UK.

Alternatively, a variety of non-invasive prototypes are being adopted to assess severity of AH, predict prognosis and advise decisions for commencement of the primary treatment GC [137-143].

To date, the Maddrey's Discriminant Function (mDF) and Glasgow Alcoholic Hepatitis Score (GAHS) remain the most used scoring model for disease prognosis in AH as they are easy to calculate at the bedside [137-143]. Patients with a MDF score ≥ 32 or GAHS score of ≥ 9 are predicted to have severe AH and represent 28-day survival rate between 41% and 67% [140-141]. GAHS is the only model that takes into consideration white cell count which represents an inflammatory index [140-141].

In terms of assessing clinical responsiveness to GC therapy, Lille and Neutrophil to lymphocyte ratio (NLR) remain most widely used [142-144]. After 7 days of GC therapy, patients who have a Lille score of more than 0.45 are categorized as GC non-responders, meaning failure to respond to GC or have

suboptimal therapy response [142, 143]. This phenotype predicts a mortality risk of 75% at 6 months [142, 143]. More recently, Lille score at day 4 was evidenced to be as useful as Lille score at day 7 in predicting 28 days mortality and could reduce prolonged use of GC and its potential complications [142, 143].

Incorporation of NLR into a modified GAHS score has proven to be a good predictive tool to assess the therapeutic implications of GC in patients with severe AH [141, 144]. Patients with NLR of 5-8 were more likely to benefit from GC and have improved 90 days mortality [141, 144].

When prognosticating a patient with AH, Model for End-Stage Liver Disease-Sodium (MELD Na) score has been validated and used to predict survival in liver diseases and also a metric for organ transplant allocation. MELD-Na is a clinical scoring system that incorporates patient's dialysis status, values of bilirubin, creatinine, coagulation (International normalized ratio) and sodium. This is also one of the metrics used in this research project when assessing prognosis for patients with severe AH.

In summary, these scoring systems are easy to use clinical parameters, but they often take a few days to complete, thus causing a delay in determining suitable therapy for patients [137-144]. In short, we are deprived of an easy, quick turnover laboratory/ scoring model in stratifying AH into their disease severity to enable effective individualised therapies.

1.7: Treatment of AH

Treatment options for AH differ based on the disease severity. Mild to moderate AH is managed conservatively with alcohol cessation and symptomatic management [138, 145]. For severe AH, the mainstay of treatment is GC, as it is evidenced that GC therapy can reduce short term mortality i.e. death from AH within the first 28 days [145-149]. Unfortunately, more than 1 in 3 patients do not respond to GC [145-149]. The non-responders to GC often predict death within 90 days [145-149]. Even within the GC responders, the efficacy of GC is variable due to individual GC resistance or partial responsiveness [145-149].

Accurate prediction of response to GC would enable us to select appropriate treatment plan for patients before exposing them blindly to GC. It would be highly beneficial even for the eligible patients, as a predictor of non-responsiveness as this could prevent adverse effects from GC like development of new infections, gastrointestinal haemorrhage, and hyperglycaemia [148-150].

A Cochrane systematic review conducted several years ago revealed that GC are not only ineffective for AH patients, but GC also predispose them with serious systemic infections and subsequently multi-organ failure [150, 151]. As many as 25% of patients with severe AH are admitted with or developed infection whilst in hospital [150, 151]. Active infection regardless of the origin precludes the patients from getting treated with GC, which acts as a form of immunosuppression [138,139]. Patients who are ineligible for GC are then left with conservative management and limited alternative therapeutic options [138, 150, 151].

Over the years, there have been a few other potential pharmacotherapies trialled clinically for AH patients.

The dominant signalling pathway in instigating inflammatory response in AH is the LPS-TLR4 pathway [152, 153]. LPS-TLR4 binds to Kupffer cells and activates the nuclear factor kappa-light-chain- enhancer of activated B cells (NF-κB) signalling and expression of NF-κB genes [152-154]. As a result, pro-inflammatory cytokines, and chemokines like TNFα, Interleukin-6 (IL-6), and Interleukin-1 (IL-1) are produced and released to the system [152-154].

The release of TNFα, can be further amplified by the positive feedback loop in Kupffer cells, resulting in upregulation of C-C chemokine ligand 2 (CCL2) and C-X-C motif ligand 1 (CXCL1) [152-155]. This process precipitates the peripheral recruitment of macrophages and neutrophils in propagation of liver inflammatory responses [152-155].

Given these observations, anti-TNFα agents were trialled for treatment of AH [157, 158]. Unfortunately, the use of Infliximab not only did not show improvement in terms of hepatocellular inflammation and injury; there were no differences in terms of Maddrey's scores at any time point between the cohort of patients treated with intravenous infliximab and the placebo cohort [157, 158]. The RCT trial of Infliximab was prematurely stopped by the Safety Monitoring Committee and sponsor because of high predominance of severe infections in those patients who received both infliximab and prednisolone [157, 158].

Additionally, there were also evidence to show that infliximab perpetuated liver injury by suppression of the TNFα-mediated liver regeneration property [157, 158]. Another well-known RCT trial demonstrated that Etanercept (a TNFα receptor blocker) was associated with a consequentially higher fatality rate after 6 months due to its profound immunosuppression property [159]. In conclusion, treatment with anti-TNFα agents were deemed inappropriate and could even be harmful to AH patients [157-159].

Another agent that has been extensively investigated was pentoxifylline, a nonselective phosphodiesterase (PDE) inhibitor [160, 161]. Pentoxifylline has shown to increase cellular cAMP, rendering a reduction in TNFα and other pro-inflammatory cytokine production [160, 161]. A factorial designed clinical trial (STOPAH) was conducted to examine the effects of pentoxifylline alone and in combination with prednisolone [161]. However, pentoxifylline failed to demonstrate mortality benefit, despite showing lower incidence of hepatorenal syndrome- which is a renal complication of severe hepatic injury [160, 161].

Nonetheless, given the pivotal role of oxidative stress in the development of ArLD, antioxidants in particular N-acetylcysteine (NAC) garnered some attention few years ago [162, 163]. A randomized controlled trial of treating AH patients with NAC infusion in combination with prednisolone showed early mortality benefit at 30 days when compared to prednisolone alone and NAC also reduced risk of infection [162, 163]. However, there was no sustainable survival benefit beyond 3 months [162, 163].

In a systematic review and meta-analysis, therapeutic efficacy of GC, pentoxifylline, and NAC, alone or simultaneously with each other were compared [164]. In patients with severe AH, the use of GC alone or in combination with pentoxifylline or NAC was beneficial in reducing the short-term mortality [164]. The addition of NAC to GC is superior to GC alone in reducing short term mortality [154]. However, none of the treatment above was efficacious in improving the medium or long-term survival [164].

Along the line of antioxidants, vitamin E (a fat-soluble vitamin with antioxidant

property) was tested in patients with mild to moderate AH in a double-blind placebo controlled randomized trial [164, 165]. However, Vitamin E therapy did not improve the biochemical derangements in AH patients despite showing beneficial effect in reducing the concentration and level of serum HA (Hyaluronic acid) [164, 165]. HA plays a vital component in the extracellular matrix and is a marker of hepatic fibrogenesis [164, 165].

On the note of investigating alternative therapies for AH, I led a systematic review and meta-analysis on zinc treatment on clinical outcomes in patients with ArLD [166]. Findings were constrained due to limited eligible studies and considerable diversity in the nature of patient cohort and therapy choice [166]. Within these limitations, zinc supplementation is not statistically connected with better survival rate or long term prognosis in patients with ArLD cirrhosis. No similar work was done in AH patients [166].

More recently, there was a clinical trial (ISAIAH) which aimed to investigate the prognostic advantages of IL-1β signal inhibition with canakinumab in AH [167]. IL-1β is an important intermediary of hepatic inflammation and canakinumab is a monoclonal antibody to reverse the adverse effects of cytokines in patients with AH [167]. However, preliminary data from the trial demonstrated that inhibition of IL-1β signalling did not improve the long-term prognosis for AH patients, despite obvious histological improvement [167].

On the other hand, the DASH trial compared a combination therapy of Anakinra (which is a recombinant human IL-1R antagonist), pentoxifylline and zinc versus GC [168]. Results of the trial showed that the combination conferred a short term survival benefit similar to prednisolone, although a head to head comparison between anakinra and GC remains unavailable [168].

Furthermore, another randomized phase 2 trial was conducted to test the efficacy of pegfilgrastim in patients with severe AH [169]. Pegfilgrastim is a long acting recombinant granulocyte colony stimulating factor (GCSF) which stimulates the bone marrow to generate and mobilise neutrophils and haemotopoietic stem cells (CD34+) into the bloodstream [169]. In animal studies, this can induce regeneration of liver cells and improve survival in acute liver failure [169]. Unfortunately, this trial showed no survival benefit at 90 days in AH patients who received Pegfilgrastim and GC when compared to GC alone [169]. The incidences of infection, renal failure and encephalopathy were also similar in both treatments [169].

In recent years, there is increasing evidence that the modulation of gut microbiota by faecal microbiota transplantation could be therapeutic for alcohol related liver disease and in particular, alcohol related hepatitis.

An initial clinical trial showed that faecal microbiota transplantation conferred improvements in 12 months survival rate in patients with severe AH when compared to historical controls (87.5% vs 33.3%) [170]. In addition, another RCT in India affirmed that patients who received faecal microbiota transplantation have improved 90 days survival rate and reduced infection rate when compared to patients who only received GC [171].

If we look beyond pharmacotherapies, liver transplantation remains an efficacious therapy for AH [172]. However, liver transplantation for these patients remains debatable. This is due to the scarcity of liver as a resource and concern about return to harmful alcohol use post transplantation [172].

1.8: Glucocorticoid (GC) signalling pathways

Glucocorticoids (GC) are a type of steroid hormones [173, 174]. GC is commonly used to treat autoimmune, allergic, inflammation and lymphoproliferative diseases [173, 174]. GC binds to and is transported by either the corticosteroid-binding globulin (CBG) or albumin in the bloodstream [173, 174]. CBG's affinity to GC is much higher than albumin although there is higher concentration of albumin in the bloodstream [173, 174].

At sites of inflammation, the reactive centre loop (RCL) region of CBG is cleaved by endogenous proteases [173, 174]. This reduces CBG's affinity to GC, allowing free GC to diffuse into the surrounding cells [173, 174].

Following diffusion into the cell, GC is activated by combining with the glucocorticoid receptor (GR) which is attached with chaperone proteins like heat shock protein (HSP) 90, p23, histone deacetylase (HDAC) 6, c-Src and the immunophilin FKBP51[174-176]. Once GC is activated, FKBP51 is switched to FKBP2 [174-176] and assists the translocation of GC-GR complex into the nucleus [174-176].

Upon nuclear translocation, the chaperone proteins dissociate from the GC-GR complex [174-176]. Upon dissociation, the chaperone proteins activate inflammatory pathways such as, Phosphoinositide 3-kinase (PI3K), protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways [174-176]. Further, the GC-GR complex binds to the glucocorticoid-responsive elements (GREs) to mediate gene expression either by activating or repressing the given genes [174-177]. This process can be either via the GC-GR complex, tethering or composite mechanisms as shown in figure 6 [174-176].

There is also evidence that the GC-GR composite can activate the GREs in a ligand-independent manner in response to certain endogenous or exogenous stressors [174-176]. Figure 6 below summarises the GC signalling pathways from its affinity to CBG in the bloodstream, to its nuclear translocation and binding to GRE.

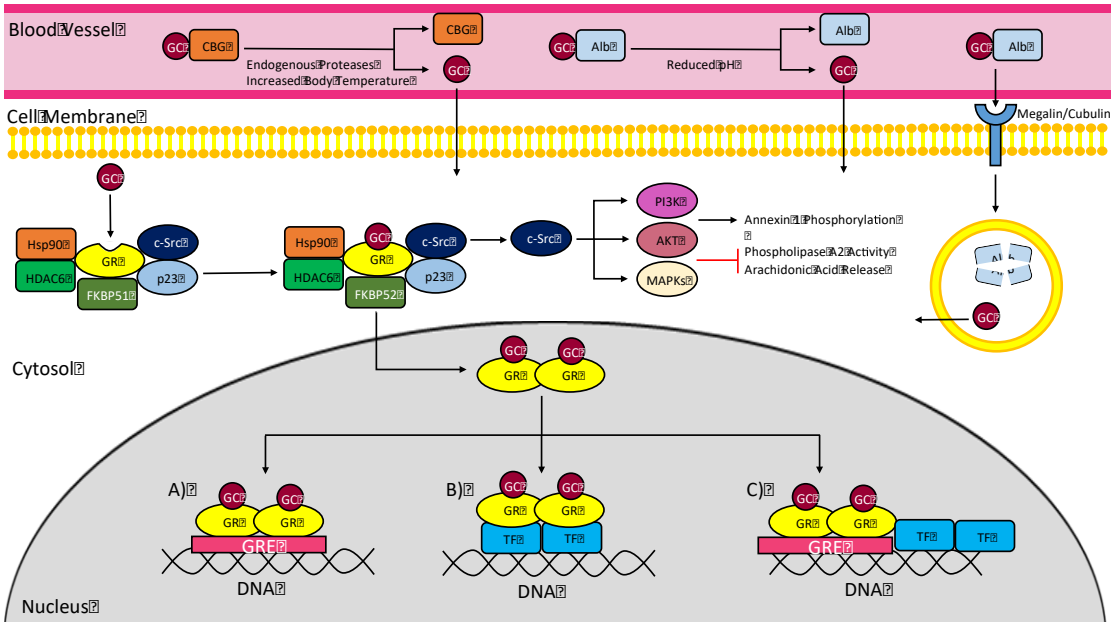


Figure 6 (own creation) depicts the GC signalling pathway from blood vessel to nucleus. GC- Glucocorticoid; CBG- Corticosteroid binding globulin; Alb-Albumin; Hsp90- Heat shock protein 90; HDAC6- Histone deacetylases 6; FKBP51- FK506 binding protein 5; GR- Glucocorticoid receptor; c-SRC- cellular-SRC; p23- p23 protein; PI3K- Phosphatidylinositol 3-kinase; AKT- Protein kinase B; MAPK- Mitogen activated protein kinase; TF- Transcription factor. A) Direct GRE binding, B) = Tethering, C) Composite element binding).

Looking closer into the nucleus, the GC-GR complex influences gene activation and repression (Figure 6) [176-180]. GC-GR tethers to the DNA- bound transcription factors to alter their signalling pathways [181,182]. Amongst the various transcription factors, the TLRs are the most well-known PAMP receptors that initiate the innate immune system [181, 182]. GR directly activates the toll-like receptor 2 (TLR2) gene, and this activation is cooperated with signal transducer and activator of transcription 5 (STAT5) [180-182].

The binding of GC-GR to the TLR promoter site suppresses gene activities of nuclear factor κB (NF-κB), activator protein 1 (AP-1), mitogen-activated protein kinase (MAPK) and IFN [182, 183]. Of which, the NF-κB and MAPKs are pivotal in the initiation of a pro-inflammatory response via downstream stimulation of IL-4, IL-10, Per1, Gilz, Fkbp5, Dusp1, β-casein, IL-2, FOXP3, and TLR2 genes [182, 183].

On the other hand, the deacetylation of GR via HDACs (particularly HDAC 2) inhibits the NF-κB pathway, thereby suppresses the output of pro-inflammatory transcription factors like TNFα, IL-1β, IL-6 and IL-12 [183-186].

In addition, the interaction of GC-GR complex with GR interacting protein 1 (GRIP1) reduces their availability for interferon regulatory factor 3 (IRF3) [185, 186]. As a result, the transcription of inflammatory genes that rely on the bindings

of IRF3 onto their promoters is inhibited [185, 186]. Thus, targeting the GRIP1:IRF3 interaction can be a potential target for suppressing inflammation and improve glucocorticoid immunosuppression [185, 186].

Figure 7 present examples of GC-GR complex interactions inside the nucleus which mediates either gene activation or gene repression.

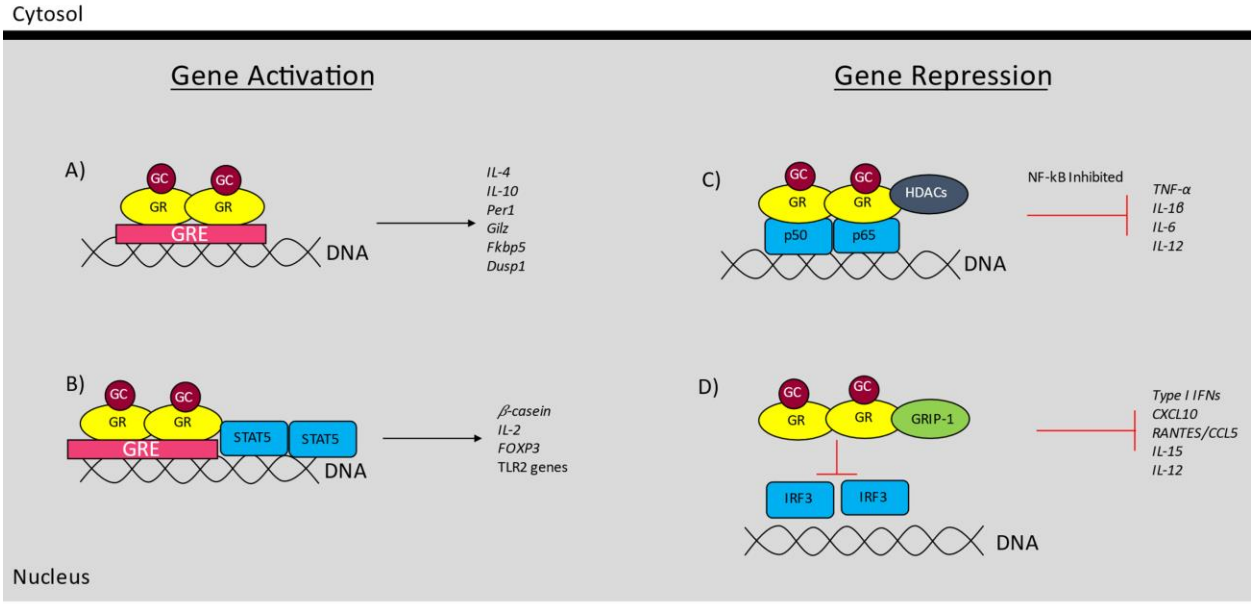


Figure 7 (own creation) illustrates GC-GR complex interaction with resultant gene activation or gene repression. Diagrams A-D: GC-GR complex binding in the nucleus may result in either gene activation or repression dependent on the binding loci. Diagram A: GC-GR complex activating a series of inflammatory genes e.g IL-4, IL-10, Per1, Gilz, Fkbp5, Dusp1. Diagram B: GR directly activates TLR2 gene, and this activation involves its cooperative action with STAT5. Diagram C: GC-GR complex binds with HDAC and thereby inhibits NF-κB and downstream inflammatory genes like TNFα, IL-16, IL-6 and IL-12. Diagram D: Grip1 is important for inhibiting Type 1 IFNs, CXCL10, RANTES/CCL5, IL-15, IL-12 by association with GC-GR. The GC-GR inhibits IRF3 activity by making GRIP1 unavailable to IRF3.

GC- Glucocorticoid; GR- glucocorticoid receptor, GRE- glucocorticoid responsive element; STAT 5- Signal transducer and activator of transcription 5; HDAC- histone deacetylases; p50- protein50; p65- protein65; GRIP-1- GR interacting protein 1; IRF3- interferon regulatory factor 3, IL-4- interleukin 4; IL-10- interleukin 10; Per1- period circadian regulator 1; Gilz- Glucocorticoid-induced leucine zipper; Fkbp5- FK506 binding protein 5; Dusp1- Dual-specificity phosphatase 1; β-casein- Beta casein; IL-2- interleukin 2; FOXP3- Forkhead box P3; TLR2 genes- Toll like receptor 2 genes; NF-κB- nuclear factor κB; TNFα-Tumour necrosis factor alpha; IL-1β- Interleukin-1beta; IL-6- Interleukin 6; IL-12- Interleukin 12; Type 1 IFNs- Type 1 Interferons; CXCL10- C-X-C motif chemokine ligand 10; RANTES (also known as CCL5)- Regulated upon activation, normal T cell expressed, and secreted; IL15- Interleukin 15; IL-12- Interleukin 12.

1.9: Glucocorticoid resistance

The occurrence of GC resistance has been explicitly described in many inflammatory diseases like chronic obstructive pulmonary disease (COPD), uveitis, and ulcerative colitis (UC) [187-192]. Up to a third of patients with these diseases show poor or absent responses to GC [187-192]. Patients with GC resistance or insensitivity are left with conservative management or alternative anti-inflammatory treatments which are likely to carry a plethora of side effects [190-192]. For instance, in ulcerative colitis, GC resistant patients may end up requiring a colectomy (surgical removal of the large bowel) [190-192]. There is no doubt GC resistance is a major barrier in many medical specialties and it incurs substantial healthcare costs when managing GC resistant patients.

Studies on GC resistance within the respiratory and gastrointestinal models have suggested several molecular mechanisms [193-195]. These include dysregulated HPA axis, the stimulation of mitogen-activated protein (MAP) kinase pathway, reduced histone deacetylase 2 (HDAC2) expression, glucocorticoid resistant GRβ receptors, glucocorticoid resistant Th17 cells and T cell subpopulation [62, 188, 189, 195, 196]. It is likely that GC resistance in AH shares similar mechanism.

There have been attempts, with variable results, in reversing GC resistance by modifying these underlying mechanisms. Examples of this would be the use of theophylline (which is an antioxidant and phosphoinositide-3-kinase-δ inhibitor) to restore HDAC 2 expression in treatment for COPD patients who are resistant to GC [85, 187].

Currently, there are no well-defined methods to verify GC resistance, although there are validated *in vitro* biomarkers of glucocorticoid responsiveness [197-201]. Of these, lymphocyte GC sensitivity has been extensively used to assess GC sensitivity clinically in non-infectious inflammatory diseases [197-201]. In recent years, the development and performance of a novel *in vitro* bioassay utilizing cellular proliferation and bromodeoxyuridine (BrdU) has produced great clinical translational ability in predicting GC responsiveness [197-201]. The BrdU incorporation in lymphocyte steroid sensitivity assay (BLISS) assay has therefore been adopted in my research project when assessing *in vitro* GC responsiveness/ resistance [198-201].

In humans, exogenous GC mimics the endogenously produced cortisol which are responsible in mediating adaptive responses to stress [202]. GC works by suppressing the liver polymorphonuclear neutrophil (PMN) infiltrates and pro-inflammatory arbitrators like TNF-alpha (TNF-α), interleukin 6 (IL-6) and interleukin (IL-8) in the hepatocytes, and also arbitrated through suppression of gene transcription [203, 204].

Dysregulated HPA axis

The hypothalamic–pituitary–adrenal (HPA) axis is a crucial neuroendocrine system which modulates multitudinous feedback loops to sustain homeostasis in mammals [195, 205]. Secretion of endogenous glucocorticoids (cortisol) is regulated by the HPA axis [195, 205]. Corticotrophin releasing factor (CRF) produced in the hypothalamus triggers the anterior pituitary to release adrenocorticotropin hormone (ACTH). ACTH then triggers release of cortisol from the adrenal cortex [195, 205].

Cortisol is released rhythmically and is susceptible to change with diurnal rhythm; physical and emotional stress, and abnormalities in the chaperones and cochaperone proteins [196, 205]. Under normal circumstances, there is a negative feedback loop in the anterior pituitary and hypothalamus to inhibit the secretion of ACTH and CRF when exogenous glucocorticoids are detected in the system [205].

An observation from an animal study is that in a state of inflammation or chronic stress, GR is more susceptible to dissociate from its co-chaperone protein, FKBP51 which regulates GR sensitivity [195, 205, 206]. The dissociation of FKBP51 from GR leads to failure of the negative feedback loop [195, 205, 206]. Such dysfunctional negative feedback response results in the failure to adequately terminate endogenous cortisol secretion [195, 205, 206]. The excessive amount of cortisol further desensitizes the CRF receptors and GR sensitivity to exogenous glucocorticoids [195, 205, 206].

Based on this observation, restoration of the dysregulated HPA axis should overcome GC resistance. However, this concept of therapy has never been tested or trialled clinically, possibly due to the unfeasibility to reverse the complex HPA axis.

Over activated mitogen-activated protein (MAP) kinase

In the alveoli of asthmatic patients who are GC resistant, pro-inflammatory cytokines like Interleukin 2 and Interleukin 4 are over-expressed [207-209]. These cytokines reduce the functionality of glucocorticoid receptors and their nuclear translocation via phosphorylation of the receptors with the help of p38 MAP kinase [207-209]. This theory was validated with the consistent finding of over activated p38 MAP kinase in asthmatic patients who do not mount clinical or biochemical response to GC [207-213]. In in-vitro animal studies, c-Jun N-terminal kinase (JNK), a type of MAP kinase which acts by directly phosphorylating the GR, resulting in GC resistance in T cells [209-213]. When MKP1 (an inhibitor of MAP kinase and glucocorticoid responsive gene) was knocked out in mice, the macrophages in these mice show reduce responsiveness to GC, again validating the theory [209-213].

In addition, Interleukin 2 activation causes paradoxical reduction in GR nuclear translocation via the signal transducer and activator of transcription 5 (STAT 5)/ Janus kinase (JAK3) signalling pathway [209-213]. Further, there are affirmations to manifest that GR nuclear translocation and GC-GR-GRE affinity (as described in the chapter above) are reduced in PBMCs of patients who are GC resistant [209-213]. This phenomenon explained by the phosphorylation of GR [209-213].

Altered histone acetylation expression

Studies on patients with COPD and patients with asthma who are active smokers have identified a key role player in GC resistance [187-189]. The expression and operation of HDAC2 are diminished in PBMCs and alveolar macrophages in these patients who are resistant to GC [187-189].

Cigarette smoking generates oxidative stress, and this impairs the HDAC 2 activity and expression [187-189, 214-216]. GC works by engaging HDAC 2 which counteracts the activity of histone acetylation stimulated by the NF-κB and other transcription factors [187-189, 214-216]. In event of HDAC 2 deficiency, GC can no longer turn off the histone acetylation and this renders disinhibited of the NF-κB pathway and transcription of inflammatory genes like the tumour necrosis factor α (TNFα), Interleukin 8 (IL-8) and granulocyte-macrophage colony stimulating factor (GM-CSF) [187-189, 214-216].

In animal models, medications like theophylline, has shown positive effects by enhancing the HDAC 2 activity and thereby reversing glucocorticoid resistance [187-189, 215-218, 219, 220]. Previous studies suggested that theophylline works effectively to restore HDAC2 via inhibiting PI3Kδ, which was activated by oxidative stress [187-189, 215-218, 219, 220]. In this context, selective PI3Kδ inhibitor should be applicable in treating GC resistance in inflammatory diseases.

Whether or not this mechanism is also followed by other inflammatory diseases like AH, is yet to be investigated. Figure 8 [188] shows the pathway where oxidative stress modulates the reduction in HDAC 2 activity in GC resistant respiratory patients [188].

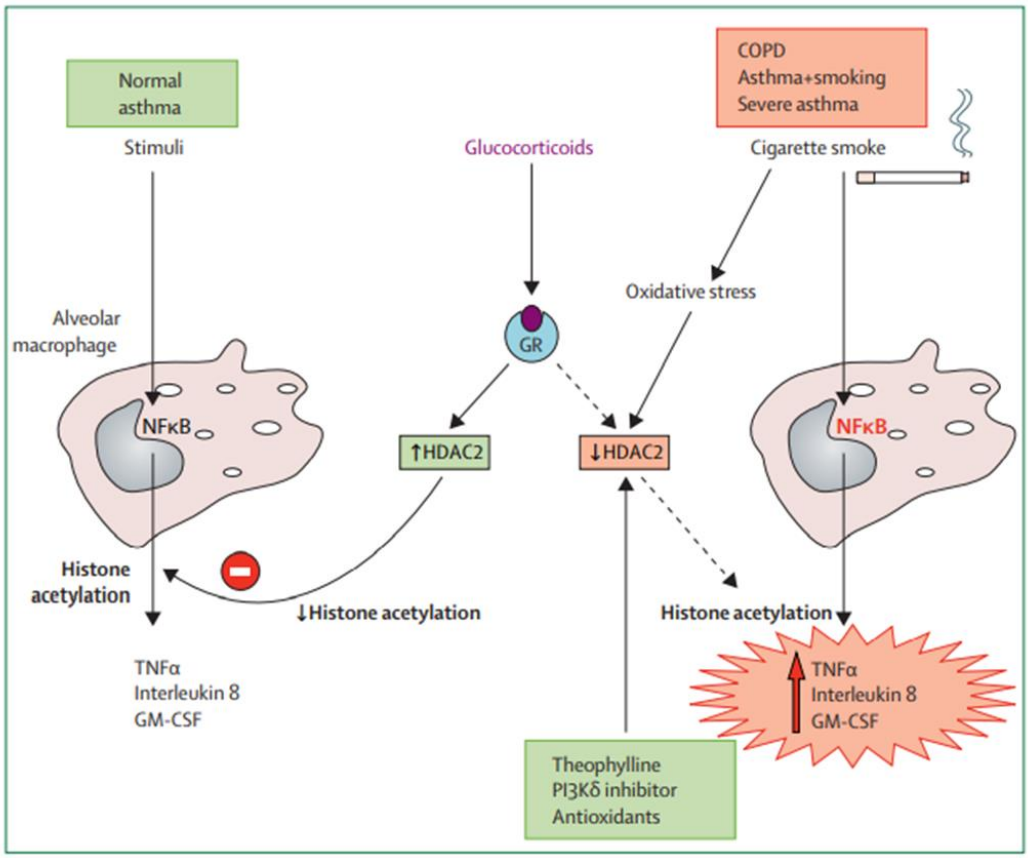


Figure 8 [188] shows the proposed mechanism behind GC resistant respiratory patients which is triggered by oxidative stress. GR- Glucocorticoid receptor; NF-κB- Nuclear factor kappa-light-chain-enhancer of activated B cells.
HDAC2- histone deacetylase 2; TNFα- Tumour necrosis factor α; GM-CSF- Granulocyte-macrophage colony stimulating factor; PI3Kδ inhibitor- Phosphoinositide 3-kinase inhibitor.

<u>Glucocorticoid resistant GRβ receptor</u>

The overall actions of glucocorticoids are mediated by the glucocorticoid receptor (GR) [213-214]. The human GR gene sits within chromosome 5 and encodes two splicing isoforms alpha (α) and beta (β) for their usage of different terminal exon 9α and 9β [209, 210, 213, 214]. GRα is the definitive receptor, which attaches to GC and manages activities of GC [209, 210, 213, 214]. In contrast, GRβ does not attach to GC but competes with GRα for the binding of GRE, thus functions as a negative inhibitor of GRα-induced trans-repression activity of the tumour necrosis (TNF) α and interleukin (IL)-6 genes [209, 210, 213, 214]. The enigmatic GRβ isoform also negatively influences the transcriptional activity of several GC reactive genes for instance, myocilin, mitogen-activated protein kinase phosphatase-1 (MPK-1), and fibronectin [209, 210, 213, 214].

Studies of the GR revealed that GRβ isoform is prompted by pro-inflammatory cytokines such as IL-1β, 2, 4, 6, 7, 8, 18, TNFα, interferons α and γ [209-214]. There is evidence to show that expression of GRβ is upregulated in GC resistant conditions like rheumatoid arthritis and inflammatory bowel disease [209-214]. In in vitro studies where they knock out the GRβ receptors from the alveolar macrophages, GRα nuclear translocation enhances and glucocorticoid responsiveness increases [209-214]. Unfortunately, this GC resistant GRβ theory is not supported in all inflammatory diseases [209-214]. The physiologic activities of GRβ rationalises further elucidation with future research advances.

Glucocorticoid resistant T cell subpopulation

The mechanism of GC resistance has been extensively studied in other diseases. A study on inflammatory uveitis and colitis has identified a population of $CD4^+$ T cells, which proliferate despite treated with high dose dexamethasone [59, 60]. Further investigations have been undertaken to determine whether the whole population of T cells or a certain subtype of CD4+ T cells with CD25 surface expression mediated glucocorticoid resistance [59, 60]. The studies show that $CD4^+CD25^-$ T cells are responsive to GC whereas $CD4^+CD25^{int}$ cells are highly resistant to GC [59, 60].

In PBMCs from patients with ulcerative colitis, anti-CD25 monoclonal antibodies (basiliximab) suppressed T cell proliferation whereas treatment with dexamethasone alone did not [192]. In this context, one could argue that T cell subpopulation which express intermediary levels of CD25 are fuelling a group of lymphocytes which perpetuate inflammation and are resistant to glucocorticoids [59, 60, 192].

The disparity in responses to GC could be related to how the immune cells responds in an inflammatory setting. The different effects of anti-CD25 and glucocorticoids on T cell proliferation provide an insight that different immune cell subpopulation have potential to render glucocorticoid resistance [59, 60, 192].

Although this concept of glucocorticoid resistant phenotype may not manifest uniformly in all autoimmune and inflammatory diseases, but there is clear potential for using T cells while testing glucocorticoid resistance in AH.

Glucocorticoid resistant Th17 cells

Variations in the adaptive immune system determine an individual's responses to glucocorticoid. Increasing evidence suggests that a distinct effector memory T-helper cells called Th17 cells play a critical role in GC resistance [61, 62, 191]. These $CD4^+$ lineage is characterised by its secretion of Interleukin 17A, 17F, Interleukin 22 and chemokine receptor 6 (CCR6) [61, 62, 191]. These GC resistant

Th17 cells are found in abundance in subjects with autoimmune diseases who are refractory to GC therapy [61, 62, 191].

From in vitro and in vivo murine models, Th17 cells consistently exhibit resistance to glucocorticoids at a genome wide level [61, 62, 191]. This refractoriness is not confounded by any variation in the GR isoforms or translocation [209-214].

Animal studies have demonstrated that these GC resistant Th17 cells are sensitive to calcineurin inhibitors like cyclosporin and tacrolimus [191]. These observations are consistent with clinical findings where GC resistant patients are often sensitive and therefore effectively treated with calcineurin inhibitors [191].

Of note, these calcineurin inhibitors act by selectively attenuating the Th17 cell division and suppressing the transcription of IL-2 and IL-17 [191]. This further reaffirm the potential of targeting Th17 cells or the effector memory $CD4^+$ T cell populations in the context of GC resistance [191].

With these proposed mechanisms in mind, many drug development and clinical trials have succeeded in attempts to reverse GC resistance or restore GC sensitivity. For instance, Basiliximab (anti-CD25 monoclonal antibody) for ulcerative colitis and theophylline (phosphodiesterase inhibitor) for COPD [192, 218-220].

These mechanisms for GC resistance are limited to molecular level and may only be applicable to specific inflammatory or autoimmune diseases. More human tissue based clinical trials are yet to be performed to explore therapeutic options. In the future, novel drugs could be developed to target AH once the major signalling pathway that regulate AH and glucocorticoid resistance is better understood.

1.10: Thesis rationale, hypotheses and objectives

Rationale

AH is a distinctive condition which typically develops shortly following a period of excessive alcohol intake and characterized by severe inflammatory changes peripherally and within the liver [9,21,22,25]. AH can superimpose on any stage of ArLD. Severe AH carries a risk of mortality rate of 30% within 90 days after presentation [9, 21, 22, 25].

Rationale for this research is to gather novel data about the inflammatory pathways in AH, which can be used to establish biomarkers to prognosticate AH and to identify patients with GC resistance to better guide therapeutic options. These findings could ultimately be utilized in minimising mortality from AH and other similar severe inflammatory disorders.

For several decades now, many therapies have been trialled for AH, but GC remains the primary treatment for AH [138, 145-149]. Advocates of GC cite benefit in short term prognosis, but there are still uncertainties especially with regards to the side effect profile and lack of long-term benefit [138,145-149].

Further, up to 30% of patients diagnosed with AH are either resistant or have suboptimal response to glucocorticoids, leaving them with very limited treatment options [138, 145-149]. Similar findings in the field of inflammatory/autoimmune diseases further highlight the importance of identifying the underlying driver for GC resistance so we can better research for alternative therapy options or ways to reverse GC resistance [187-190].

Several mechanisms of glucocorticoid resistance have been proposed with the most compelling data demonstrating the impact of oxidative stress and epigenetic regulation in glucocorticoid signalling pathways [28, 46, 80, 220]. Studies on glucocorticoid resistant respiratory models confirmed that glucocorticoid resistance is driven by oxidative stress and reduced HDAC2 activity [187-190].

Excessive oxidative stress stimulates the downstream phosphoinositide-3-kinase delta (PI3Kδ)/ Akt inflammatory signalling pathway which then enables unrivalled inflammatory processes [187-190, 220]. Medications targeted to inhibit PI3Kδ like theophylline and nortriptyline have successfully shown to reverse oxidative stress and consequently, restore glucocorticoid sensitivity [85, 218, 219].

It is widely known that alcohol metabolism gives rise to the production and accumulation of ROS and DNA adducts, resulting in oxidative stress [10, 11]. However, the effects of oxidative stress and its subsequent ramifications on histone modifications have not been studied in AH.

Therefore, my research aim was to determine whether there is overproduction of ROS in AH and to delineate an easy method for measuring oxidative stress which could be reproduced in clinical settings. These experiments were performed on immune cell subsets of interest (T cells and monocytes).

In addition, I aimed to assess any differences in the overall expression of histone genes, and in particular the activity of histone acetylation and de-acetylation when comparing AH patients of different disease severity.

I planned to conclude my research with in vitro experiments assessing the potential of several therapeutic interventions in improving glucocorticoid resistance. Data gathered from AH about oxidative stress, histone modifications and glucocorticoid resistance may also benefit other inflammatory diseases of which mechanisms are not fully understood.

Hypotheses

a) Measurement of oxidative stress can predict 90 days mortality of patients with severe AH.
b) Patients with severe AH have altered epigenetic regulation of glucocorticoid signalling i.e altered expression of HAT/ HDAC/ PI3Kδ and this is driven by oxidative stress.
c) Drugs targeting downstream signalling pathways of oxidative stress e.g N-acetylcysteine, Zinc, short chain fatty acid, HDAC inhibitor can improve glucocorticoid sensitivity.

Objectives

a) Measure oxidative stress in peripheral blood from patients with AH.
b) Determine most reproducible and reliable biomarker of oxidative stress in peripheral blood from patients with AH.
c) Characterise gene expression of all histone acetyltransferases (HATs) and histone deacetylases (HDACs) comparing healthy volunteers with AH patients.
d) Measure functional activity of HAT/HDAC/PI3Kδ.
e) Investigate potential therapies to improve glucocorticoid resistance by using in vitro cell culture method.

Chapter 2: Experimental Methods

2.1: Patient recruitment

All studies were conducted according to Good Medical Practice (GMP) in research standards and the Declaration of Helsinki.

The study was ethically approved by the NHS Health Research Authority (reference numbers: 15/LO/1501 and 19/LO/0829) and conducted according to the principle of the International Conference on Harmonisation: Good Clinical Practice (ICH-GCP).

Written consent was acquired from all participants with full mental capacity to consent. Seventeen out of the fifty five AH patient cohort recruited for this research work lacked mental capacity to consent due to hepatic encephalopathy or delirium tremens. Therefore, assent was acquired from the nominated consultee or next of kin. Two out of the seventeen patients regained capacity and subsequently gave full consent to participate in the research. The rest of the patients had mental capacity to consent at time of inclusion.

Alcohol related hepatitis

Adult patients admitted as inpatients to University Hospitals Plymouth NHS Trust with severe AH between March 2019 and November 2022 were recruited.

AH is characterized by recent development or worsening of jaundice in the with bilirubin level of >30umol/L [128-129] with less than 60 days of abstinence from alcohol before onset of jaundice. Patients with AH often present with elevated liver enzymes for instance, the alanine aminotransferase (ALT) and aspartate transaminase (AST) of more than 50 IU/L [128, 129]. The individual AST and ALT values typically do not exceed 400 IU/mL and AST/ALT ratio is expected to be >1.5 in AH [128, 129]. Levels out of this range raise suspicion of other differential diagnoses like drug induced liver injury (DILI) and ischaemic hepatitis [128, 129]. Additional laboratory parameters to look out for include raised total white cell counts with elevated subsets (leukocytes and neutrophils), coagulopathy (prothrombin time>12seconds or INR>1.2) [128, 129].

The definition of heavy alcohol use is >60g/7.5units of alcohol per day for men; and >40g/4 units of alcohol per day for women [128-130].

In addition, the formula (4.6x {prothrombin time-control time}) + serum bilirubin in mg/dl is utilized to quantify a patient's Maddrey's discriminant function (DF) score [141]. Patients with Maddrey's discriminant function of greater than 32 are classified as severe AH and those with Maddrey's discriminant function less than 32 are classified as non-severe AH.

Serology blood tests including viral hepatitis, autoimmune liver screen, and liver ultrasound are routinely performed to exclude confounding diagnosis like viral hepatitis, autoimmune liver disease, obstruction in the biliary tree and hepatocellular cancer, which may present similarly to AH [9, 42].

50mls of peripheral blood was drawn after consent form signed, where possible within first 3 days of hospital admission. Blood samples were taken before patient was exposed to GC. Patients who were deemed suitable for GC therapy would then proceed to be started on oral prednisolone and event documented. Baseline clinical laboratory parameters like bilirubin, ALT, AST, ALP, albumin, Platelets, clotting function, renal function, full blood count were documented. These were then checked again at day 7, day 14, day 28 and day 90. If patients were discharged, they would have been followed up with a telephone or face-to-face clinic appointment with blood tests at day 28 and day 90. No further research related follow up was conducted beyond 90 days.

Patient demographic and baseline characteristic data were collected. These included age, gender, ethnicity, relevant medical history, development of infection, signs of liver decompensation. The primary study outcome was 90 days mortality, but patients' survival at 6 months and 1 year are recorded.

Experimental methods using patients' peripheral bloods are being described below.

Non-AH Alcohol related liver cirrhosis (ArLD cirrhosis) controls

Adult patients admitted as inpatients to University Hospitals Plymouth NHS Trust with decompensated alcohol related liver (ArLD) cirrhosis between March 2019 and November 2022 were recruited. ArLD patients without features of alcohol related hepatitis clinically and histologically are recruited as controls. Patients with decompensated ArLD cirrhosis who had features of ACLF were excluded before recruitment.

Patients with liver cirrhosis can be asymptomatic depending on whether they are clinically compensated or decompensated from their cirrhosis. Therefore, we diagnose liver cirrhosis in accordance to clinical, radiology and histology findings.

Patients with decompensated liver cirrhosis usually present with a combination of liver enzyme dysfunction and portal hypertension [42, 128-130]. Their signs and symptoms include ascites, jaundice, hepatic encephalopathy (which is a temporary of neuropsychiatric disorder), bacterial infection of ascitic fluid, bleeding, hepatorenal syndrome [42, 128-130]. The most indicative findings of a cirrhotic liver on a liver ultrasound are the surface nodularity, heterogeneity, coarsening of the hepatic architecture [42, 221]. Signs of portal hypertension can

also be picked up on the ultrasound e.g., splenomegaly and abdominal varices [42, 221].

On the other hand, transient elastrography with fibroscan is a speedy and non-intrusive way to quantify liver stiffness, especially useful for patients who are not suitable/ contraindicated for more invasive investigations [222-223]. A cut off value of 20kPa was utilized to establish the presence of cirrhosis [222-223].

Histologically, liver cirrhosis is signified by the transformation of injured liver cells into collagenous scars and formation of fibrous bands around regenerative nodules [224]. Fibrosis is commonly classified into different staging in accordance to METAVIR scoring system [224].

Healthy volunteer controls

Healthy volunteers (HVs) were recruited from laboratory staff of University of Plymouth laboratory, Derriford Research Facility and hospital workers who provided consent and self-declaration with absence of acute and chronic health problems (HRA reference number: 1703). They were also assessed by recruiter to be free of active infection. Blood samples obtained from HVs were used as controls.

2.2: Peripheral blood mononuclear cells isolation (PBMC)

Patient's peripheral blood samples were taken via venepuncture either by me or a trained research nurse from University of Plymouth NHS Trust. 45mLs of blood was taken and contained in 5x9ml green plasma separating tubes (BD) and an additional 5mls of blood taken into a gold serum separating tube (BD).

Bloods were then transferred into 50mls Falcon tube (BD) and diluted with equal amount of RPMI 1640. The mixture was then layered on top of Ficoll-Paque PLUS solution (GE Healthcare, Cardiff UK) at a ratio of 2:1. Tubes were then centrifuged for 20minutes at 2000rpm (brake off). The mixture would have been separated into three layers, with the uppermost layer consisting of plasma, the second layer which consists of PBMCs and the third layer consisting of erythrocytes and polymorphonuclear leukocytes such as neutrophils, eosinophils and basophils.

The PBMC layer was then carefully pipetted out using a 1ml pipette into a clean 50mL Falcon tube. Then, PBMC was washed twice with RPMI 1640 and centrifuged for 10minutes at 1700 rpm (brake on). Following the washing steps, supernatant was removed carefully. The remnant PBMC pellet was then mixed thoroughly in 1mL of pre-constituted complete media solution.

2.2.1: Cell counting

A small amount of 10µL worth of cells were suspended with 90uL of 0.4% Trypan blue (Sigma-Aldrich). 10µL of the cell suspension was pipetted out and put on both sides of the haemocytometer chamber. Cells were then viewed and counted under microscope using x10 magnification using handheld cell counter.

Figure 9 below is an example of the magnified field of haemocytometer slide. All the cells within the chosen 25 squares with triple border were counted.

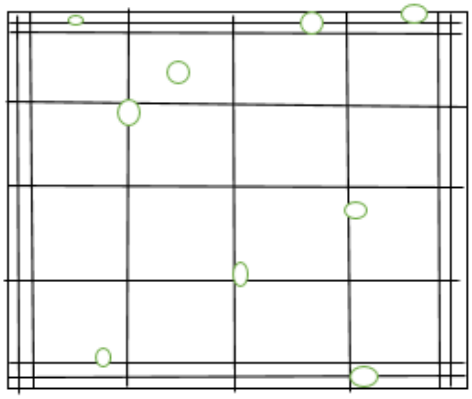


Figure 9 (own creation): example of magnified field of haemocytometer slide for cell calculation. Green circles- cells eligible for counting.

Figure 10 below is a representative image of a field of viable PBMC cells under the microscope using 10x magnification. Viable cells with intact cell membranes do not take up the colour of Trypan blue whereas damaged cells take up Trypan blue due to their disrupted membrane, therefore appear blue in the haemocytometer.

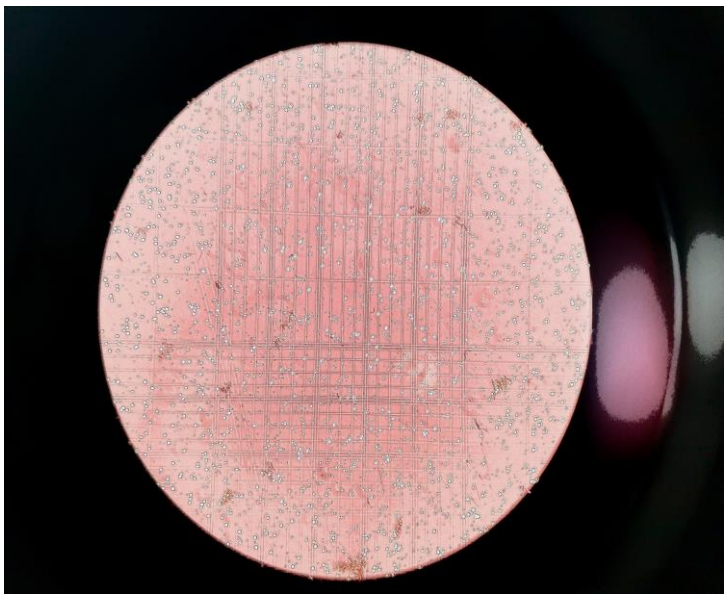


Figure 10 (own photograph): PBMC cells stained with Trypan blue and viewed under the microscope x10 magnification.

Total cell number was then calculated using equation (n/10 x5 x10^6). Cells that were not used immediately were stored inside a cryo freezing container like Mr.Frosty within a minus 80°C freezer. After 24 hours, these samples typically get transferred out from the freezer to a liquid nitrogen storage tank for longer storage and future usage.

2.3: BrdU incorporation in lymphocyte steroid sensitivity assay (BLISS)

The hepatology research group I worked with has developed a new laboratory assay (BLISS assay) to measure intracellular GC resistance based on leukocytes' response to GC. The assay takes three days to complete and has previously been tested for accuracy and reproducibility in identifying GC responders and non-responders.

PBMCs were first isolated from peripheral bloods. A density of 6.5x10^6 cells were then taken out from the total PBMC to be processed for BLISS assay.

Day 1

Phytohaemagluttinin (PHA) and Dexamethasone (Dex) are made up in advance from stock solutions. 150µL RPMI added to one aliquot of 100µL of PHA (concentration at 400ug/mL). 990µL RPMI added to one aliquot of 10µL Dex (concentration of 10^{-3} M). In a new 15mL conical tube, 6.5x10^6 cells were suspended in 3mL of RPMI (cells at 2 x10^6/mL). The cells were then plated out following the layout as shown in table 1 below:

	1	2	3	4	5	6	7	8	9	10	11	12
A		Cells Alone							Media control			
B												
C		Cells +PHA							Media +BrdU			
D												
E		Cells +PHA		+Dex					Media +BrdU POD			
F												

Table 1: Plate layout for BLISS assay. PHA- Phytohaemagluttinin; Dex- Dexamethasone; BrdU- Bromodeoxyuridine; BrdU POD- Bromodeoxyuridine with peroxidase.

Firstly, the cells were plated into 5 wells (B2-6) with 170µL/well labelled cells alone. 30µL of complete media then added to each of these wells. Next, 10µL of PHA was added to the stock 15mL conical tube and resuspended with the stock cells. 180µL /well of the stock +PHA solution then plated into D2-6 wells labelled 'Cells+PHA'. 20µL complete media then added to each of these wells. Lastly, 20µL of Dex was added into the same 15mL conical tube and resuspended with the remaining stock cells. Next, 200µL /well plated into F2-6 wells labelled 'Cells+PHA+Dex'.

200µL of complete media added into all control wells (B9-11, D9-11, and F9-11). After addition of all media, plate was covered and placed in the incubator at 37'C with 5% CO2. The plate was incubated for 48 hours.

Day 3

BrdU solution was first diluted to 1:100 from stock by adding 10µL to 990µL RPMI. 20µL of BrdU mixture was added into all wells containing cells, and the control row labelled 'Media + BrdU' at D9-11. Plate then covered and incubated for further 6 hours in the incubator with 5% CO2. After 6 hours of incubation, contents of all wells were transferred into a 96 flat well plate using the same plate plan. Next, the plate was spinned at 600rpm for 10minutes, and supernatant flicked off from plate into a waste container.

The plate was then dried in a 60°C oven for 1 hour. Then, 200µL FixDenat was added into every well of the plate. Plate was then placed in a room temperature incubator for 15 minutes. After incubation, supernatant flicked off from plate into the waste container.

Next, BrdU-POD was diluted with antibody diluent solution at 1:100 concentration. 100µL of the antibody-BrdU-POD solution was then added into each well containing cells and the control wells labelled 'Media+BrdU-POD'. Following that, plate incubated at room temperature for a further 90 minutes. Supernatant flicked off from plate into waste container. Following that, plate was washed three times with 200µL per well of washing solution. Said washing solution was pre-diluted at concentration of 1:10 with distilled H_2O. Following that, plate was washed for a final time with equal amount per well of PBS solution. Solution then flicked off from plate into the waste container. Finally, 100µL of substrate solution was put into each well before incubation at room temperature for 5 minutes.

Following incubation, plate was read under a plate reader at the absorbance wavelengths of 370nm and 492nm. Final absorbance value was calculated by deducting the absorbance value at A492nm from A370nm. IMAX values are

then calculated using the formula (1-{proliferation in presence of GC}/ {proliferation in absence of GC}) x100.

2.4: *In vitro* measurement of lipid peroxidation with TBARS assay

When assessing oxidative stress, it is crucial to detect and quantify the level of ROS [126, 127]. However, direct quantification of ROS can be onerous and provide insignificant yield as ROS are volatile and short-lived which means their levels are fluctuating continuously [126, 127]. Further, most ROS probes only capture a percentage of ROS, and ROS tend to interfere with most probe molecules, making the interpretation of results challenging [126, 127]. Therefore, researchers prefer to measure the end-product of oxidative damage as a surrogate marker of oxidative stress [126, 127].

ROS is capable of degrading lipid and generate reactive lipid peroxides in a process named lipid peroxidation [115,225]. Further decomposition of these peroxides then culminate the production of several end products, in particular- malondialdehyde (MDA) [225-228]. Thiobarbituric acid (TBARS) assay kit which measures level of MDA is therefore deemed the easiest and most effective method of determining the relative lipid peroxidation and a reliable biomarker of oxidative stress content in a sample [225-228].

The TBARS assay kit used in my experiments was purchased from Cayman Chemical, USA. PBMCs were isolated as above and $20x10^6$ of the whole PBMC cells were removed for this assay and prepared according to manufacturer's manual. 8 tubes of standards were prepared by a serial dilution of 1:2 concentration from a stock solution of 125µM MDA standard.

100µL of patient sample which was pre-diluted in PBS were put into their respective labelled vials with a 100µL of sodium dodecyl sulphate (SDS) solution each vial. The mixture was allowed to mix thoroughly. Next, 4mL of colour reagent was added to the vials and mixed thoroughly. The vials were then added to vigorously boiling water to boil for an hour. After an hour, the vials were immediately removed and placed in ice bath to stop reaction.

150µL from each vial were pipetted in duplicates onto designated wells on a clear plate. The plate was then placed and read under the plate reader at absorbance wavelength of 530nm.

2.5: Magnetic activated cell sorting (MACS) positive selection

Separation of PBMCs into their subtypes of T cells and monocytes is necessary to understand their individual impact on histone modifications [229, 230]. Studies on this field has suggested that magnetic activated cell sorting (MACS) technology is the most efficient and robust way to isolate human monocytes of high quality and purity [229, 230].

Magnetic activated cell sorting beads (Miltenyi Biotec) were used to isolate monocytes and T cells from the PBMCs extracted previously. The resultant cell subtypes were incubated in MACS buffer at a concentration of $1x10^6$ per 80µL in the fridge (temperature 4°C). 20µL of positive selection CD14+ microbeads were added into the MACS mixture before incubation. Cells were then washed and suspended in an additional 1mL of MACS buffer. They were filtered through a MS column (Miltenyi Biotec) over a MACS magnet. MS column contains a matrix composed of superparamagnetic spheres in a plastic column coating. When the column is placed in a MACS Separator, the strong magnetic field within the column retains cells being labelled with the microbeads.

The column was detached from the magnet and flushed through with 1mL of MACS buffer. These cells were labeled as CD14+. The flow through (labelled CD14-) was then further suspended with 20µL of CD4+ microbeads and incubated in the fridge for 15 minutes. Cell pellet was washed thoroughly and returned to liquid medium in MACS buffer before getting filtered through a new MS column. The flow through of this step was labeled CD4-. The column removed and flushed through with 1mL of MACS buffer. These cells which were flushed through were then labeled as CD4+.

CD14+ and CD4+ cells were retained while the rest of cells were discarded. CD14+ and CD4+ cells were then washed once more before getting assessed and counted using Trypan blue solution under the microscope. This step is to ensure cell viability of cells and to ensure a fix number of cells go through to the next set of experiments. Figure 11 was extracted from the manufacturer's (Miltenyi Biotec) website. It summarises the mechanism and process of magnetic activated cell sorting (MACS) method in segregating subsets from PBMCs, predominantly T cells and monocytes.

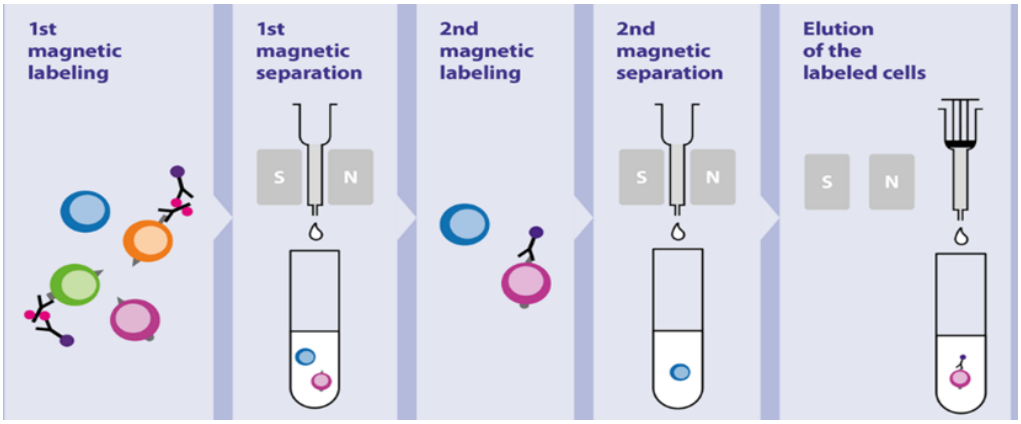


Figure 11 [229, 230]: Process of MACS method in isolating T cells and monocytes from PBMCs.

2.6: RNA isolation

RNA isolation was performed using Direct Zol RNA isolation kit from Zymo Research (USA). Before the start of protocol, reagents were prepared. 48mL of 99% ethanol was added to 12mL of RNA wash buffer concentrate whereas 15.5mL of 99% ethanol was added to 24mL of Direct-zol DNA wash 1 concentrate.

A density of $20x10^6$ PBMC cells from selected patients were extracted from the main liquid nitrogen storage and defrosted according to standard manufacturer instruction manual. Cells were then centrifuged at 1700rpm for 10minutes. Pelleted cells were mixed thoroughly with 300µL of TRIzol®. The mixture was placed in the room temperature incubator for an hour. The supernatant was then transferred into a nuclease free eppendorf.

2.6.1: RNA purification

For the RNA purification step, 300µL of 99% ethanol was added and mixed thoroughly with the supernatant in the nuclease free eppendorf. The mixture was then moved into a Zymo-Spin™ IICR column and centrifuged at maximum speed of 16,000 xg in a microcentrifuge for 30seconds. The column was then moved into a different collection tube and flows through discarded.

Next, 400µL of RNA Prep buffer was transferred into the column and the column was subsequently centrifuged at 16,000 xg for 30seconds. This step was repeated for another time. Following that, the column was washed with 700µL of RNA wash buffer and mixture centrifuged at 16,000 xg for 1 minute to remove wash buffer. Next, cleaned column mixture was transferred into a new 2mL nuclease free eppendorf tube. Further 50µL of DNase/RNase free water was put on directly to the column mixture and then centrifuged at 16,000 xg for 1

minute. The products which permeated through, contained pure RNA was then labelled and stored frozen at -20°C freezer.

2.7: cDNA reverse transcription

Before undergoing the step of cDNA reverse transcription, the RNA content and purity of each sample was measured with a Nano drop (ThermoScientific, UK). A fixed amount of 500ng high quality RNA from each sample was then separated and reverse transcribed guided by the steps stated in the manufacturer's manual (ThermoFisher Scientific, UK).

Reverse transcriptase (RT) master mix was made ready before the start of protocol with volume of each component illustrated in table 2 below.

Component	**Volume**
10X RT Buffer	2 µL
25X dNTP Mix (100mM)	0.8 µL
10X RT random primers	2 µL
MultiScribe reverse transcriptase	1 µL
RNAse inhibitor	1 µL
Nuclease free H_20	3.2 µL
Total per reaction	10 µL

Table 2: Components of reverse transcriptase master mix with their respective volume. RT- Reverse Transcriptase; dNTP – deoxynucleoside triphosphate; RNAse-Ribonuclease.

All the components were mixed gently in ice and stored aside. 10µL of the RT master mix was pipetted into a clean microcentrifuge eppendorf tube. Next, 10µL of patient's RNA sample was added into the eppendorf tube and mixed thoroughly before centrifugation at 10,000 xg for 1 minute. The tubes were then placed on ice until ready to load into the thermal cycle. Thermal cycle programme was set up as table 3 below:

Setting	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temperature	25°C	37°C	85°C	4°C
Timing	10minutes	120minutes	5minutes	∞

Table 3: Thermal cycle settings for cDNA reverse transcription with respective temperature and timing for each step. °C- degrees celsius; ∞- Infinity amount of time.

2.8: Quantitative real-time polymerase chain reaction (qPCR)

qPCR was carried out using the Roche480 LightCycler (Roche, UK). HDAC1-11, HATs and SIRT 1-7 gene primers were obtained from Eurofins Genomics UK and optimised as stated by the manufacturer's protocol. Samples were amplified at 45°C for 2 minutes, 95°C for 10 minutes and then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression of HDACs, HATs and SIRTUINs were normalized to housekeeping gene GAPDH and expressions were compared in relativity to pooled cDNA from 5 HVs.

Table 4 shows all the primers utilized in this set of experiment.

Gene	Forward sequence	Reverse sequence	Product size
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCGT	101
HDAC 1	TAAATTCTTGCGCTCCATCC	AACAGGCCATCGAATACTGG	102
HDAC 2	CAGTTGCTGGAGCTGTGAAG	AATTCAAGGATGGCAAGCAC	139
HDAC 3	CTGGTCCTGCATTACGGTCT	TATTGGTGGGGCTGACTCTC	130
HDAC 4	AGCAGAGGCTCTCCCTTTTC	GTGGCTGCTCCAGTAAGACC	126
HDAC 5	AGATGCACTCCTCCAGTGCT	GGATGATGGCAAATCCATTC	102
HDAC 6	GAACCTTGAGGCTGAAGCAC	TCCTGGATCAGTTGCTCCTT	138
HDAC 7	AGCGGAGGTGATTCTGAAAA	GAACAGGAGGCAAAAAGCTG	146
HDAC 8	CGACGGAAATTTGAGCGTAT	TGTTCCTGGGAAAAATCCTG	138
HDAC 9	GCGGTCCAGGTTAAAACAGA	GACCAGAGCCTGGAGAACTG	146
HDAC 10	TGGGAAGCTCCTGTACCTCTT	GGCTGGAGTGGCTGCTATAC	73
HDAC 11	CTTCTGTGCCTATGCGGACATC	GAAGTCTCGCTCATGCCCATTG	106
Sirtuin 1	TAGACACGCTGGAACAGGTTGC	CTCCTCGTACAGCTTCACAGTC	117
Sirtuin 2	GCCCTTTACCAACATGGCTG	TTCGTACAACACCCAGAGCG	120
Sirtuin 3	AGAAGAGATGCGGGACCTTG	GGTCCATCAAGCCTAGAGCAG	105
Sirtuin 4	GGCAGGAATCTCCACCGAAT	GCACTCCGGACAAAATCACC	111
Sirtuin 5	GGTGTTCCGACCTTCAGAGG	GTGGTAGAACTCCCACACCC	114
Sirtuin 6	AGTCTTCCAGTGTGGTGTTCC	TCCATGGTCCAGACTCCGT	94
Sirtuin 7	GGTGGAGCGGGAATAGTCAG	CTGGGATAGACGCTGCACAT	99
GCN5	AAGGACCCCGACCAGCTCTA	GGGAAGCGGATGACCTCGTA	120

PCAF	AGGCACCATCTCAACGAAGA	AAGACCGAGCGAAGCAATGT	120
P300	GGGACTAACCAATGGTGGTG	ATTGGGAGAAGTCAAGCCTG	120
CBP	GAGGTTTTTGTCCGAGTGGTGG	TGGGTGGCAATGGAAGATGTAA	122
SRC-1	CATGGTCAGGCAAAAACCTT	GCTTGCCGATTTTGGTGTAT	120
SOD-1	ACGGGGTGCTGGTTTGCGTC	TTCAGCACGCACACGGCCTT	120
SOD-2	TCCCAAGGGAAACACTCGGCTTT	AAACCACTGGGTGACATCTACCAGA	123
Catalase	CGTGCTGAATGAGGAACAGA	AGTCAGGGTGGACCTCAGTG	120
GPX-1	TCCCTGCGGGGCAAGGTACTAC	TTCGTTCTTGGCGTTCTCCTGATG	124
BCL2	TGTGGAGAGCGTCAACCGGGAG	ATCAAACAGAGGCCGCATGCTG	122
BAX	TGGACTTCCTCCGGGAGCGG	CTGGGGGCCTCAGCCCATCT	120
NFKB-1	CCTGGATGACTCTTGGGAAA	TCAGCCAGCTGTTTCATGTC	120
HSPA4	AGCAGCGCTCTCGGTTGCAG	AGACAGGACACGGACCCCCG	120
HSPA5	TGCTGCTGCCCAACTGGCTG	GAACACGCCGACGCAGGAGT	120
EIF2A	ACGCCGCTCTTGACAGTCCG	TTGCCCCAGGCAAACAAGGTCC	122
EIF2A3K	CCCCAACAAGGCCAGCCTGG	GGACAGCCAGCCGTGTT CCC	120

Table 4: Gene primers name with forward sequence, reverse sequence, and respective product size.

2.9: Flow cytometry

Flow cytometry was conducted using BD ARIA II. 7-aminoactinomycin D (7-AAD) stains were used with all samples to assess cell viability during analysis. Fluorescence minus one (FMO) control were used in the pilot experiments to determine the negative population to allow best gating strategy going forward. Thereafter, single colour compensation controls were used.

Table 5 shows a list of antibodies used for flow cytometry experiments.

Antibody	**Supplier**	**Product number**
7AAD viability staining solution	eBioscience	E000031-1639
CD3 PE anti human isotype mouse IgG 2α,κ	Biolegend	317308
CD14 anti Hu APC	Invitrogen	2071261

Table 5: A list of antibodies with respective supplier and product number.

2.9.1: Intracellular measurement of ROS with dichloro-dihydro-fluorescein diacetate (DCFH-DA)

To quantify and appreciate the differences in level of ROS in HV versus AH patients and between different severity of AH, I have chosen to measure the end products of oxidative damage with a cell permeable and fluorescent probe named 2, 7-dichlorofluorescein diacetate (DCFH-DA, Sigma Aldrich UK).

DCFH-DA is a fluorogenic dye that directly measures the hydroxyl, peroxyl and other ROS products within the cell [231-234]. After diffusion into the cell, DCFH-DA is deacetylated by cellular esterase to a non-fluorescent compound, which is later oxidized by into highly fluorescent 2, 7-dichlorofluorescein (DCF) [231-234]. DCF can be picked up by flow cytometry and quantified with excitation emission at 535nm [231-234]. The fluorescent peak in cells that contained hydrogen peroxide (H_2O_2) shift right compared to the peaks in controls [231-234].

To allow flexibility in experimental timings, I tested whether DCFH-DA staining worked on fixed cells. However, no signal was seen in any condition stained with DCFH-DA on fixed cells. Evidently, these experiments must be done on fresh samples.

The effect of DCFH-DA staining on cell viability was assessed using 7-AAD, which is a ready-made nucleic acid dye solution to exclude non-viable cells during the data analysis in flow cytometry. There was minimal spectral overlap between emissions of antibodies. CD3 and CD14 antibodies were used on top of DCFH-DA to differentiate T cell and monocyte populations.

The antibodies used in this experiment have distinctive fluorescence to ensure minimal overlap during flow cytometry. Table 6 below shows the antibodies with their respective fluorescent probes which are used in the flow cytometry.

Antibody	Fluorescent probes
DCFH-DA	FIT-C
CD14	APC
CD3	PE
7-AAD	PerCP cy5.5

Table 6: Types of antibody and respective fluorescent probes.

For the flow cytometry experiments, PBMCs were isolated from peripheral bloods as protocol above. 2 x 10^6 cells were extracted and transferred into FACS tube and washed with fluorescent antibody cell sorting (FACS) buffer.

The cells were spun down, supernatant removed, and the pellet is stained with CD3 PE, CD14 APC and DCFH-DA antibodies for 30 minutes in at 4°C.

Following that, cells were washed once again, and the pellet was suspended in 200µL of FACS buffer. Analysis of samples were done with the BD ARIA II flow cytometry (BD Biosciences, Oxford, UK). Cells produced different mean fluorescence value as they expressed different amount of labelled antibodies in the context of CD3, CD14, and DCFH-DA. Dead cells were excluded as they were labelled actively with 7-AAD stain.

2.10: Nuclear and Cytoplasmic extraction

$1x10^6$ of $CD14^+$ Monocytes and $1x10^6$ of $CD4^+$ T cells magnetically sorted from PBMC were used for this nuclear and cytoplasmic extraction protocol (PIERCE, ThermoFisher UK). Each cell subtype was processed separately to avoid contamination but followed the same steps below.

Immediately before use, 1µL of protease inhibitor was added to CER I and NER from concentrated stocks.

Cells were transferred into a clean Eppendorf tube and washed twice with 1mL of ice-cold PBS, and then centrifuged at 500g for 5minutes. Supernatant removed carefully with pipette, leaving a dry cell pellet at the bottom of Eppendorf. Then, 100µL of ice-cold CER I was re-suspended with the cell pellet. The tube was then spinned at the highest setting for 15seconds before 15 minutes of incubation in the ice.

Next, 5.5µL of ice-cold CER II was mixed into the tube and tube vortexed for further 5 seconds on the highest setting. The tube was then incubated on ice for 1 minute. After the incubation, the tube was centrifuged at 16,000 xg for 5 minutes. Following that, the supernatant which represents the cytoplasmic extract, was transferred to an eppendorf and stored at -80°C.

The insoluble cell pellet that contains the nuclei was further suspended in 50µL of ice-cold NER and then vortexed on the highest setting for 15 seconds. The sample then placed on ice and vortexed for 15seconds every 10minutes, for a total of 40minutes. After the vortex, the Eppendorf was centrifuged in the microcentrifuge for 10minutes at maximum speed of 16,000 xg. The remnant supernatant which was the nuclear extract was transferred to an Eppendorf and then set aside in a freezer of -80°C.

2.11: Hoechst Assay

To unify the DNA density of the nuclear and cytoplasmic extracts for my histone experiments, I used a fluorescent blue dye, Hoechst stain purchased from ThermoScientific, UK.

Lambda DNA (at a concentration of 0.3µg/µL) was used as standards. They were diluted at 1:20 to make a standard curve. Nuclear extracts were washed gently twice in 200µL of PBS. Meanwhile, Hoechst stain was prepared at 4µg/mL in 2x TNE buffer. 50µM of DNA standard and samples were pipetted into a black microtitre plate in duplicates. 50µL of Hoechst stain was added to each well.

Plate was gently shaken for 30minutes on an orbital shaker. Fluorescence of samples and standards were measured in a fluorescent plate reader at excitation emission of 360nm and at 460nm.

2.12: Histone Acetyltransferase activity assay (HAT)

Before undergoing the HAT activity assay (Abcam, UK), the reagents were optimised according to the manufacturer's manual. HAT Substrate I, Substrate II were reconstituted with 550µL of HAT reconstitution buffer. A master mix was drawn up in preparation. Master mix contained a total of 68µL (50µL 2X HAT Assay buffer, 5uL HAT Substrate I, 5µL of HAT Substrate II and 8µL of NADH Generating Enzyme).

50µg of nuclear extract was prepared according to the nuclear extraction protocol above and diluted with 40µL of water. Positive controls were prepared by adding manufacturer's 10µL of cell nuclear extract at concentration of 4mg/mL to 30µL of water.

Finally, the master mix was pipetted carefully into each well, and mixed with 50µg of nuclear extract (in 40µL of water). Plate incubated at 37°C for 2 hours and then read in a plate reader at 440nm. HAT activity level was expressed in the format of relative optical density value per µg.

2.13: Class 1 histone deacetylase activity assay (HDAC)

Histone Deacetylase Class 1 (HDAC) activity assay was purchased from Amsbio, UK and experiment was performed in accordance to the manufacturer's instruction manual.

Before the start of assay, Trichostatin A (concentration of 200µM) was diluted at a concentration of 1:10 with HDAC Assay Buffer to make up a 20µM solution. Fluorogenic HDAC substrate 3 (concentration of 5mM) was diluted 1:25 with

HDAC assay buffer to make up a 200μM solution while HDAC 2 was diluted in HDAC assay buffer to make up to a 1ng/μL solution.

Next, the master mix was prepared (consisted of 5μL of Fluorogenic HDAC substrate 3 + 5μL of BSA at 1mg/mL concentration + 30μL HDAC Assay Buffer). 40μL of this master mix was pipetted into to every well of a microtitre black plate. 5μL of patient sample (standardized at a concentration of 50ug nuclear extract) was added to their respective wells. 5μL of Trichostatin A was added to the negative control wells; whilst 5μL of the HDAC Assay Buffer was added to the blank wells. Lastly, 5μL of diluted HDAC2 enzymes was added to the patient sample wells, positive control and negative control wells. All samples were plated in triplicates.

Plate was incubated at 37°C for 30minutes. Next, 50μL of HDAC Assay Developer was put into each well. Plate further incubated at room temperature for 15 minutes. Finally, plate was read in a microtiter-plate reader at wavelength of 350nm and 460nm. Class 1 HDAC Activity calculated using formula: $14.6-0.01847x + 8.827e^{-6}x^2$.

2.14: Phosphoinositide-3-Kinase Catalytic Delta Polypeptide (PI3Kδ) activity assay

PI3K δ activity ELISA kit was purchased from Biomatik, UK. Before the start of assay, 7 tubes of standards were reconstituted with serial triple dilution from standard stock solution (concentration at 1000pg/mL). Each tube was mixed thoroughly and kept aside at room temperature.

Next, the reagents were reconstituted in accordance to manufacturer manual. Detection reagent A was first reconstituted with 150μL of Reagent Diluent and then diluted at 1:100 with Assay Diluent A. Detection Reagent B was also diluted at 1:100 with Assay Diluent B.

Samples were standardized at a density of 50μg of nuclear extract. Samples were then diluted with 100μL of PBS. 100μL of each dilution of standard, blank and samples were pipetted into their respective well in a pre-coated 96 wells plate. Plate was sheltered and incubated at 37°C for an hour.

Following that, the supernatant of each well was removed. 100μL of Detection Reagent A was added to each well and plate underwent further incubation at 37°C for an hour. Subsequently, solution was drawn out carefully and each well was washed with the pre-made wash solution using a multi-channel pipette. Wash step was repeated 3 times.

Next, 100µL of Detection Reagent B was pipetted to each well. Plate covered and incubated at 37°C for 30minutes. Subsequently, 90µL of substrate solution was mixed into each well simultaneously with a multi-channel pipette. Plate was incubated at 37°C for 10 minutes. Finally, 50µL of stop solution was put into each well and plate tapped gently to ensure thorough mixing. Finally, the plate was read under the microplate reader at 450nm.

2.15: Cell culture

After PBMC extraction, cells were suspended in PBS reagent and cultured in 96 wells U shaped plate. A density of 200,000 cells were cultured in each well. One plate was set up as baseline BLISS, whilst a second plate was set up with different treatment conditions. All cultures received PHA to stimulate cell proliferation. All samples were plated in five replicates. Table 7 below shows the supplemental treatment choices and their respective concentration for cell cultures.

Treatment type	**Concentrations/ well**
Zinc sulphate (Zn)	50µM
Sodium Butyrate (NaBu)	20mM
N-acetylcysteine (NAC)	10mM
Vorinostat	10mM

Table 7: Cell culture supplemental treatment types with concentrations used per well in a 96 wells plate. Zn- Zinc, NaBu- Sodium Butyrate, NAC-N-acetylcysteine, uM-micromolar, mM-millimolar.

Concentration of each supplement was decided based on the cell toxicity experiment results gained from another member of the hepatology research group. All cells were proliferated with PHA. Cells were separated into two groups. First group received only the desired treatment supplement; whilst the second group received the treatment supplement with additional dexamethasone. They were then plated into their labelled wells in two different 96 well U plates. Both plates were incubated for 48 hours before undergoing the BLISS assay as described in paragraph above. Final absorbance value was obtained through plate reader and final IMAX values were calculated according to the formula stated in the BLISS paragraph.

2.16: Statistical analysis

All the statistical analysis in my research work was conducted with GraphPad Prism version 8 (GraphPad, Software Inc, California, USA). Mean and standard deviation were calculated for all variables.

All the data analyzed in this thesis were non-parametrically distributed, due to small-scale sample size. Student's t test was utilized to differentiate values of 2 independent sample cohorts to a single relative control value.

Correlations were performed using the Pearson correlation coefficient with an online calculator at Pearson Correlation Coefficient Calculator (socscistatistics.com). Regression analysis was conducted to determine the relationship between two independent variables. The nearer the r value is to zero, the weaker the correlation relationship.

A p value of equal to or less than 5% ($p \leq 0.05$) was categorized to be significant and represented by one asterisk (*); whereas a p value of equal or less than 1% ($p \leq 0.01$) was considered extremely significant and symbolized with two asterisks (**).

I performed receiver operating characteristic (ROC) curve analysis to identify whether percentage of maximal oxidative stress could be an unconventional, but reliable predictor of infection occurrence and disease outcome. The more elevated the AUC score is, the better the experimented model is in distinguishing subjects into their distinct categories. By general standard, AUC value of between 0.9-1 is excellent; 0.8-0.9 is good; 0.6-0.7 is poor but acceptable. AUC value below 0.6 is no better than a model that performs random guessing.

Chapter 3: Oxidative stress is increased in Alcohol related Liver Disease (ArLD) and Alcohol related Hepatitis (AH)

Introduction

Alcohol generates products of ROS via the CYP2E1 pathway [10-13]. The overproduction of ROS, which tips the homeostasis to oxidative stress contributes to histone modifications which then play a role in GC resistance [11, 86, 216].

Oxidative stress can be assessed in one of three approaches: (1) direct quantification of free radicals, (2) assessment of the damage caused by free radicals and (3) identification of compounds that counteract free radicals for instance, antioxidant levels [126, 127]. In my research project, I adopted two commonly used methodologies and that is to directly measure the amount of ROS and the product of lipid peroxidation.

The direct measurement of ROS involves quantifying ROS compounds superoxide anion or hydrogen peroxide [126, 127, 231-234]. These compounds are eliminated by enzymatic reaction, and they further oxidize biological molecules intracellularly [126, 127, 231-234]. An overwhelming amount of these compounds leads to the inability of antioxidants to counteract. On the other hand, lipid peroxidation is a well investigated marker of ROS-mediated damage to cell membranes, lipoproteins and lipid containing molecules [225-228].

Most reagents used for measuring ROS or product of ROS fluctuate easily with environmental variables such as light, oxygen, and temperature changes [126, 127]. It is therefore crucial that the storage and usage of these reagents and samples are performed meticulously, and experiments performed in a timely fashion. This limitation explains the time constraints and limited number of samples processed in my experiments.

Of note, there is appreciable difference in subject's age between the AH and HV group for the oxidative stress assays [235-237]. This is due to recruitment challenges for suitable HV. Genetic animal studies have confirmed that increased oxidative stress and impaired oxidative stress defence is detected in aged tissue [235-237]. Furthermore, gender difference is a vital factor to be considered when analysing oxidative stress [235-237]. In animal studies, male subjects have a higher production of ROS and less anti-oxidant defence mechanism [235-237]. No studies of such has been performed on human subjects. These limitations have been taken into account when interpreting results from the oxidative stress assays.

TBARS assay

Lipid degradation by ROS is called lipid peroxidation [225-228]. Lipid peroxidation process generates highly reactive end products [225-228]. These include Malondialdehyde (MDA) and Thiobarbituric acid reactive substances (TBA) [225-228]. MDA reacts with TBA at boiling temperature in acidic to generate a pink/red-coloured adduct, named MDA-TBA adduct [225-228]. These adducts can then be quantified under a spectrophotometer [225-228].

A well-validated and reproducible TBARS assay kit was purchased from Cayman Chemicals and was adopted in my research to quantify the MDA-TBA adducts in patient samples. Figure 12 illustrates the chemical reaction between MDA and TBA and product MDA: TBA adducts.

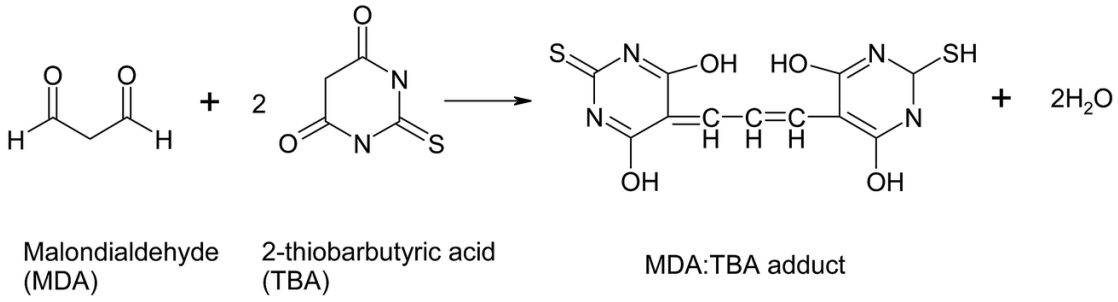


Figure 12: Chemical formulae extracted from the manufacturer's website and illustrates the chemical reaction between MDA and TBA. MDA- Malondialdehyde; TBA- 2-thiobarbutyric acid; H_20- water.

Results from this assay are presented as malondialdehyde concentration (micromole). Higher MDA levels reflect higher oxygen radical activity, and diagnostic of higher lipid peroxidation. Thus, MDA level is functional as an indicator of oxidative stress.

DCFH-DA staining

As described in the methodology chapter, 2, 7-dichlorofluorescein diacetate (DCFH-DA stain) is a fluorogenic stain that directly measures the ROS abundance within the cell [231-234]. After dissemination intracellularly, DCFH-DA is deacetylated by intracellular esterases and hydroxide to generate a non-fluorescent compound [231-234]. Said non-fluorescent compound are then oxidized by free radicals into fluorescent 2, 7-dichlorofluorescein (DCF) [231-234]. Figure 13 illustrates the chemical process from $DCFH_2$-DA to the eventual product DCF.

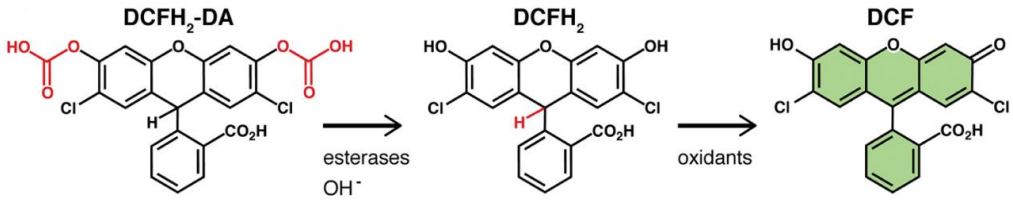


Figure 13 [231-234]: Chemical formulae extracted from the manufacturer's website and illustrates the chemical reaction between DCFH-DA and esterases which then transform into DCFH. Upon oxidation, DCF is formed.
$DCFH_2$-DA- dichlorofluorescein diacetate; OH^- - hydroxide; $DCFH_2$-dichlorofluorescin; DCF-2′7′-dichlorofluorescein.

Results from this experiment are presented as percentage of maximal oxidative stress with the fluorescence of positive controls using Tert-butylhydroperoxide (TBH) as denominator.

Results- TBARS assay

For the TBARS assay, twenty-two subjects were recruited.

Ten were AH patients (60% male; mean age 46.6; mean MELD 13.9; mean DF 45.6); five were patients with ArLD and were included as controls (40% male; mean age 57.6; mean MELD 22.6). There were more male patients in the AH cohort compared to the ArLD cohort. There was no significant dissimilarity between the age of ArLD and AH patients recruited into this experiment. Within the ArLD control group, 4 out of 5 patients decompensated with jaundice and ascites. No evidence of infection identified and therefore they were not commenced on antibiotics. All the patients (inclusive of AH and ArLD patients) survived beyond 90 days.

Maddrey's discriminant function test scores (DF) was applicable to patients with AH, whereas MELD score was applicable to both ArLD and AH patients.

Patient characteristics are presented in table 8.

	All (n= 15)	**ArLD (n=5)**	**AH (n=10)**
Age	50.2 (12.3)	57.6 (16.5)	46.6 (8.3)
Gender (%male)	8 (53.3%)	2 (40%)	6 (60%)
DF	-	-	45.6 (21.2)
MELD	16.8 (7.4)	22.6 (4.5)	13.9 (7)

Table 8: Patient characteristics with individual age, gender, DF score, MELD score. ArLD- Alcohol related liver disease; AH- Alcohol related hepatitis; DF- Maddrey's Discriminant Function; MELD- Model for End Stage Liver Disease.

The remaining seven subjects were HVs (42.8% male; mean age 25.8). None of the HVs had any acute or chronic health comorbidities. HV characteristics are presented in table 9. Results are presented in mean values with standard deviation.

	HV (n=7)
Age	25.8 (3.5)
Gender (%male)	3 (42.8%)

Table 9: Characteristics of healthy volunteers with age and gender. HV- Healthy Volunteers.

The timing of both TBARS and DCFH-DA assays were within 4 hours after blood samples were taken.

Results from the TBARS assay are presented in Figure 14. Mean value of MDA concentration for HVs was 17 with 95% CI [11.78, 22.22]; ArLD was 38.85 with 95% CI [16.98, 60.71]; and AH was 43.74 with 95% CI [31.94, 55.53]. This result demonstrates that MDA concentration was notably higher in the AH patients in contrary to HVs with mean value of 43.74 vs 17 and $p<0.01$. MDA concentration was also remarkably more elevated in patients with ArLD in contrast to HVs (mean 38.85 vs 17; $p<0.05$). There is no significant difference between the MDA concentrations AH and ArLD (mean 43.74 vs 38.85; $p=0.33$).

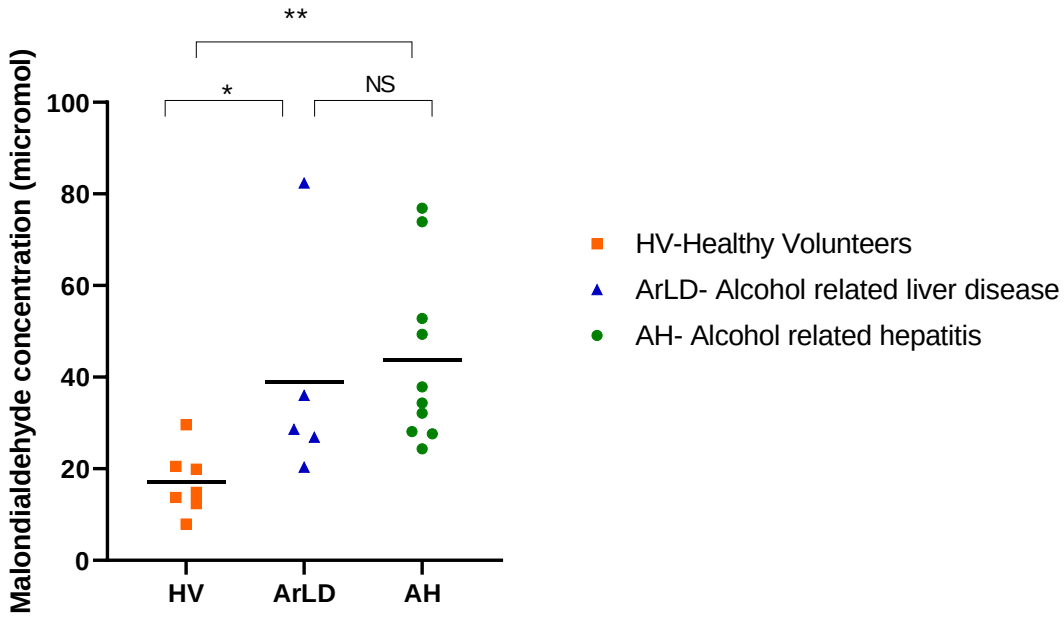


Figure 14: Malondialdehyde (MDA) concentration in micromol values for healthy volunteers (HVs), Alcohol related liver disease (ArLD) and Alcohol

related hepatitis (AH) patients. Oxidative stress as quantified by surrogate marker lipid peroxidation is evidently upregulated in patients with ArLD and AH in contrast to HVs. Statistical significance: NS- Not significant, *-p≤0.05, ** - p≤0.01.

In both AH and ArLD patients, there was no clear connection between MDA level and MELD score (R=0.13; R^2 is 0.018; p= 0.63). Figure 15 shows the scatterplots between the MDA concentrations (x-axis) and MELD score (y-axis). Each point in the scatterplot is a subject. The correlation coefficient is shown in the figure.

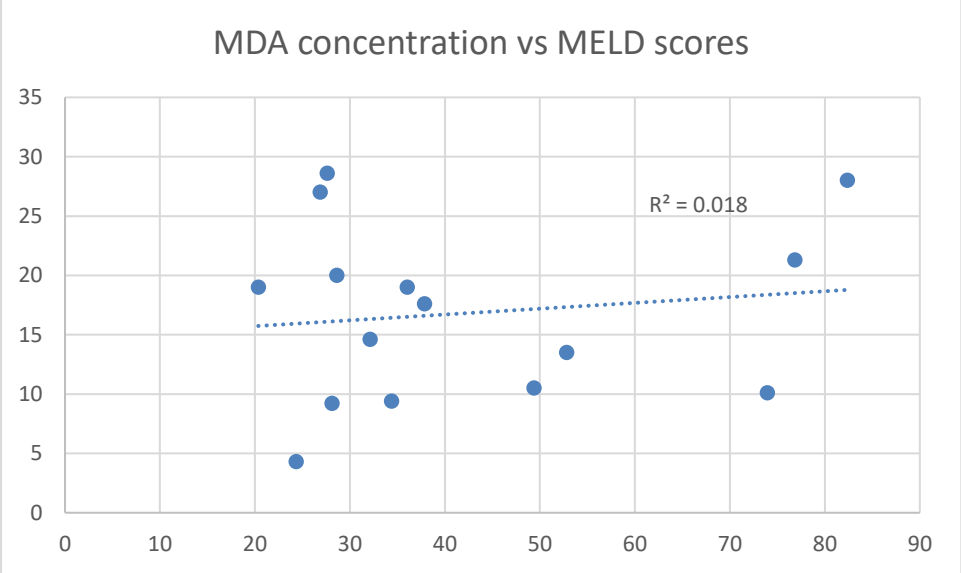


Figure 15: Scatterplots between the MDA concentrations (x-axis) and MELD scores (y-axis). R^2 -correlation coefficient.

In AH patients, there was no strong correlation between MDA level and DF score (r= 0.56; r^2 is 0.32; p= 0.09). Figure 16 shows the scatterplots between the MDA concentrations (x-axis) and DF score (y-axis).

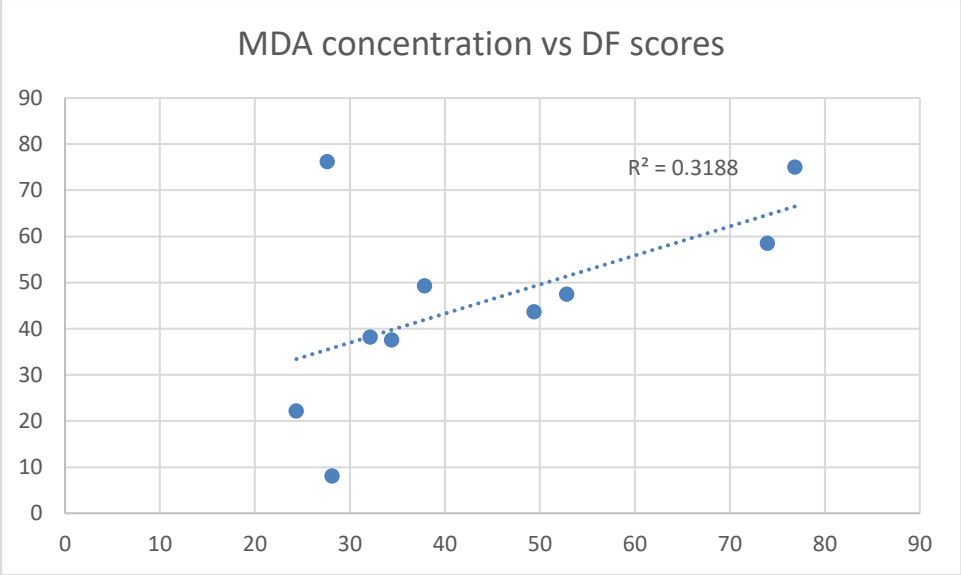


Figure 16: Scatterplots between the MDA concentrations (x-axis) and DF score (y-axis). R^2 -correlation coefficient. Each point in the scatterplot is a subject. The correlation coefficient is shown in the figure.

Results- DCFH-DA staining

To ascertain optimum dose of DCFH-DA, dose titration experiments were conducted. Figure 17 below show a series of flow cytometry histograms with different dosages of DCFH-DA.

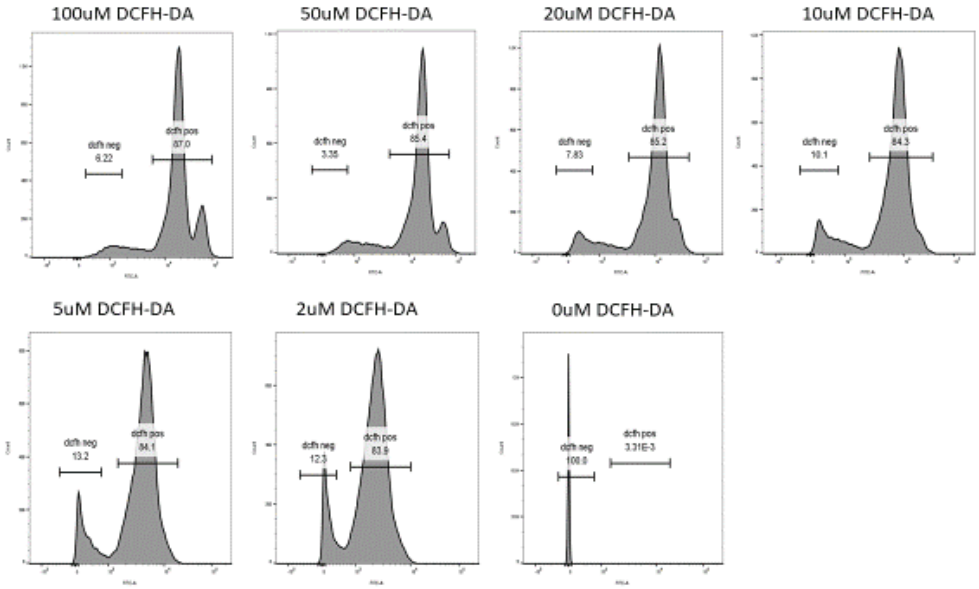
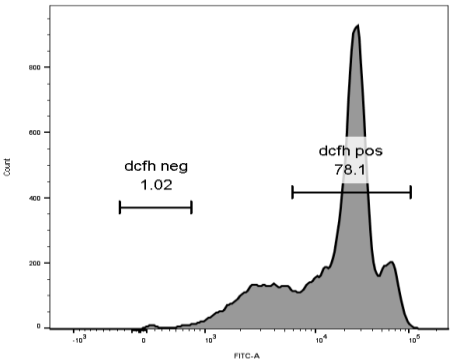


Figure 17: Representative flow cytometry histograms of DCFH-DA dose titration.

High doses of DCFH-DA (100μM, 50μM and 20μM) present with two peaks, and give no clear demarcation between DCFH-DA positive and negative. 5μM presents as the concentration with most optimum signal.

With regards to positive controls, Tert-butylhydroperoxide (TBH) is a product of reaction between hydrogen peroxide and tert-butyl alcohol in the presence of sulphuric acid. It is good as positive control because the addition of TBH would produce a high concentration of ROS within the PBMCs. Figure 18 below demonstrates that 50μM TBH when paired with 5μM DCFH-DA effectively eliminates the double positive peak and has a good negative peak too, making this dose an optimum dose for TBH as positive control. Therefore for the rest of my research, the optimized concentration of DCFH-DA (5μM) and TBH (50μM) were used.

500μM TBH+ 5μM DCFH-DA



50μM TBH+ 5μM DCFH-DA

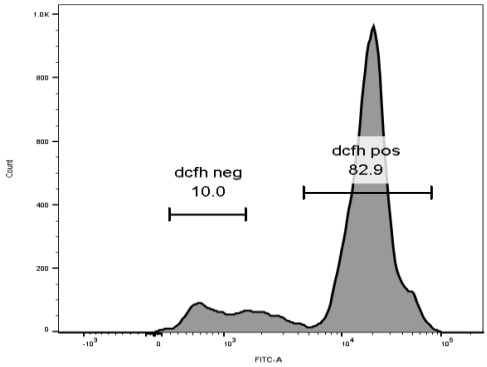


Figure 18 shows two representative flow cytometry histogram of TBH dose titration. Left figure shows the combination of 500μM TBH and 5μM of DCFH-DA where there is double positive peak; whilst right figure shows the combination of 50μM TBH with 5μM DCFH-DA.

Figure 19 below are representative histograms of the gating strategy used during flow cytometry analysis. Specific gating was used to segregate population of non-classical monocytes, intermediate monocytes, and T cells. Oxidative stress was quantified by the measurement of geometric mean of cells stained with DCFH-DA and results are presented as a percentage of maximal oxidative stress. All data were subsequently analysed using FlowJo v10 software.

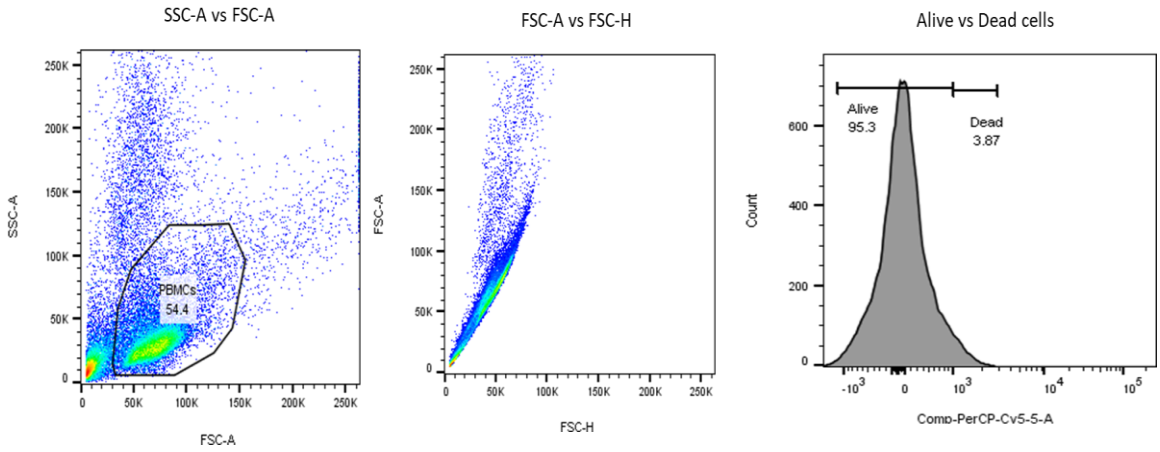


Figure 19: Example of gating strategy for PBMCs. PBMCs were selected from the forward side scatter and doublets and then dead cells were excluded.

For this part of experiment, forty-seven AH patients were recruited (59.6% male, mean age 53.5, mean DF 72.3, mean MELD 22.6). Twelve out of the forty-seven patients (25.5%) died within 90 days of hospital admission with AH. Of which, 17% of the forty-seven recruited patients died within a short period of 28 days. Within the non-survivors' group, seven were male; mean age 54.7, mean MELD 29.7, and DF 139.9.

There were no noteworthy dissimilarities in the age and gender of survivors and non-survivors. DF and MELD scores were greater in the non-survivors group indicating this group has more severe disease. Peripheral blood samples from patients with AH were obtained at hospital admission before any potential treatment with GC. Four out of forty-seven (8.5%) of the AH patients was treated with GC at day 3 of hospital admission. One out of the four patients (25%) treated with GC did not survive beyond 90 days of hospital presentation.

Patient characteristics are presented in table 10. Results are presented in mean values with standard deviation.

	All (n= 47)	**Survivors at Day 90 (n=35)**	**Non-survivors at Day 90 (n=12)**
Age	53.5 (9.5)	53 (9.8)	54.7 (8.4)
Gender (%male)	28 (59.6%)	21 (60%)	7 (58.3%)
DF	72.3 (76.5)	49.2 (30.4)	139.9 (122.3)
MELD	22.6 (6.9)	20.2 (4.6)	29.7 (7.7)
		Mortality rate	
Day 28 mortality		8 (17%)	
Day 90 mortality		12 (25.5%)	

Table 10: Patient characteristics with individual age, gender, DF score, MELD score, Day 28 and Day 90 mortality. DF- Maddrey's Discriminant Function; MELD- Model for End Stage Liver Disease; D28- Day 28; D90- Day 90. Eleven HV (4 male, mean age 37.8) were studied. None of the HV has health comorbidities. HV characteristics are presented in table 11. Results are presented in mean values with standard deviation.

	HV (n=11)
Age	37.8 (11.4)
Gender (%male)	4 (36.3%)

Table 11: Characteristics of healthy volunteers with age and gender. HV- Healthy Volunteers.

Percentage maximal oxidative stress was quantified in both cell subtypes (T cells and monocytes). These cell subtypes have been separated using their distinctive surface markers with antibodies and flow cytometry. For example, monocytes were stained with CD14+ antibodies; whilst T cells were stained with CD3+ antibodies.

Within the CD14+ monocytes, the percentage maximal oxidative stress in AH patients were significantly higher than in HV in CD14+ monocytes (63.2% vs 41.9%, p=0.0005), but not in CD3+ T cells (57.5% vs 52.7%; p=0.24).

Within AH patients, percentage maximal oxidative stress was significantly higher in non-survivors compared to survivors in both cell subtypes. In CD14+ monocytes (70.9% vs 60.5%; p=0.02); whilst in CD3+ T cells (67% vs 54.8%; p=0.05). Results are presented in the figures below. Figure 20 represents the results of percentage of maximal oxidative stress within CD14+ monocytes for HV, AH survivors and non-survivors.

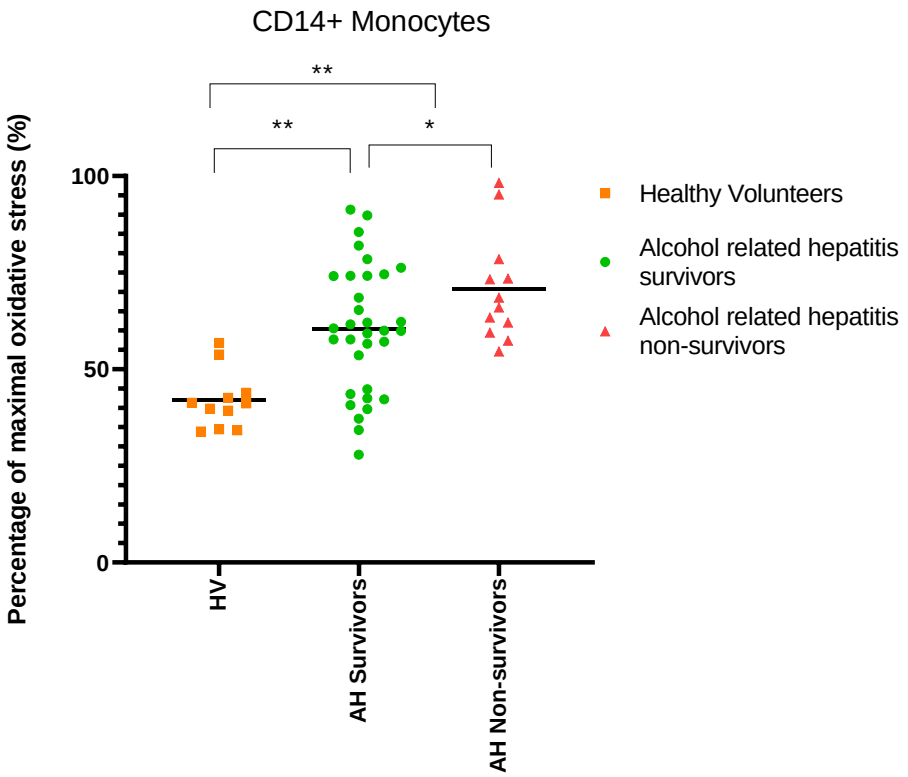


Figure 20: Percentage of maximal oxidative stress in CD14+ monocytes of HV, AH survivors, AH non-survivors. HV- Healthy volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: *- p≤0.05; **- p≤0.01.

Figure 21 represents the results of percentage of maximal oxidative stress within CD3+ T cells for HV, AH survivors and non-survivors.

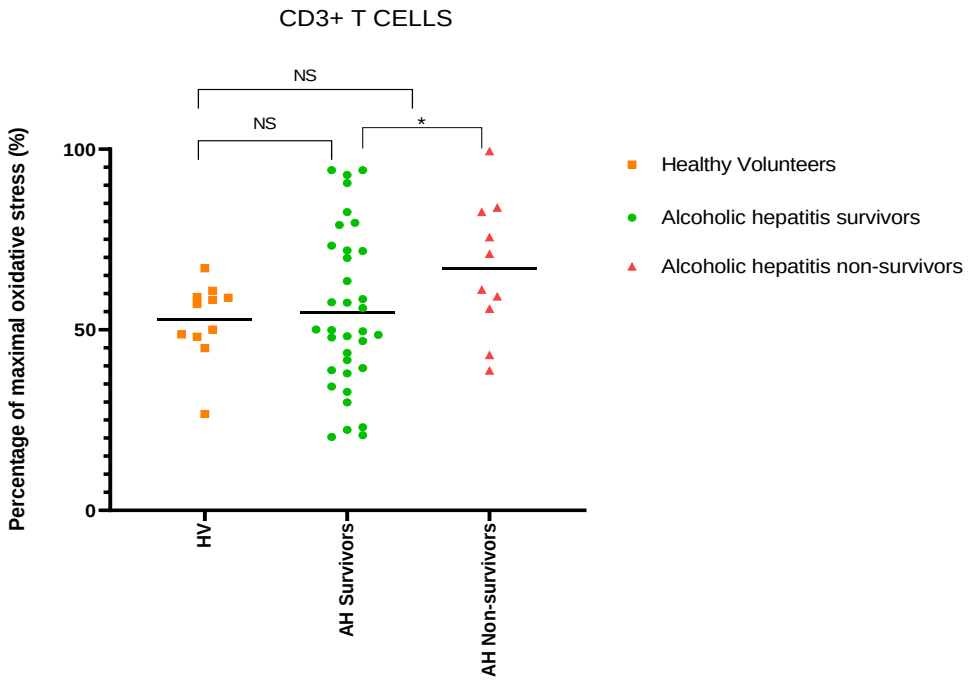


Figure 21: Percentage of maximal oxidative stress in CD3+ T cells of HV, AH survivors, AH non-survivors. HV- Healthy volunteers; AH survivors- Alcoholic hepatitis survivors; AH non-survivors- Alcoholic hepatitis non-survivors. Statistical significance: NS- Not significant, *-p≤0.05, ** - p≤0.01.

Percentage maximal oxidative stress as prognostic marker

Stratifying the severity of AH in clinical settings is challenging, especially with variable clinical presentations and course of disease. Early identification of patients with poor outcome will help avoid inappropriate use of GC and could produce beneficial consequences on patients' long-term morbidity and mortality rate.

Here, I am assessing the performance of percentage maximal oxidative stress as a surrogate 90 days outcome prognosticator with ROC curve analysis. This is done in comparison with Model for end stage liver disease (MELD) score which has been established clinically as a good prognostic marker in patients with liver disease.

CD14+ Monocytes

Percentage of maximal oxidative stress vs Day 90 mortality

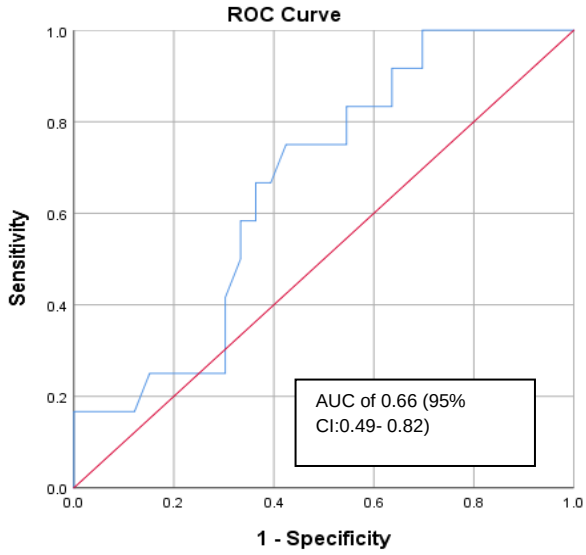


Figure 22

CD3+ T cells

Percentage of maximal oxidative stress vs Day 90 mortality

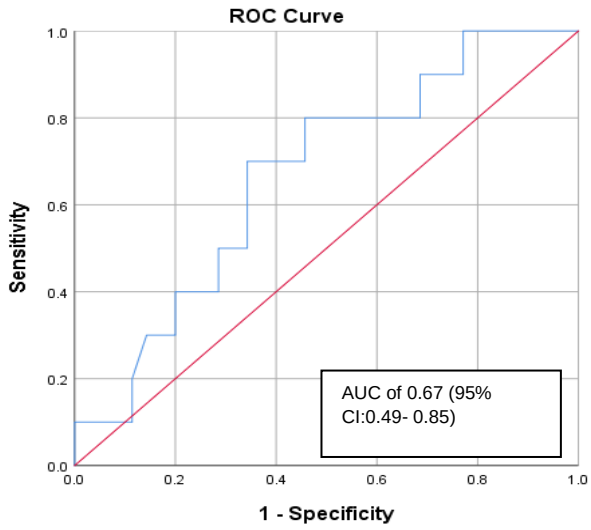


Figure 23

Figure 22 & 23: ROC curves of percentage of maximal oxidative stress vs Day 90 mortality in CD14+ Monocytes and CD3+ T cells respectively. ROC- receiver operating characteristic analysis; AUC- area under the ROC curve; 95% CI- 95% Confidence interval.

Figure 22 shows the percentage of maximal oxidative stress within CD14+ monocytes in predicting day 90 mortality with a result AUC of 0.66 (95% CI:0.49- 0.82). Similarly, Figure 23 shows the percentage of maximal oxidative stress within CD3+ T cells and day 90 mortality with a result AUC of 0.67 (95% CI:0.49-0.85). Overall, percentage of maximal oxidative stress with AUCs of 0.66 and 0.67 respectively, are too low to suggest meaningful usefulness in clinical practice. There is no significant difference when analyzing results between CD14+ monocytes and CD3+ T cells.

When compared to more conventional prognostic marker e.g. MELD score, GAHS and DF scores, it appears that these remain superior. Figure 24 demonstrates the results of ROC analysis for MELD scores and day 90 mortality in the same cohort of patients with a more superior AUC of 0.85 (95% CI: 0.72-0.98). Figure 25 and 26 demonstrate the results of ROC analysis for GAHS (AUC of 0.82 CI:0.75-0.91), DF scores (AUC of 0.80 CI:0.70-0.91) and day 90 mortality.

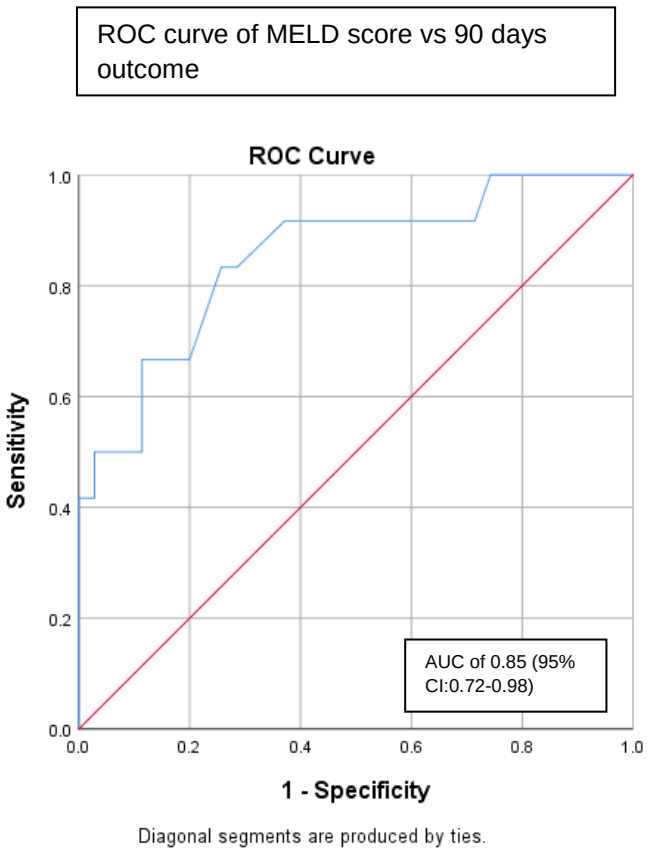


Figure 24

Figure 24: ROC curves of MELD scores vs Day 90 mortality. ROC- receiver operating characteristic analysis; AUC- area under the ROC curve; 95% CI- 95% Confidence interval.

Figure 25

ROC curve of GAHS score vs 90 days outcome

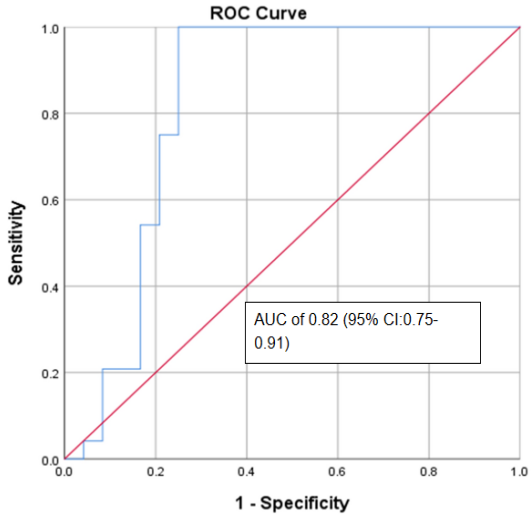


Figure 26

ROC curve of DF score vs 90 days outcome

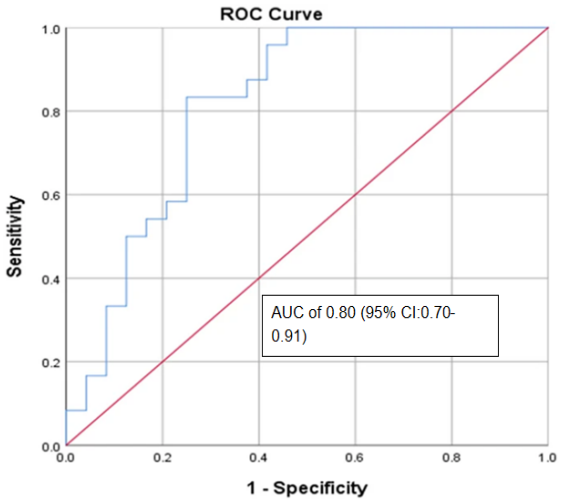


Figure 25 & 26: ROC curves of GAHS and DF scores vs Day 90 mortality. ROC-receiver operating characteristic analysis; AUC- area under the ROC curve; 95% CI-95% Confidence interval.

In addition, there was no strong correlation between percentage of maximal oxidative stress with MELD scores (R= 0.072; R^2 is 0.0052; p= 0.09) or with DF scores (R= 0.1574; R^2 is 0.0248; p= 0.301) in CD14+ monocytes. This is also consistent in CD3+ T cells and MELD scores (R= -0.0107; R^2 = 0.0001; p= 0.948) and DF scores (R= -0.0046; R^2 = 0; p= 0.979).

The lack of correlation suggests that oxidative stress may be independent of liver disease severity. Despite lack of correlation, percentage of maximal oxidative stress can be a supplementary to MELD/GAHS/ DF scores in recognizing AH patients with significant risk for early mortality.

Percentage maximal oxidative stress as predictor marker for infection

Here, I am determining if percentage maximal oxidative stress can be used as a predictor for occurrence of infection. Onset and source of infections for the patients were recorded prospectively. Eight (17%) patients had infections at admission. Sources of infection were 4.3% chest; 2.1% ascites; 2.1% bacteraemia; and the remaining 8.5% source was unknown. A further thirteen patients (27.6%) developed infection during their time in hospital (2.1% chest; 8.5% urine, 2.1% ascites, 2.1% bacteraemia and 12.7% source unknown) (Table 12).

Infection source	Baseline	Later
Chest	2 (4.3%)	1 (2.1%)
Urine	0	4 (8.5%)
Ascites	1 (2.1%)	1 (2.1%)
Bacteraemia	1 (2.1%)	1 (2.1%)
Unknown	4 (8.5%)	6 (12.7%)

Table 12: Number (percentage) and source of infections at baseline and during hospital admission for AH.

When assessing the diagnostic performance of percentage of maximal oxidative stress for any infection occurrence (both at baseline and at later stage of hospitalisation), AUC of 0.5 (CI 0.44-0.75) indicates that percentage maximal oxidative stress has a poor predictive ability for occurrence of infection.

Next, I assessed the correlation between percentages of maximal oxidative stress with several conventional inflammatory biomarkers for infection e.g. absolute numbers of white blood cell counts (WCC), lymphocyte counts and neutrophil counts which are WCC subpopulation. I also assessed the correlation between percentages of maximal oxidative stress with the neutrophil to lymphocyte ratio (NLR) to determine if percentage maximal oxidative stress can be used in conjunction or in replacement of these markers when assessing severity of infection/ inflammation.

Within the CD14+ monocytes population, there was no strong correlation between percentage of maximal oxidative stress with WCC values (r= 0.097), lymphocytes (r= -0.082), neutrophils (r= 0.0948) and NLR (r=0.085). Similarly in the CD3+ T cells population. There was also no strong correlation between percentage of maximal oxidative stress with absolute values of WCC and its subpopulations lymphocytes, neutrophils (r= 0.0628, r= -0.1563, r= 0.052 and r=0.061).

In summary, percentage maximal oxidative stress was not good predictor of infection. It did not correlate with a number of inflammatory markers like WCC, lymphocytes, neutrophils and NLR. However, these markers are part of the primary mediators of a complex processes of acquired immunity in response to infection. Therefore, even though this oxidative stress marker is not correlated with the numbers of inflammatory markers, further investigation needs to be done on the correlation of oxidative stress marker with the functionality of inflammatory markers, especially white cell count.

In an acute inflammatory state like AH, it is pertinent to understand the machinery of antioxidant defence mechanism [238-241]. Cellular antioxidant defence mechanism consists of a series of antioxidant enzymes which act to maintain homeostasis [238-241]. The antioxidants do so by counteracting or restricting the production of excess ROS intracellularly [238-241]. Antioxidant enzymes includes four major groups: a) secretory function b) antioxidant defence, c) apoptosis, and d) endoplasmic reticulum stress response [238-241]. All these antioxidant enzymes act by either detoxifying hydrogen peroxide (H_2O_2), inhibition of lipid peroxidation or restrict protein folding to chain break the ROS pathways [238-241].

To assess the antioxidants, I started by assessing the expression of antioxidant genes excluding the secretory function group as they are not applicable to AH.

Among the antioxidant genes, the secretory function group consists of INS (Insulin), GCK (Glucokinase), PCK1 (PC1/3, Proprotein convertase subtilisin/kexin type 1), PCSK2 (PC2, Proprotein convertase subtilisin/kexin type 2) and GJA1 (Connexin 43A, Gap junction protein 1) [238-241].

The antioxidant defence genes consist of SOD1 (Superoxide dismutase 1), SOD 2 (Superoxide dismutase 2), CAT (Catalase), GPX-1 (Glutathione peroxidase 1) [238-241].

Apoptosis antioxidant genes consist of BCL2 (B-cell CLL/lymphoma 2), BAX (BCL2- associated X protein) and NF-κB 1 (Nuclear factor of kappa light polypeptide gene enhancer in beta cells 1) [238-241].

Endoplasmic reticulum stress response antioxidant genes include HSPA4 (Heat shock protein 70 kDa protein 4), HSPA5 (Heat shock protein 7-kDa protein 5), EIF2A (Eukaryotic translation initiation factor 2A) and EIF2AK3 (Eukaryotic translation initiation factor 2 alpha kinase 3) [238-241].

For this study, I have selected to examine the expression of SOD1, SOD2, CAT, GPX-1, BCL2, BAX, NF-κB 1, HSPA4, HSPA5, EIF2A, and EIF2AK3 [238-241].

Figure 27 show that in CD14+ monocytes, SOD 2, CAT, NF-κB 1, HSPA5 were significantly overexpressed in the survivor group whereas BCL2 and EIF2A were significantly under expressed in the survivor group. Both AH survivor and non-survivor groups have not expressed other genes differently. AH patient characteristics (survivors =10; non survivors =5) of this part of experiment is in table 13 that comes in next chapter.

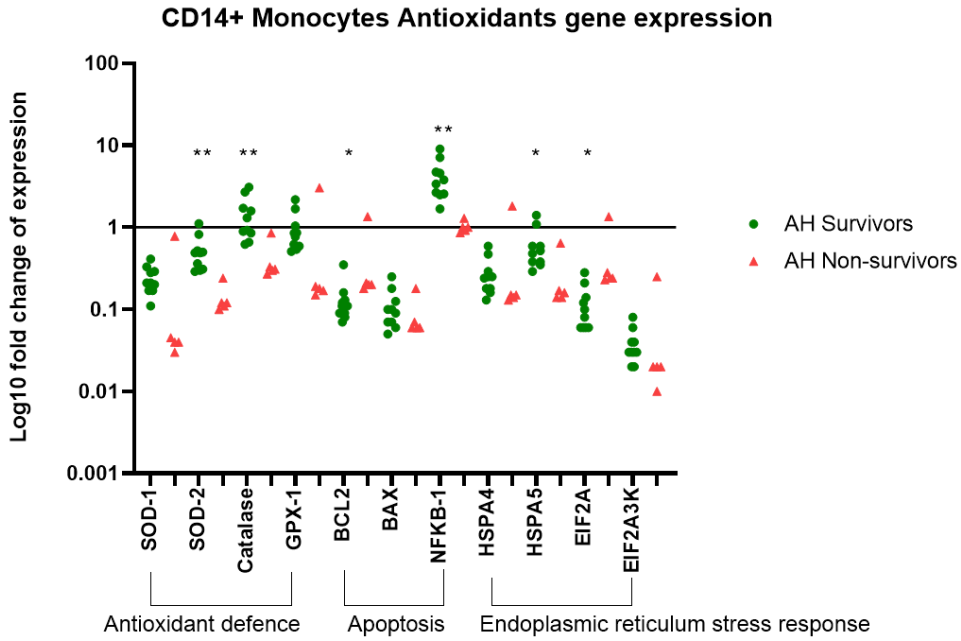


Figure 27 shows Log10 fold change of expression for antioxidant genes in CD14+ Monocytes. SOD1- Superoxide dismutase 1; SOD 2- Superoxide dismutase 2; CAT-Catalase); GPX-1-Glutathione peroxidase 1; BCL2- B-cell CLL/lymphoma 2, BAX- BCL2- associated X protein, NF-κB 1- Nuclear factor of kappa light polypeptide gene enhancer in beta cells 1; HSPA4- Heat shock protein 70 kDa protein 4; HSPA5- Heat shock protein 7-kDa protein 5; EIF2A- Eukaryotic translation initiation factor 2A; and EIF2AK3- Eukaryotic translation initiation factor 2 alpha kinase 3; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: *-p≤0.05, ** - p≤0.01.

On the other hand, in figure 28 in CD4+ T cells, SOD 1, BCL2, NF-κB 1, HSPA4, HSPA5, EIF2A and EIF2A3K were significantly overexpressed in the non-survivor group when compared to the survivor group. Expression of the rest of genes were not significantly different in AH survivors and non-survivors.

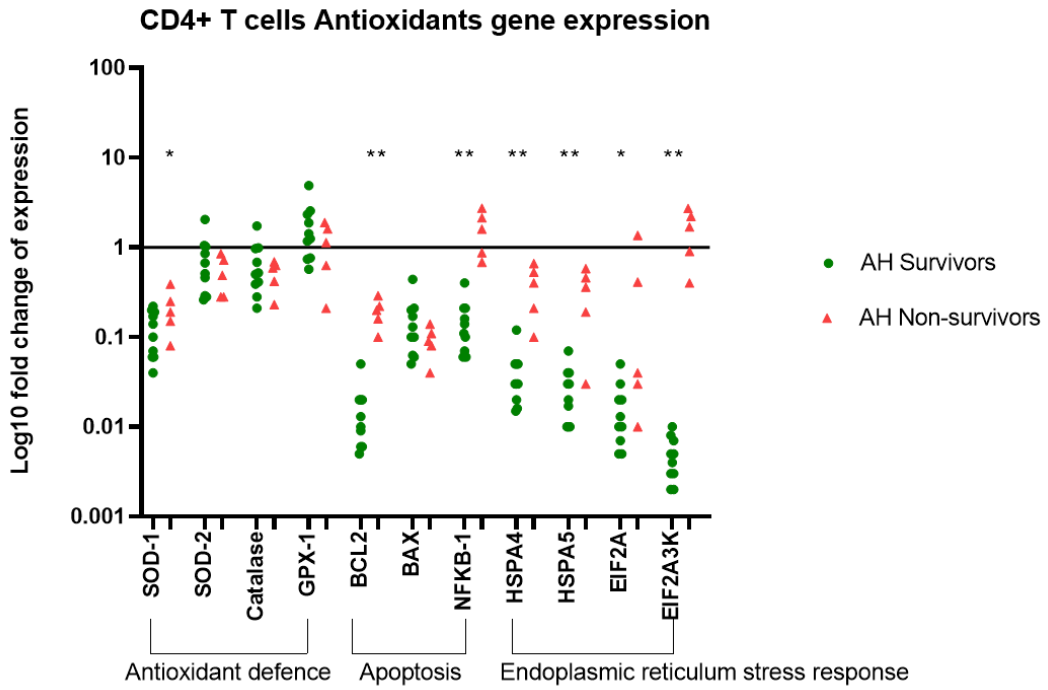


Figure 28 shows Log10 fold change of expression for antioxidant genes in CD4+ T cells. SOD1- Superoxide dismutase 1; SOD 2- Superoxide dismutase 2; CAT-Catalase); GPX-1-Glutathione peroxidase 1; BCL2- B-cell CLL/lymphoma 2, BAX- BCL2- associated X protein, NF-κB 1- Nuclear factor of kappa light polypeptide gene enhancer in beta cells 1; HSPA4- Heat shock protein 70 kDa protein 4; HSPA5- Heat shock protein 7-kDa protein 5; EIF2A- Eukaryotic translation initiation factor 2A; and EIF2AK3- Eukaryotic translation initiation factor 2 alpha kinase 3; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: *-p≤0.05, ** - p≤0.01.

Overall, there are considerable heterogeneity in the assessment of antioxidant genes. These results are difficult to interpret as the results show contradictory results within the cell subtypes. However, the AH non-survivors had shown trend of diminished expression of the antioxidant genes but upregulated expression of apoptosis and ER reticulum stress response genes. This pattern is more apparent in the CD14+ monocytes.

Discussion

MDA concentration as quantified by the TBARS ELISA assay was notably higher in the AH patients in contrary to HVs with mean value of 43.74 vs 17 and $p<0.01$. In addition, percentage maximal oxidative stress as ascertained by DCFH-DA staining was significantly higher in AH patients compared to HV in CD14+ monocytes (63.2% vs 41.9%, p=0.0005), but not in CD3+ T cells (57.5% vs 52.7%; p=0.24). These findings indicate that there is a significant difference in level of oxidative stress in individuals who drink excessive amount of alcohol to the extent of having AH when compared to individuals with no diagnosis of AH.

Within the group of AH patients, non-survivors expressed higher levels of oxidative stress in both monocytes (70.9% vs 60.5%; p=0.02) and T cells (67% vs 54.8%; p=0.05). These findings demonstrate that patients who did not survive had close to maximal level of oxidative stress and this was true in both CD3 T cells and CD14 monocytes.

These findings corroborated with the theory that if excessive alcohol gets metabolized via the CYP2E1 oxidative pathway, excessive ROS and consequential end products of lipid peroxidation, DNA and protein damage are generated [10, 11, 19]. This then results in enhanced oxidative stress and subsequent cellular death [10, 11, 19].

My findings also further highlight that both monocytes and T cells which belong to the innate immune system are activated and involved in the inflammatory process. Oxidative stress as measured by MDA level was significantly apparent in monocytes, but not in T cells. On the other hand, the difference in oxidative stress as measured by DCFH-DA staining was significant in both monocytes and T cells. These differences can be explained by individualistic immune paralysis and inability of T cells/ monocytes to mount a response with the development of tissue damage within the liver [29, 51]. The deleterious effects of alcohol on these immune cells can bring about downstream epigenetic changes which I will discuss in the next chapter.

Overall, DCFH-DA staining via flow cytometry seems to be an easy and reproducible method of measuring oxidative stress. From my data, although percentage maximal oxidative stress as quantified by DCFH-DA staining (AUC of 0.66- 0.67) were not as good as MELD/ GAHS/ DF scores in predicting survival, they could act as supplementary parameters in triaging AH patients with more severe phenotype.

My data has further shown that percentage maximal oxidative stress has a poor predictive ability for occurrence of infection. When assessing the diagnostic

performance of percentage of maximal oxidative stress for any infection occurrence (both at baseline and at later stage of hospitalisation), the AUC is 0.5.

Similar insignificant pattern is observed when trying to establish for correlation between percentage of maximal oxidative stress with absolute values of white cell count, WCC subpopulations and NLR. This finding could be explained by the common phenomenon of dysfunctional leukocyte and extravasation in severe sepsis [131]. During sepsis, activated PAMPs would trigger leukocyte extravasation out of the circulatory system towards the affected site of damage/ infection [131]. This is followed by production and release of ROS in order to clear the pathogen and for host survival [131]. This process acts as double-edged sword as it defends the host, but also precipitates cellular death [131]

During the later stages of severe sepsis when leukocytes extravasation intensifies, the neutrophils in particular would become dysfunctional and start attacking host tissue [131]. At this stage, more ROS is released and contributes to more pronounced inflammation and tissue damage [131]. In summary, leukocyte/ white cell count becomes non-representative in severe systemic inflammation as they are heavily impacted from oxidative stress.

On the other hand, endogenous antioxidants are defense system for oxidative stress. Antioxidant status were found to be diminished in patients with severe sepsis due to prolonged oxidative stress [238-241]. Antioxidants like SOD and GPX are crucial in ROS elimination [238-241]. For example, SOD catalyzes the dismutation of superoxide and forms H_2O_2; and then GPX acts as electron donor to convert H_2O_2 into water for elimination [238-241]. Therefore, if the antioxidants are diminished or non-functional, the ability to counteract ROS is essentially removed.

My results from the comparison of patients who survived and didn't survive their course of AH, showed the depletion of antioxidant gene expression in the non-survivors group. The significantly affected antioxidant genes in the non-survivors monocytes were SOD 2, CAT, NF-κB 1 and HSPA5. This is however, inconsistent with T cells where SOD 1, BCL2, NF-κB 1, HSPA4, HSPA5, EIF2A and EIF2A3K were significantly upregulated in the non-survivor group when compared to the survivor group. In both monocytes and T cells of the survivors and non-survivors, there were no significant difference in the expression of GPX genes.

Due to genetic variability, it is unlikely that all patients with severe AH will bear the same affected antioxidant genes. My piece of data is a novel step into better understand the underlying factors in counteracting alcohol induced oxidative stress.

Limitations to my study would be the small-scale cohort I was experimenting with and the observational nature of my study. Also, the healthy volunteer cohort is unmatched with the patient cohort in terms of age and gender. My data is insufficient in defining the cause-and-effect relationship of oxidative stress with individual biochemical markers.

There is currently absence of data with oxidative stress and antioxidants in the field of AH. Further studies with larger patient population and more comparator groups are needed. Further flow cytometry analysis into the intrinsic property of both monocytes and T cells is pertinent to clarify the potential biologic mechanism between oxidative stress and biochemical inflammatory markers.

Chapter 4: Differential histone gene expression and activity in survivors and non-survivors from severe alcohol related hepatitis

Introduction

In respiratory diseases, chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease that causes airway obstruction in both restrictive and obstructive pattern [82, 85, 187]. It is distinguished by gradual accumulation of inflammation in the airways and lung tissues [82, 85, 187]. This process is mediated by the increased expression of inflammatory genes [82, 85, 187]. In the context of normal physiology, the increased expression of these inflammatory genes is suppressed by histone deacetylase 2 (HDAC2) to achieve equilibrium [82, 85, 187]. In COPD, the activity and expression levels of HDAC 2 were dampened down by oxidative stress, which consequentially amplified inflammatory response [82, 85, 187].

GCs are anti-inflammatory medications utilized for the therapy of COPD [85, 180, 187-189, 194]. They act by engaging GR which are recruited by HDAC 2 to function [85, 180, 187-189, 194]. The reduction in HDAC2 in the context of increased inflammation negatively impact on the functionality of GR, causing GC resistance in COPD [85, 180, 187-189, 194]. It has been postulated that the reduced HDAC 2 expression is secondary to increased oxidative stress in COPD [85, 180, 187-189, 194].

From the hepatology perspective, AH is similarly a disease driven by inflammation and GC resistance is commonly seen. In my previous chapter, my data highlights that oxidative stress is notably more elevated in patients with AH in contrast to HVs; and within patients with AH, more elevated in the non survivors when compared to survivors.

Reduced HDAC 2 expression is seen commonly in respiratory patients who are resistant to glucocorticoids [85, 180, 187-189, 194]. This phenomenon is evidenced both peripherally in the PBMCs and in the alveoli of these patients [85, 180, 187-189, 194]. The proposed mechanism is oxidative stress activates PI3Kδ activity, and that gives rise to phosphorylation and inactivation of HDAC2 [84, 218-220]. These inactivated HDAC2 are then ubiquitinated and degraded in the system [85, 218-220]. In addition, nitrative stress generates peroxynitrite, which then results in formation of nitrates (NO) and tyrosine residues (Tyr) to inhibit HDAC2 activities [85, 218-220]. Figure 29 [189] illustrates the mechanism of reduction of HDAC2 expression in patients with severe asthma who smokes and patients with COPD [189].

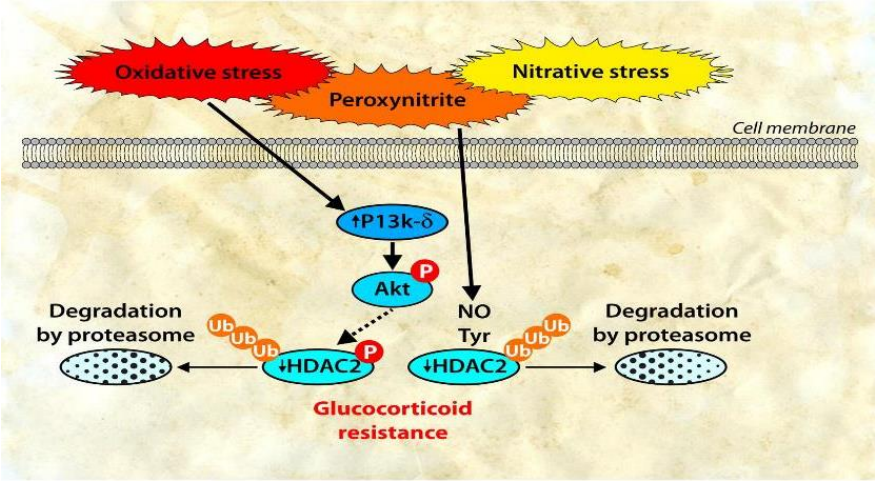


Figure 29 [189]: Proposed mechanism behind glucocorticoid resistance in patients with smokers with severe asthma and COPD patients. PI3Kδ - phosphoinositide 3-kinase delta; Akt- Protein kinase B; HDAC2- Histone deacetylases 2; Ub- Ubiquitination; P- Phosphorylation.

Therefore, it is pertinent to investigate if GC resistant AH patients too, follow this pathway of mechanism of reduced HDAC2 and increased PI3Kδ activity.

Peripheral blood obtained from AH patients at baseline, was magnetically sorted into subsets of CD4+ T cells and CD14+ monocytes as protocol described in the methodology chapter. Their respective cDNA was synthesized for the qPCR experiments. HV cDNA were collected from 5 healthy individuals with no known health comorbidities.

Expression of all eighteen HDAC genes (HDAC1-11, SIRT 1-7) and five HAT genes (GCN5, PCAF, p300, CBP, SRC-1) were assessed in each patient's T cells and monocytes. Control expression levels were from housekeeping gene GAPDH and expression levels of AH patients were compared to expression levels of pooled cDNA from 5 HVs.

The aim of this part of research is to establish differences in HDAC and HAT genes expression as a downstream effect from increased oxidative stress. These results are not aimed to establish an association or comparison with the pre-existing standard prognostic scores.

Results

Fifteen AH patients were selected randomly from the total cohort of AH. Within the recruited patients, mean age was 51.6; mostly male 66.7%; mean DF was 54.3, mean MELD 21.8 (table 12). Samples from AH patients were obtained at baseline before treatment with GC (if any). Two out of fifteen patients (13.3%)

died within 28 days of hospital admission with AH; whilst five out of the fifteen patients (33.3%) died within 90 days (table 12).

Within the survivors' group, 80% were male; mean age was 49; mean DF score 56.5 and mean MELD score 21. In the non-survivors' group, 40% were male; mean age 56.8; mean DF score 50.5 and mean MELD 23.4 (table 12).

In summary, the non survivors cohort was slightly older than the survivors' cohort ($p<0.04$). There was no noteworthy difference in the disease severity of the survivors and non-survivors as measured by their DF and MELD scores. Patient characteristics are presented in table 13 below. Results are presented in mean values with standard deviation.

	All (n=15)	**Survivors at Day 90 (n=10)**	**Non-survivors at Day 90 (n=5)**
Age	51.6 (8.5)	49 (7.2)	56.8(9.2)
Gender (%male)	10 (66.7%)	8 (80%)	2 (40%)
DF	54.5 (24.9)	56.5 (25.3)	50.5 (26.4)
MELD	21.8 (4.5)	21 (4.9)	23.4 (3.8)
Mortality rate			
D28 mortality		2 (13.3%)	
D90 mortality		5 (33.3%)	

Table 13: Patient characteristics with age, gender, DF, MELD, D28 and D90 mortality. DF- Maddrey's Discriminant Function score; MELD- Model for End Stage Liver Disease score; D28- Day 28; D90- Day90.

In addition, cases of infection at baseline or at later stage during hospital stay were recorded prospectively. Six (26%) patients had infection at presentation to hospital. Among the sources of infection, 4.3% were chest; 4.3% ascites; 4.3% bacteraemia and 13% source unknown. A further eight patients developed infection during their stay in hospital (13% urine; 4.3% ascites, 4.3% bacteraemia and 13% source unknown) (Table 14).

Infection source	Baseline	Later
Chest	1 (4.3%)	0
Urine	0	3 (13%)
Ascites	1 (4.3%)	1 (4.3%)
Bacteraemia	1 (4.3%)	1 (4.3%)
C.Diff	0	0
Unknown	3 (13%)	3 (13%)

Table 14: Number (percentage) and source of infections at baseline and during hospital admission for AH.

None of the fifteen patients recruited for this set of experiment was given glucocorticoids. This is either due to ineligibility based on their DF score or the occurrence of infection at baseline.

Figure 28 below show that in CD14+ monocytes, HDAC 2, 3, 8, SIRT 1, 2, 5, 6, and 7 were significantly upregulated in the survivor group (Figure 30). The rest of HDAC genes are not differentially expressed in both cohort of AH survivors and non-survivors.

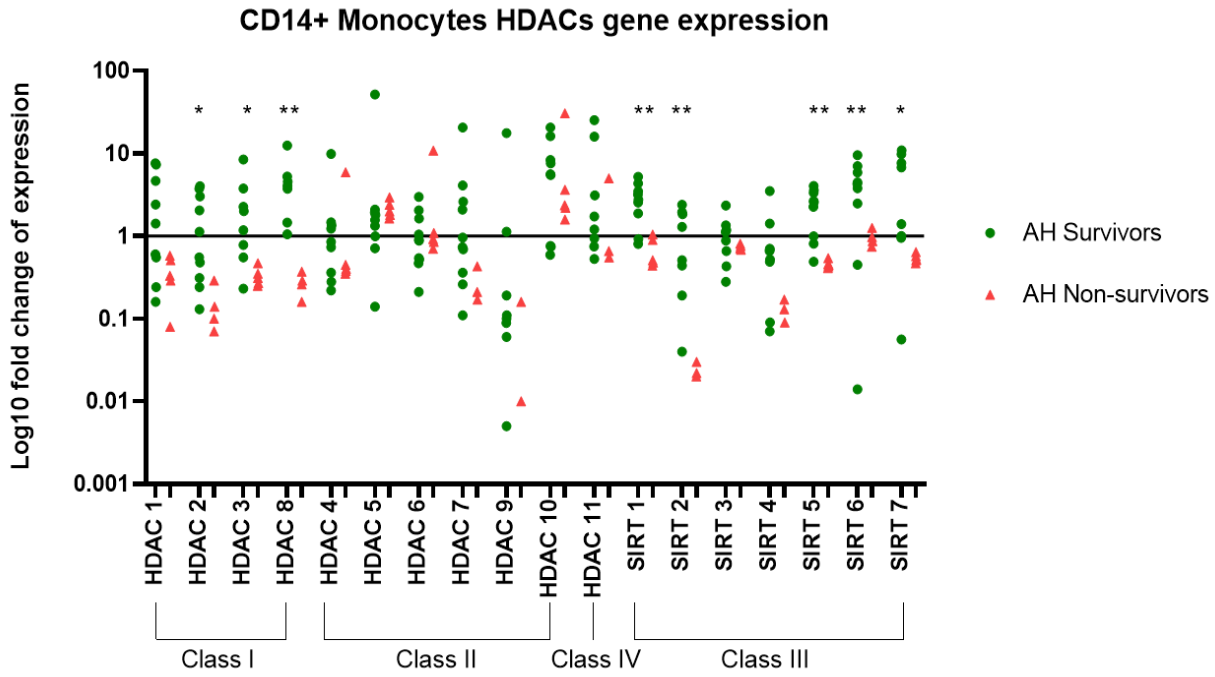


Figure 30 shows Log10 fold change of expression for HDAC genes in CD14+ monocytes. HDAC- Histone deacetylases; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors; Statistical significance: *-p≤0.05, ** - p≤0.01.

From figure 31 below, it is evident that in CD4+ T cells, the expression levels of HDAC 2, 3 and 8 were significantly higher in the survivor group, while only HDAC 11 was significantly higher in the non-survivor group. Expression of rest of the genes were not significantly different in both AH survivors and non-survivors.

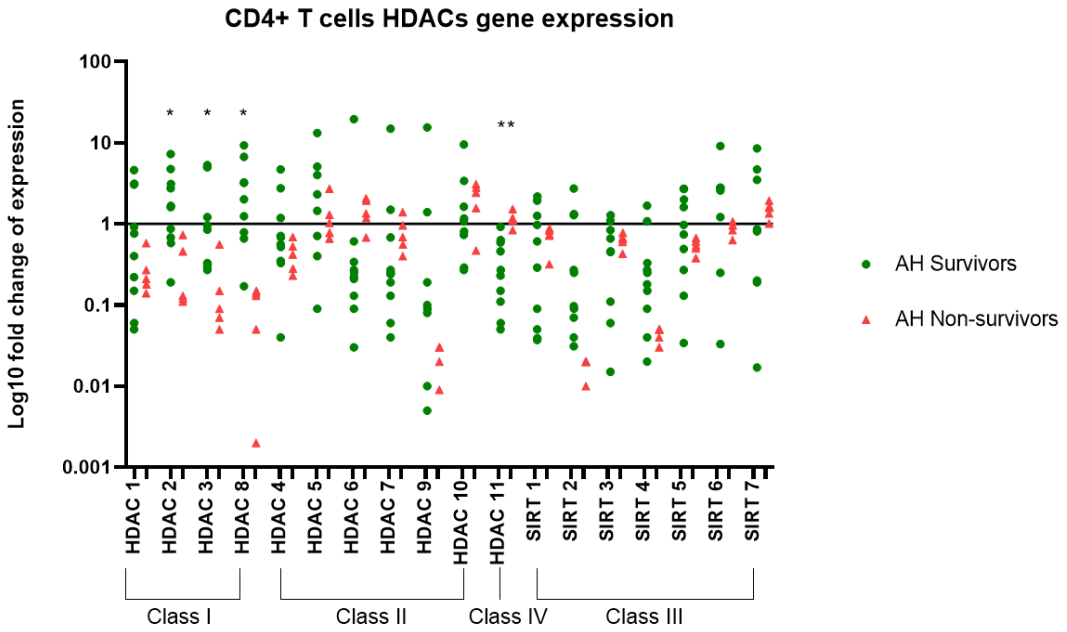


Figure 31 shows Log10 fold change of expression for HDAC genes in CD4+ T cells. HDAC- Histone deacetylases; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors; Statistical significance: *- p≤0.05, ** - p≤0.01.

Of note, the commonly affected HDAC genes for both monocytes and T cells were HDAC 2, HDAC 3 and HDAC 8. These three affected HDAC genes which belong to class I HDACs were significantly diminished in the AH non-survivor group.

On the other hand, when investigating the HAT genes which typically act in opposition to HDAC genes, my data showed that GCN5, and SRC-1 genes were significantly over-expressed in CD14+ monocytes in AH survivors in contrast to non-survivors (Figure 32). The rest of the HAT genes shown were not significantly different in both AH survivors and non-survivors (Figure 33).

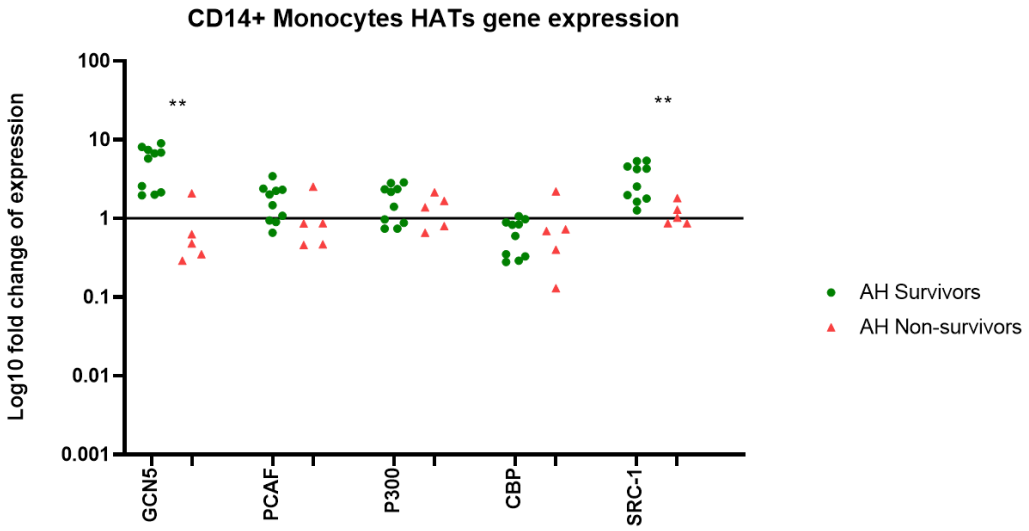


Figure 32 shows Log10 fold change of expression for HAT genes in CD14+ Monocytes. HATs- Histone acetyltransferases; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors; Statistical significance: ** - p≤0.01.

On the other hand, in CD4+ T cells, AH non survivors had significantly over-expressed HAT genes including that of GCN5, p300, CBP and SRC-1 (Figure 33).

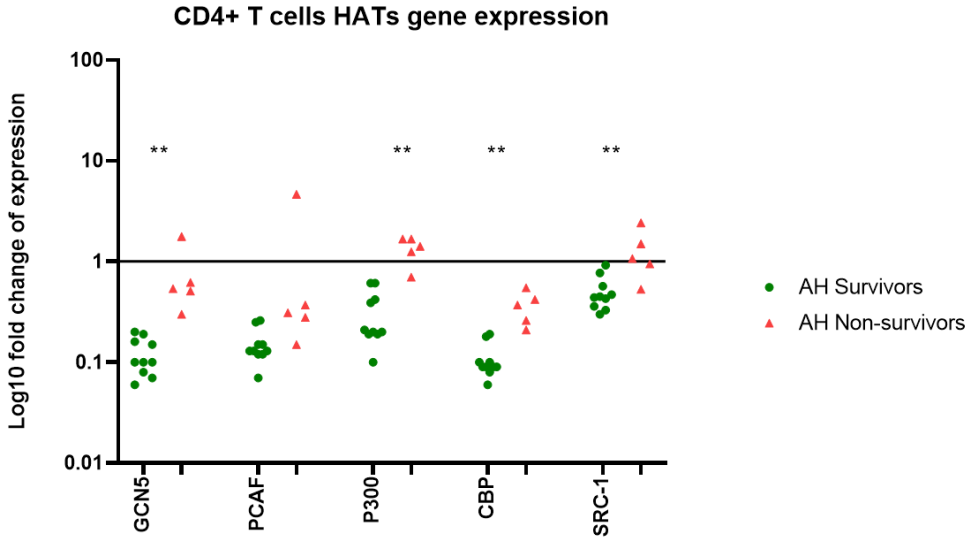


Figure 33 shows Log10 fold change of expression for HAT genes in CD4+ T cells. HATs- Histone acetyltransferases; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors; Statistical significance: ** - p≤0.01.

Here, the findings between monocytes and T cells are conflicting. Gene expressions of GCN5 and SRC-1 were downregulated in non survivors

compared to survivors in monocytes whereas T cells showed contradictory pattern in these two genes.

To further verify the presence of differences in HAT and HDAC activity, I assessed the overall functional activity of HDAC, HAT and downstream inflammatory pathway mediated by PI3Kδ.

A total of twenty-one patients with AH were recruited for these experiments (61.9% were male, mean age 52.2, mean DF 77.6, mean MELD 24.3) (table 14). Fifteen patients of this group were the same patients recruited for the PCR experiments to allow further data analysis at later stage. Eight out of twenty-one patients (38%) died within 90 days. Within the non-survivor group, 37.5% were male; mean age 55; mean DF score 112 and mean MELD score 29.3 (table 15). The non-survivor group had more severe disease phenotype than the survivor group as evidenced by their significantly higher DF and MELD scores. Ten healthy volunteers were recruited as controls (40% male; mean age 34.9) (table 16). None of the healthy volunteers have health comorbidities.

Results are presented in mean values with (standard deviation).

	All (n=21)	**Survivors at Day 90 (n=13)**	**Non-survivors at Day 90 (n=8)**
Age	52.2 (8.6)	50.5 (7.2)	55 (10.5)
Gender (%male)	13 (61.9%)	10 (76.9%)	3 (37.5%)
DF	77.6 (71.9)	56.4 (23.8)	112.2 (107.8)
MELD	24.3 (7.4)	21.2 (4.4)	29.4 (8.7)
Mortality rate			
D28 mortality		-	-
D90 mortality		8 (38%)	8 (38%)

Table 15: Patient characteristics with age, gender, DF, MELD, D28 and D90 mortality. DF- Maddrey's Discriminant Function score; MELD- Model for End Stage Liver Disease score; D28- Day 28; D90- Day90.

	HV (n=10)
Age	34.9 (13.8)
Gender (%male)	4 (40%)

Table 16: Characteristics of healthy volunteers with age and gender. HV- Healthy Volunteers.

Class 1 HDAC functional activity was quantified for HVs, AH survivors and non-survivors using a commercially available ELISA kit (Amsbio, UK). For this

assay, CD14+ and CD4+ nucleus were utilized for the HDAC activity whereas the cytoplasm were used as negative controls.

In the CD14+ monocytes, class 1 HDAC activity was significantly higher in HVs (n=10) than AH patients (n=21; 81.1% vs 43.6%; p<0.01) and in AH survivors than non-survivors (55.4% vs 24.6%; p<0.01) (Figure 34).

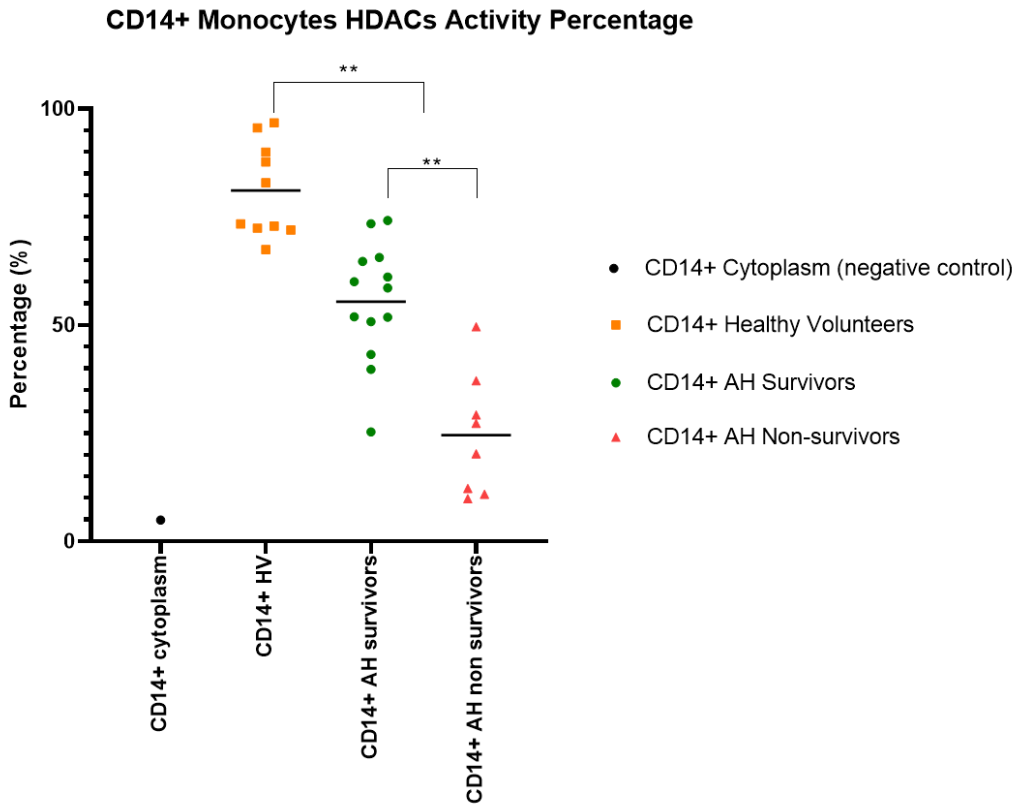


Figure 34 shows class I HDAC activity within the CD14+ monocytes. HDAC- histone deacetylases; HV- Healthy Volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: *-p≤0.05, ** - p≤0.01.

Similarly, in CD4+ T cells, class 1 HDAC activity was significantly higher in HVs when compared to AH patients (80.6% vs 44.5%; p<0.01) (Figure 35). Class 1 HDAC activity was again significantly higher in AH survivors compared to AH non-survivors (58.2% vs 22.1%; p<0.01) (Figure 35).

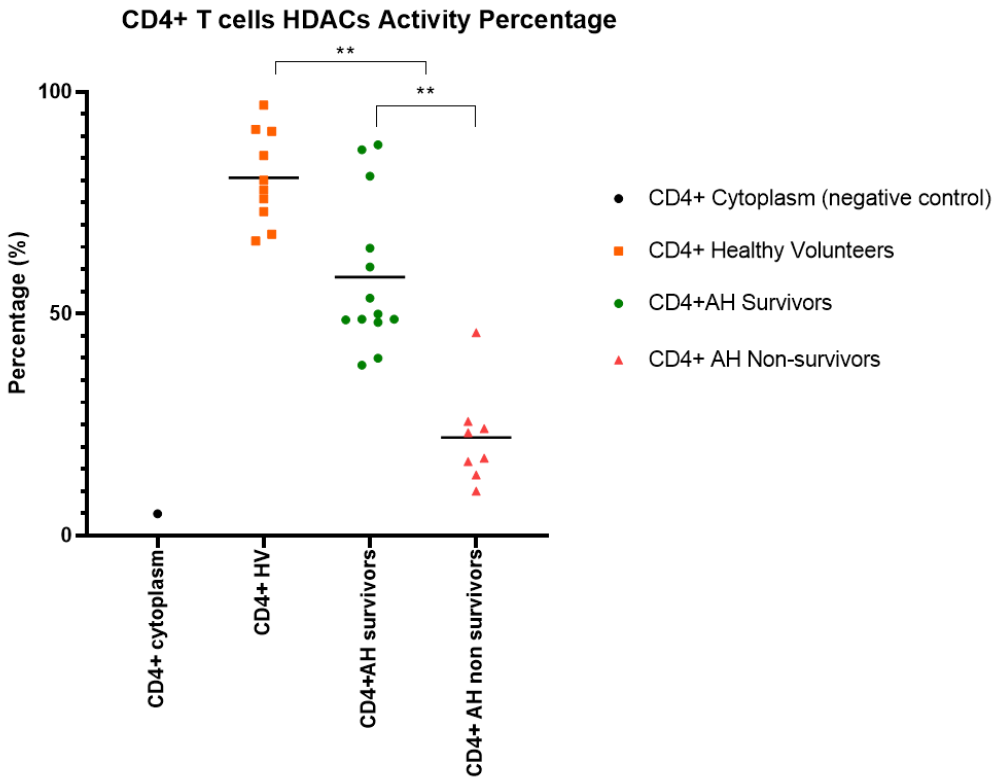


Figure 35 shows class I HDAC activity within the CD4+ T cells. HDAC- histone deacetylases; HV- Healthy Volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: *-p≤0.05,
** - p≤0.01.

On the other hand, functional activity of histone acetyltransferases (HATs) was quantified with a commercially obtainable ELISA kit (Abcam, UK). Experiment data were expressed in relative optical density per microgram (ug). When investigating HATs activity within the CD14+ monocytes, HVs (n=10) has significantly higher HATs activity when compared to AH patients (n=21; 1.47 vs 0.53; p<0.01) (Figure 36). HATs activity were not significantly dissimilar between AH survivors (n=13) to non-survivors (n=8; 0.56 vs 0.47; p=0.19) (Figure 36).

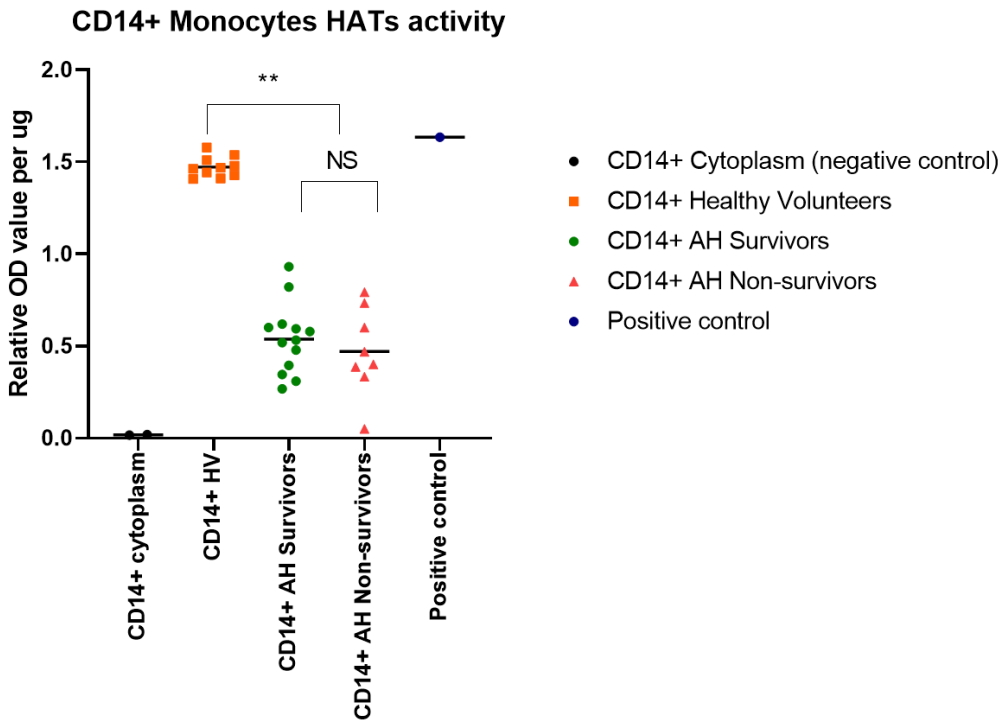


Figure 36 shows HATs activity within the CD14+ monocytes. HATs- histone acetyltransferases; HV- Healthy Volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: NS- Not significant; *-p≤0.05; ** - p≤0.01.

Whereas in CD4+ T cells, HVs has significantly higher HAT activity than AH patients (1.31 vs 0.37; p<0.01) (Figure 37). Similar results observed for the CD4+ T cells subset when comparing AH survivors to non-survivors which shows no significant differences (0.43 vs 0.28; p=0.06) (Figure 37).

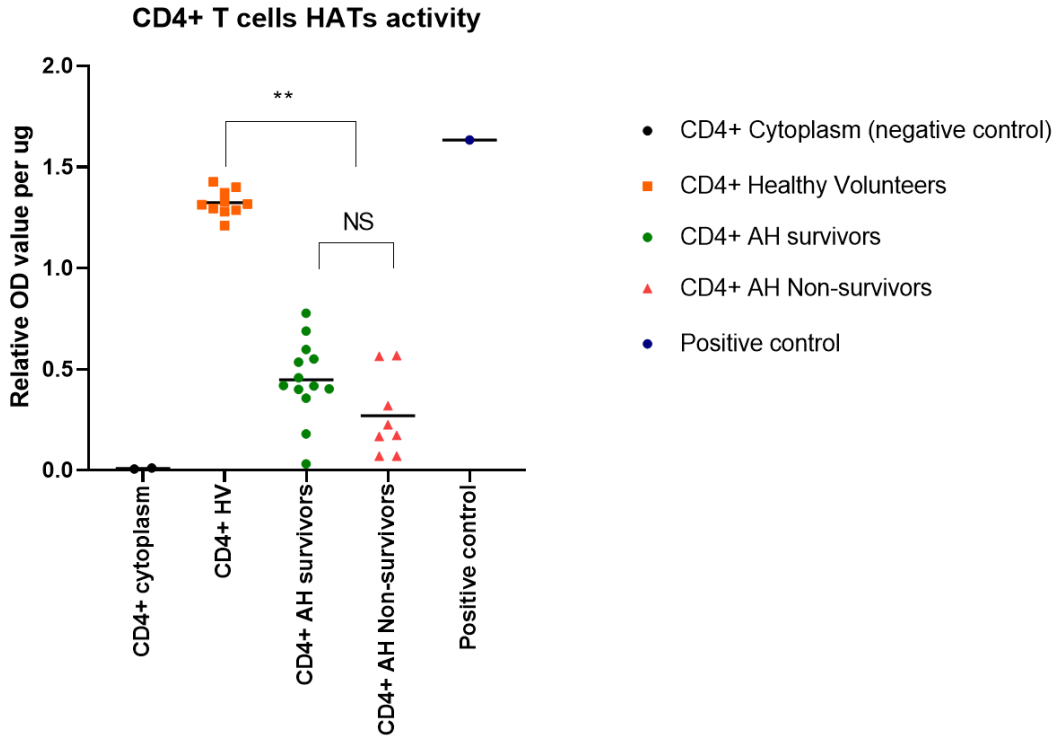


Figure 37 shows HATs activity within the CD4+ T cells. HATs- histone acetyltransferases; HV- Healthy Volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: NS- Not significant; *-p≤0.05; ** - p≤0.01.

Lastly, PI3Kδ activity was assessed with commercially available kit (2bScientific, UK). Results are expressed in PI3Kδ concentration (ng/ml).

In the CD14+ Monocytes, PI3Kδ activity was remarkably more elevated in HVs (n=10) than AH patients (n=21; 18.8 vs 12.9; p<0.01) and in AH survivors than non-survivors (16.3 vs 7.3; p<0.01) (Figure 38).

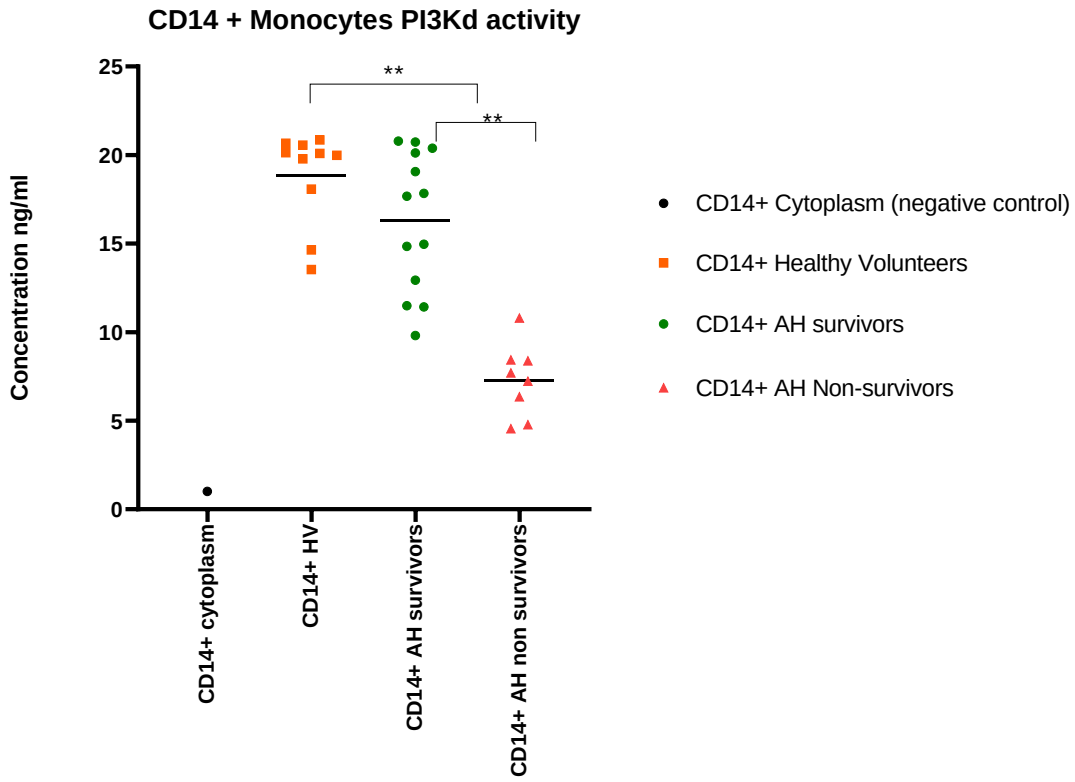


Figure 38 shows PI3Kδ activity within the CD14+ monocytes. PI3Kδ - phosphoinositide 3-kinase delta; HV- Healthy Volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: ** - p≤0.01.

In CD4+ T cells, PI3Kδ activity was significantly higher in HVs when compared to AH patients (18.1 vs 12.9; p<0.01) (Figure 39). PI3Kδ was again notably more upregulated in AH survivors compared to AH non-survivors (16.7 vs 6.9; p<0.01) (Figure 39).

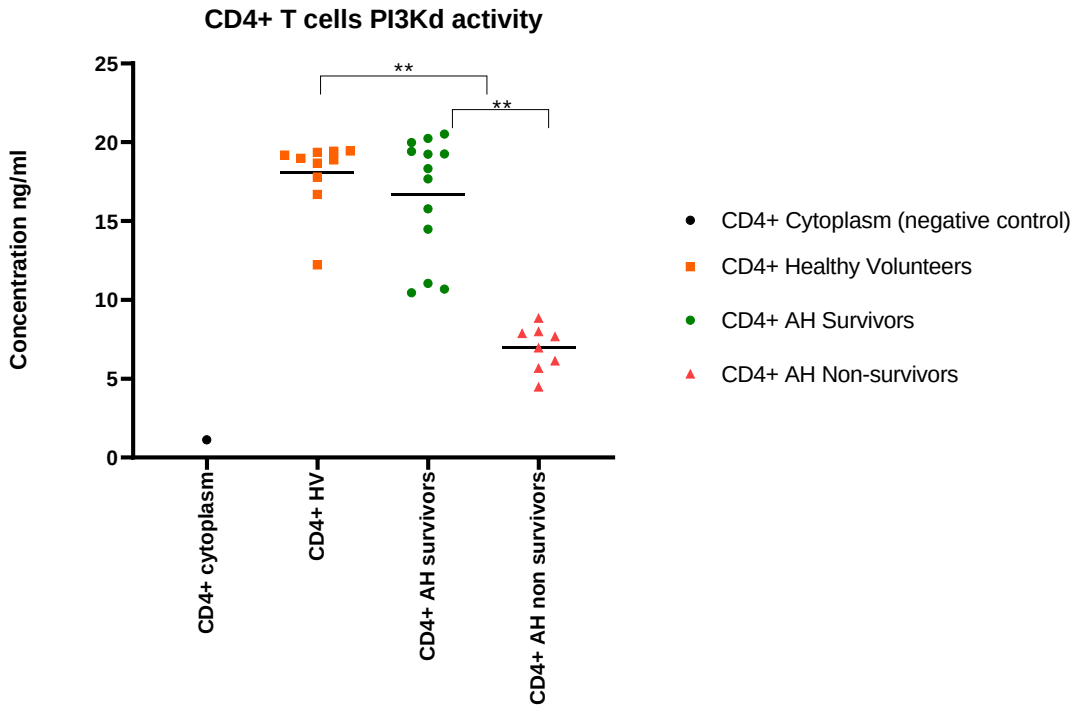


Figure 39 shows PI3Kδ activity within the CD4+ T cells. PI3Kδ -phosphoinositide 3-kinase delta; HV- Healthy Volunteers; AH survivors- Alcohol related hepatitis survivors; AH non-survivors- Alcohol related hepatitis non-survivors. Statistical significance: ** - p≤0.01.

Discussion

An equilibrium between histone acetylation and deacetylation in cells is vital to maintain a state of balance, and any perturbation can overdrive the pro-inflammatory transcription factors [66-69]. In many intracellular processes, HAT and HDAC activities are regulated by the accessibility of co-factors, enzymatic moderation of different types of protein and subsequent protein-protein interplay [66-69].

From my experiments, three key histone genes were significantly under-expressed in the AH non-survivor group for both monocytes and T cells. They were HDAC 2, HDAC 3 and HDAC 8, all of which belonged to class I HDACs. There is very little knowledge about the roles of these HDAC genes in liver disease. However, wisdom can be gathered from animal models and experiments done on other inflammatory diseases.

In accordance to respiratory models, specific knockdown of HDAC 2 expression stimulates NF-κB mediated gene expression, which resulted in glucocorticoid resistance [187-190]. Therefore, reinstitution of HDAC 2 can restore glucocorticoid sensitivity in some COPD patients [85, 219, 220].

On the other hand, HDAC 3 has been found to be critical in various diseases due to its heavy involvement in chromatin remodelling and gene transcription [72, 79, 80]. In mice with fatty liver, HDAC 3 inhibition suppressed Keap 1 translation and as a result, upregulated antioxidants [72, 79, 80]. Unsurprisingly, this process alleviated oxidative stress in the mice [72, 79, 80].

However, subsequent animal research then demonstrated that HDAC 3 exacerbated fat accumulation within the liver via interaction with with prospero-related homeobox 1 (PROX1) [72, 79, 80]. These contradictory observations highlighted that HDAC 3 can act as a double-edged sword whereby both its up-regulation and down-regulation can cause liver injury.

On the other hand, there is little to know about HDAC 8. In AH mice models, over-expression of HDAC 8 aggravated alcohol induced inflammation and liver injury [72, 79, 80]. Therefore, MiR-451a induced inhibition of HDAC 8 can suppress secretion of inflammatory transcription factors e.g. TNF-α and IL-β, which in turn improved alcohol induced hepatotoxicity [72, 79, 80]. However, the aetiology behind this mechanism remains unclear.

Some considerations must be borne in mind when interpreting data on HDAC genes as it is often a group of genes which correspond to progression of a disease, rather than an individual culprit gene. Emphasis should be shifted to identify the underlying role of class 1 HDACs as a group when attempting to understand the regulation of inflammatory reactions in AH.

It is known that class I HDACs are involved in innate immunity by contributing to the inflammatory reactions, in contrast to class II which are mainly involved in adaptive immunity [72-74, 80]. Animal studies have suggested that alcohol induced inhibition of class I HDAC activities, by expanding accessibility of cellular acetate levels, and thereby stimulating HAT activity which oppose HDACs [79-81]. In mice study, binge alcohol induced changes in HDAC gene expression and downregulation of HDAC activity were responsible for hepatic fat accumulation and hepatotoxicity [79-81].

My data showed that class 1 HDAC activities were significantly downregulated in AH when comparing to HVs (43.6% vs 81.1% with $p<0.01$) in monocytes, and (44.5% vs 80.6% with $p<0.01$) in T cells. Within the AH samples, HDAC activities were significantly reduced in patients who did not survive their disease in contrast to those who overcame their disease (24.6% vs 55.4% with $p<0.01$) in monocytes, and similarly in T cells (22.1% vs 58.2% with $p<0.01$).

On the other hand, when investigating HAT activity within the CD14+ monocytes, HVs (n=10) had significantly higher HATs activity when compared to AH patients (n=21; 1.47 vs 0.53 with $p<0.01$). No noteworthy difference was observed in the HATs activity when comparing between AH survivors (n=13) to non-survivors (n=8; 0.56 vs 0.47 with $p=0.19$). Whereas in CD4+ T cells, HVs

had significantly higher HAT activity than AH patients (1.31 vs 0.37; p<0.01). Similar result observed in the CD4+ T cells subset when comparing AH survivors to non-survivors which showed no significant differences (0.43 vs 0.28; p=0.06).

In summary, whilst I expected the downregulation of HDAC activities will be associated with upregulation of HAT activities [72-74, 80], my data has clearly shown that both HDAC and HAT activities were significantly downregulated in both monocytes and T cells. Within the HAT activities, whilst there were notable differences when comparing AH patients to HVs, this was not the case when comparing AH survivors to non-survivors.

This may be explained by the existence of several key players in histone modifications where just measuring their activities is unable to improve our understanding of these intracellular processes.

These results are difficult to interpret, as there is a diversity of gene co-activators within the HAT and HDAC family, which can affect the overall histone activity. The functional assays measure global activity but cannot discriminate between individual HDACs/ HATs. The heterogeneity of different HDAC and HAT gene function makes it challenging to determine the dominant key gene. In this context, further investigation into the functional effects of HDAC/ HAT with ChIP-seq or ATAC-seq will be useful.

When I explore further into the downstream effects from HDAC modifications, I gathered that under-expressed HDACs can cause rogue activation of PI3K signaling pathway [242-245]. PI3Ks are a family of intracellular lipid kinases that are categorized into three classes [242-245]. Class I PI3Ks garnered the most attention as research on various inflammatory diseases have suggested that activation of class I PI3Ks, would stimulate the Akt pathway along with the NF-κB signalling [85, 180, 242-245], further precipitating systemic inflammation. Naturally, PI3K inhibition became a drug target when treating glucocorticoid resistant respiratory diseases [85, 180, 242-245]. Of note, class I PI3Ks consist of isoforms PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ [242-245].

From my experiments, PI3Kδ activities were significantly diminished in AH when compared with HVs for both monocytes (12.9 vs 18.8; p<0.01) and T cells (12.9 vs 18.1; p<0.01). More profound reduction in PI3Kδ activities were seen in monocytes of AH non survivors in contrast to survivors (7.3 vs 16.3; p<0.01) and T cells (6.9 vs 16.7; p<0.01). These findings contradict the anticipated theory where reduced HDAC expression would trigger upregulation of PI3K signalling [85, 184]. However, my findings could be explained with the global profound immune dysfunction in patients suffering from severe AH which suggests that this may not be amenable to drug targeting.

Much of my work has been performed with peripheral bloods of a small cohort of patients. Therefore, it highlights the need for further studies using *ex-vivo* liver tissue or liver infiltrating immune cells to better understand the differential histone modifications in AH. This was not a randomised controlled trial, so the groups are not comparable. Importantly, the data on PI3Kδ signalling activities is the realisation that single pathway targeting for systemic inflammatory diseases like AH will exhibit limited efficacy, due to concurrent activation or repression of other inflammatory related signalling pathways.

Chapter 5: *In vitro* cell therapeutic targets in severe alcohol related hepatitis

Introduction

This is the domain of my research that aims to explore the potential impact of several therapies on cell responsiveness to glucocorticoids (GC). My previous chapters have elucidated that oxidative stress is significantly elevated in PBMC's of patients with severe alcohol related hepatitis. Antioxidants, which serve to counterbalance the effects of oxidants, are thus being considered to have beneficial effects in the management of AH [238-241]. The use of antioxidants has generated conflicting outcomes in clinical trials [162, 163].

Malnutrition is common in patients who misuse alcohol and is one of the markers of poor prognosis in patients with AH. *In vitro*, dietary supplementation with trace elements like zinc, niacin, and short chain fatty acids, has been shown to reinstate the gut microbiome symbiosis and gut barrier integrity [38-47, 246-252]. Trace elements and micronutrients are also pivotal in antioxidant pathways [246-252]. In light of this, I have chosen to pulse PBMCs of severe AH patients with antioxidants Zinc, NAC, and sodium butyrate (a type of short chain fatty acid) respectively to determine whether these potential therapies would reduce the intracellular oxidative stress levels and therefore, increase the responsiveness to GC.

HDAC inhibitors currently are being exploited as anti-cancer and anti-inflammatory agents [253-255]. Effects of these drugs were applauded in cancer research due to their ability to suppress HDAC activity, which directly affects the ensuing expression of transcription factors and inflammatory genes [253-255]. In mouse models of inflammatory bowel disease, one of the HDAC inhibitors named suberoylanilide hydroxamix acid (SAHA), which is also called-Vorinostat was able to attenuate inflammatory changes by suppressing secretion and mobilization of inflammatory cytokines and chemokines [253-255]. In my experiment, I utilized SAHA (Vorinostat) as a positive control where further reduction in HDAC activity will make the cells more resistant to GC.

In my study, PBMC cells of AH patients were isolated and prepared fresh. PBMCs were incubated with individual treatment for 48 hours before undergoing BLISS assay to assess the cells' *in vitro* GC sensitivity. The required dose for each experimental treatment was selected based on previous pharmacokinetic studies and observed efficacy for these compounds in *in vitro* models.

GC sensitivity was determined using BLISS assay, which gives a final IMAX value (maximum percentage inhibition of proliferation) [197-199]. IMAX was calculated using formula (1– [proliferation in presence of GC] / [proliferation in absence of GC]) x100 [197-199]. The IMAX value suggests a percentage of suppression of cellular proliferation by GC, thus representing cellular sensitivity/ responsiveness to GC [197-199]. Therefore, a low IMAX value is suggestive of glucocorticoid resistance, and my experiments were intended to highlight any intervention which will enhance the IMAX value [197-199].

Results

Five AH patients were consecutively recruited and experiments performed on fresh ex-vivo samples. These patients have mean age of 50.6; all males; mean DF 54.6, mean MELD 23.6 (table 10). Samples from AH patients were obtained at baseline before treatment with GC (if any). Three out of five patients (60%) died within 90 days of hospital admission with AH.

Within the survivor's group, mean age was 58; mean DF score 28.3 and mean MELD score 16.5 (table 10). In the non-survivor's group, mean age 45.6; mean DF score 72.2 and mean MELD 28.3 (table 10).

In summary, the non-survivor group was slightly younger than the survivor group. The mean DF and MELD scores were significantly higher in the non survivors when compared to survivors indicating a more severe disease phenotype.

Patient characteristics are presented in table 17 below. Results are presented in mean values (standard deviation).

	All (n=5)	**Survivors at Day 90 (n=2)**	**Non-survivors at Day 90 (n=3)**
Age	50.6 (8.8)	58 (5.6)	45.6 (7.1)
Gender (%male)	5 (100%)		
DF	54.6 (33.6)	28.3 (9.4)	72.2 (32.6)
MELD	23.6 (9.6)	16.5 (0.7)	28.3 (10.1)
Mortality rate			
D90 mortality		3 (60%)	

Table 17: Patient characteristics with age, gender, DF, MELD, D28 and D90 mortality. DF- Maddrey's Discriminant Function score; MELD- Model for End Stage Liver Disease score; D28- Day 28

None of the five patients recruited for this set of experiment was given GC. Table 18 below shows the baseline IMAX values of each patient with their IMAX values post treatment.

Patient	Baseline IMAX	Post Zinc	Post NAC	Post NaBu	Post Vorinostat	90 days mortality
A	13.19	33.78	54.35	-3.82	3.65	D
B	37.26	9.67	39.49	-16.33	7.09	D
C	-2.64	11.79	-4.01	-3.01	3.62	D
D	8.06	11.82	22.25	-2.77	1.74	A
E	18.93	17.18	8.07	-0.99	7.91	A

Table 18: Patient's baseline IMAX values and values post treatment. IMAX- maximum percentage inhibition of proliferation; NAC- N-acetylcysteine; NaBu- Sodium butyrate; A- Alive; D- Dead.

Figure 40 presents the data above with patients' baseline IMAX values and IMAX values post treatment.

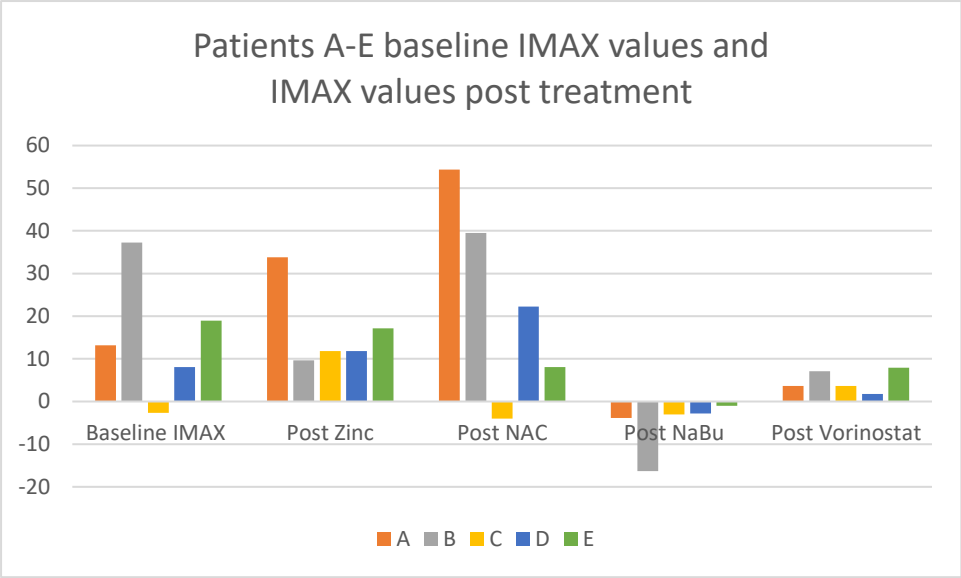


Figure 40 Patient's baseline IMAX values and values post treatment. IMAX- maximum percentage inhibition of proliferation; NAC- N-acetylcysteine; NaBu- Sodium butyrate; A- Patient A; B- Patient B; C- Patient C, D- Patient D, E- Patient E.

These results have shown great degree of heterogeneity. The cellular responses post sodium butyrate (NaBu) is particularly interesting as it appears to impose a negative effect on all patients' cellular responsiveness for GC. In certain tumour cell line studies, sodium butyrate has manifested pro-apoptotic activities through ROS formation [249, 250].

From my data, both antioxidants (zinc and NAC) have not demonstrated consistent and appreciable effect on garnering better sensitivity to GC. On the

contrary, Vorinostat therapy reduced the IMAX values of patients and in other words, made the cells less sensitive to GC.

Overall, there were no statistically significant changes in the IMAX values pre or post treatment for these five patients. Further testing with HDAC/ HAT gene expression would be worthwhile to confirm that oxidative stress directly alters epigenetic regulation.

Discussion

Zinc deficiency is apparent in numerous acute and chronic inflammatory diseases [246, 248, 251, 252]. It is one of the main trace elements associated with aberrant immune cell activation and dysfunctional immune cell regulation [246, 248, 251, 252]. In mouse models, zinc deficiency aggravates oxidative stress in the gut and precipitates the disruption of gut junction integrity [246, 248, 251, 252]. Our systematic review of zinc supplementation in ArLD has spotlighted the scarcity of prime quality studies examining its effect [166]. The cell culture experiments were intended to determine whether in vitro zinc supplementation to PBMC of patients with severe AH would improve their intracellular sensitivity to GC. However, given the limited data, I am unable to yield results with statistical significance.

NAC was considered a potential antioxidant therapy when used alone, or in conjunction with glucocorticoids [162, 163]. However, when it was trialled clinically, there was no mortality benefit at 6 months [162-164]. Since oxidative stress seems to be mediating the reduction of HDAC2 which results in GC resistance [187-190], antioxidants like NAC and Zinc could potentially be effective in reversing this mechanism, but my *in vitro* studies were unable to demonstrate any benefit.

My data further reaffirms that targeting oxidative stress alone in AH patients may not be the most effective approach. In an environment of sustained inflammation like that of AH, the cells have an exhausted phenotype and therefore likely to present an abnormal response to therapies [29]. Patients' dysfunctional immune cells may directly or indirectly affect their innate response to anti-inflammatory or antioxidant agents [29]. This indicates that rather than targeting individual level of oxidative stress, we need to develop ways to simultaneously target multiple oxidant mediated inflammatory pathways.

The pathological impact of alcohol on gut microbiome and intestinal barrier are evident in both animal and human studies [39-44]. The exact mechanism behind this is affected by multiple factors and involves oxidative stress [39-44]. In chronic alcohol users, gut dysbiosis and disrupted tight junctions are prevalent and as

a result, the immune system is over-stimulated with PAMPs and DAMPs [39-44]. This increases the amount of pro-inflammatory cytokines circulating in the system, which further breaks down the gut integrity [39-44]. The idea of supplementing PBMC cells with in vitro sodium butyrate stems from the concept that this short chain fatty acid can rebalance the intracellular inflammation and subsequently restore the cellular ability to respond to GC.

However, there is a mismatch between the concept and data yielded from my cell culture work. Sodium butyrate has shown deleterious effects on the PBMC cells of my recruited patients, although the heterogeneity makes the result difficult to interpret.

The role of class 1 HDAC inhibitors has been validated and approved for the treatment of many malignancies [253-255]. The modifiable epigenetic changes during cancer evolution makes therapies targeted at histones attractive [253-255]. However, when testing on PBMCs of AH patients, the established class 1 HDAC inhibitors (Vorinostat) does not seem to demonstrate beneficial effects on the cellular GC responsiveness. In fact, Vorinostat has further desensitised the cells and worsened glucocorticoid resistance.

The lack of HDAC agonists makes it challenging to test my hypothesis that by improving HDAC activity, the cellular glucocorticoid resistance can be improved/ reversed.

There is a clear paucity of cell culture work in the field of AH. A likely reason for this is patients with AH often present a heterogeneous phenotype, and this compound on the quality of experiments. Consequently, I have limited references to go by when it comes to pharmacokinetics of each therapy in AH. Larger *in vitro* studies are obligated to validate the potentials of each therapy.

Chapter 6: General Discussion

My research was developed around three principal concepts:

1. Oxidative stress is increased in patients with severe AH and therefore measurement of oxidative stress peripherally can predict clinical outcome of AH patients (Chapter 3)
2. Patients with severe AH have altered epigenetic regulation of GC signalling driven by oxidative stress (Chapter 4).
3. GC insensitivity/ resistance can be reversed with alternative treatments including that of antioxidants, short chain fatty acid and positive control with pan-HDAC inhibitor (chapter 5).

Each of these concepts was examined and the resulting data has the potential to be translated into clinical practice and guide future therapeutic targets.

6.1 Translating oxidative stress to clinical practice

Oxidative stress is a result of disparity between production and elimination of free radicals [11, 28]. Free radicals are inclusive of ROS and RNS [11, 28]. They are produced from the cellular redox process [11, 28]. In various studies, oxidative stress has been found to have strong correlation with inflammation [60, 61, 103, 108, 110, 184]. During inflammation, immune cells penetrate the tissues to generate products of ROS and RNS as a physiological response [108, 110]. Excess ROS stimulates transcription factors like NF-κB, which disrupts the cellular homeostasis by perpetuating inflammation and resulting in cellular injury and apoptosis [31, 103]. The extent of cellular injury from oxidative stress is usually limited by the antioxidants which act as defence system [19, 24]. It is worth highlighting that antioxidant capacity is commonly compromised in critically ill patients [19, 240, 241].

To justify measuring oxidative stress in patients with AH, I looked into the existing work on the connection between oxidative stress with other diseases which are characterized by progressive inflammation, including metabolic dysfunction associated steatotic liver disease (MASLD), inflammatory bowel diseases, uveitis and COPD [59-62, 256-258].

Oxidative stress represents an important factor in triggering persistent low grade inflammation and hepatic cells injury in the progression of MASLD [256-258]. In MASLD, oxidative stress occurs due to a combination of increased production of ROS due to lipid peroxidation by polyunsaturated fatty acid and aldehyde products and saturated antioxidant mechanism [256-258]. Diffusion of these ROS and RNS products into the cells perpetuate intracellular inflammation [256-258]. Unopposed ROS due to saturated antioxidant machinery

and impaired antioxidant capacity as a result of reduced level of antioxidants glutathione, MnSOD and catalase [256-258]. Overall, over production of ROS appears to then upregulate the pro-inflammatory cytokines and activates the hepatic stellate cells [256-258]. This event leads to chronic inflammatory responses and fibrosis generation in progressive MASLD [256-258]. Among treatments for MASLD, vitamin E and polyphenols which are antioxidants were studied intensively, but they yield inconsistent results [256-258].

Next, inflammatory bowel disease (IBD) is another inflammatory disease which is very well researched. There are two distinct types of IBD- Ulcerative Colitis (UC) and Crohn's disease (CD) [238-241]. Currently, the diagnosis of IBD is made based on clinical assessments, colonoscopy and biopsy histology [238-241]. CRP has been used as a surrogate marker of overall inflammation but lacks sensitivity to the colitis activity [259-261]. Faecal calprotectin is a sensitive tool in differentiating inflammation within the gastrointestinal tract, but this test is lacking a unified cut off point and its low specificity level remains its major limitation [259-261].

For both UC and CD, oxidative stress related protein and DNA modifications have been identified as key player in the pathogenesis and neoplastic transformation in IBD [138, 238-241]. Therefore, many studies were performed to investigate the diagnostic and prognostic utility of surrogate markers of oxidative stress in IBD patients [138, 238-241].

On the other hand, oxidative stress is involved in many diseases of the anterior segment of the eyes, in particular uveitis which is characterised by acute and active periods of inflammation [59, 228, 262-265]. The exact pathogenesis of uveitis is perplexing, but the current comprehension is overwhelming ROS leads to oxidative stress which then drives and perpetuates inflammation within the ocular tissue [59, 228, 262-266]. This then result in permanent tissue damage and eventual ocular complication of sight loss [59, 228, 262-266].

Further in COPD, there is evidence to suggest that ROS and RNS generated from cigarette smoking contribute to the disease pathological process [82,187-189]. Overwhelming levels of ROS and RNS lead to chronic oxidative stress [187-189]. This drives oxidative damage to DNA, proteins and lipid, which then perpetuates the systemic inflammation [187-189]. In respiratory models, oxidative stress induced histone modifications also lead to an array of downstream processes which contribute to GC resistance [187-189]

Based on observations of the above inflammatory diseases, I can conclude that oxidative stress is strongly correlated with inflammation. Therefore, this justifies the need for rigorous assessment and quantification of oxidative stress in patients with AH. I intend to translate my findings to clinical practice by

establishing an easy and reproducible way of measuring oxidative stress directly or by measuring the marker of oxidative stress.

6.2: Justification of ways to measure oxidative stress

To execute all the parameters of oxidative stress would be arduous and uneconomical. Various methods to measure oxidative stress have been trialled on, but generally, tests measuring single free radicals or pro-oxidant capacity did not yield consistent results [126, 127].

Among the markers of lipid peroxidation, malondialdehyde (MDA) was found to be useful in predicting severity in Crohn's disease with respectable accuracy rate and specificity rate [238-241]. In addition, previous animal studies have shown the correlation between severe intraocular inflammation and presence of MDA [228]. Strategies to inhibit MDA levels have resulted in suppression of inflammation and retinal damage [228].

Amidst all the indicators of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHDG) was assessed most often [231-234]. Studies have confirmed that intracellular DNA damage is common in IBD patients due to oxidative stress [231-234]. Therefore unsurprisingly, 8-OHDG levels were found to be high in the leukocytes of patients with UC and CD, making 8-OHDG a feasible marker for oxidative stress and a useful tool for diagnosis and disease surveillance [231-234].

On the other hand, there is large discrepancy in the results when measuring the activities of antioxidants in IBD [267-271]. This is unsurprising as several polymorphisms have been described in association with each antioxidant [267-271]. As such, antioxidants have often followed multiple pathways and functionalities to protect against oxidative stress [267-271]. Activities of antioxidants ought to be assessed in groups, rather than individually. When measured serologically, the total antioxidant capacity in both UC and CD is decreased and representative of the overall cellular ability to counteract oxidative stress [267-271].

Building on the theories stated above, I have therefore chosen to experiment with MDA and 8-OHDG to quantify oxidative stress and to assess their potential to be applied to clinical practice as diagnostic and prognostic tool. Further, we know from previous studies that excessive ROS weakens the tissue's innate antioxidant system. This led me to again suggest that measuring the antioxidant level/capacity serologically or genetically could tell us more about the disease severity. I have therefore chosen to measure the antioxidant gene expression as surrogate marker of antioxidant capacity.

6.3 Interpretation of data: Oxidative stress as marker of disease activity and prognosis

My experimental results confirmed that oxidative stress as quantified by MDA level is upregulated in patients with ArLD and AH when compared to HVs. When comparing AH patients with HVs, MDA concentration was significantly higher in the former group (mean 43.74 vs 17; $p<0.01$). MDA concentration was also significantly higher in ArLD patients when compared to HVs (mean 38.85 vs 17; $p<0.05$). There is no noteworthy difference between the MDA concentrations of AH and ArLD (mean 43.74 vs 38.85; $p=0.33$).

This demonstrates that MDA level can be a complimenting tool when diagnosing AH with the caveat that the level would be falsely elevated if measured in the presence of concomitant inflammatory diseases other than AH.

On the other hand, oxidative stress as quantified by intracellular ROS by DCFH-DA fluorescence, presented in the format of percentage maximal oxidative stress is significantly higher in the AH patients when compared to HVs in the monocyte subtype. Percentage maximal oxidative stress within CD14+ monocytes compared to HVs (63.24 vs 41.94; $p<0.01$).

Within the AH patient cohort, percentage maximal oxidative stress was notably more elevated in patients who did not survive their course of disease. T in both cell subtypes. In CD14+ monocytes (70.9% vs 60.5%; $p=0.02$); whilst in CD3+ T cells (67% vs 54.8%; $p=0.05$). The p values produced from these data highlight the strength of association between oxidative stress and disease severity in AH.

Further data to assess the prognostic utility of percentage maximal oxidative stress suggest that within the AH group, percentage maximal oxidative stress is significantly higher in the non-survivors when compared to the survivors. The percentage of maximal oxidative stress within CD14+ monocytes in predicting day 90 mortality yield a result AUC of 0.66 (95% CI:0.49- 0.82). Similarly, percentage of maximal oxidative stress within CD3+ T cells and day 90 mortality yield a result AUC of 0.67 (95% CI:0.49-0.85).

Even though the respective percentage of maximal oxidative stress with AUCs of 0.66 and 0.67 are too low to be used as a meaningful singular prognostic test in clinical practice, it could complement the existing scoring models e.g. Lille score [142, 143] when stratifying disease severity as it can be done on admission and results be available in quick turnaround time, with limiting factor of the need for specialist equipment and staff.

6.4 AH patients have differential histone activities and gene expression driven by oxidative stress

In the pathogenesis of inflammatory diseases, the involvement of histone modifications have garnered a lot of attention lately [61, 62, 63]. Histone modifications typically comprise of histone acetylation, deacetylation, methylation, phosphorylation and ubiquitination) [61, 62, 63].

Oxidative stress is thought to play an important role in histone modifications [83, 84,172].

When assessing histone modifications, there are at least two levels at which histones can be investigated [67-69]. One is to look at the total level of histone activities and to compare total histone activities in diseased vs healthy samples [78-82]. The other is quantifying histones using measurement of specific histone gene expression, using control housekeeping genes to normalise the results [78-82].

With the advantage of research on various inflammatory diseases, I have chosen to assess both the histone activities using ELISA and histone gene expressions in AH using PCR.

The PBMCs of AH patients were magnetically separated into monocytes and T cells prior to measurement of their histone modifications.

My data highlights that the HDAC activities are significantly downregulated in AH when comparing to HVs (43.6% vs 81.1%; $p<0.01$) in monocytes, and (44.5% vs 80.6%; $p<0.01$) in T cells. Within the AH samples, HDAC activities are reduced in the patients who did not survive when compared to the survivors (24.6% vs 55.4%; $p<0.01$) in monocytes, and similarly in T cells (22.1% vs 58.2%; $p<0.01$).

When looking into the individual histone gene expressions, it is worth highlighting in particular that the HDAC2 and HDAC8 gene expression were significantly downregulated in the patients who did not survive their course of disease compared to the patients who did. There is no distinct difference between the monocytes and T cells.

In the subject of GC resistance, very few convincing genetic or epigenetic determinants have ever been identified. It is suggested from the respiratory models that the epigenetic component of GC resistance is likely related to HDAC2 inhibition [187-190]. GC resistance in COPD and asthmatic patients who smoke has an association with HDAC2 inhibition, and that this is driven by oxidative stress [187-190]. Whether or not AH patients do follow suit the same oxidative driven histone alterations remains unknown. Nonetheless, my data is a step towards identifying the key histone change which affects GC sensitivity in AH.

The close linkage between oxidative stress and the regulation of histone gene expression is beyond doubt, but involves very diverse pathways. Therefore, defining the main pathway that oxidative stress accord to, is of particular interest.

In cancer and other inflammatory diseases, the role of PI3Kδ signalling has been particularly highlighted [85, 180, 268-271]. In respiratory diseases, in vitro oxidative stress activates PI3K, and in turn reduces the activity of histone deacetylase 2 and reduced GC receptor expression and thereby, inducing resistance to GC [85, 180]. The expressions of PI3Kδ and AKT phosphorylation were augmented in the macrophages of COPD patients and experiments which restored PI3K inhibition, have resulted in reversal of GC resistance [85, 180].

In UC, PI3K/AKT pathway has vital regulatory effects in the pathogenesis of the disease, by way of perpetuating oxidative stress and inflammation [267-271]. PI3K is an intracellular kinase which produces downstream effect on AKT [267-271]. Previous research showed that the PI3K/AkT signalling pathway regulates and releases pro-inflammatory cytokines like the TNFα [267-271]. In vivo treatment with 5-ASA has been found to decrease the MDA level and inhibits the activation of PI3K/Alt signalling [267-271], and in turn ameliorates the symptoms of UC [267-271]. Previous research has confirmed that this particular pathway is upregulated in UC associated colon cancer [267-271].

In uveitis, the permanent damage to eye tissue were caused by amplification of inflammation [59, 228, 262-266]. Previous research has highlighted the vicious cycle between ROS production and pro-inflammatory cytokines exacerbation [59, 228, 262-266]. The retinal pigment epithelium (RPE) which is one of the affected cells in uveitis, are found to be affected the most by oxidative stress [59, 228, 262-266]. Oxidative stress activates the PI3K/ Akt pathway which then inhibits the anti-apoptotic genes, contributing to oxidative-stress-induced cell death [59, 228, 262-266].

In an acute inflammatory liver setting, the relationship between oxidative stress and PI3K/ Akt signalling pathway in AH remains unclear.

From my research, PI3Kδ activity was significantly lower in AH when compared to HVs (monocytes AH vs HV 12.9 vs 18.8; $p<0.01$ and T cells AH vs HV 12.9 vs 18.1; $p<0.01$). It is also worth noting that PI3Kδ activity was significantly lower in AH non survivors compared to survivors both in monocytes (7.3 vs 16.3; $p<0.01$) and in T cells (6.9 vs 16.7; $p<0.01$).

Overall, my findings overcame the anticipated hypothesis in which oxidative stress exacerbates the PI3K/AKT pathway in AH, which then induces GC resistance. This is explained by the existence of several pathways driven by oxidative stress, where just assessing one pathway is unlikely to improve our understanding of these oxidative stress driven intracellular processes.

My data is limited by small sample size and the observational nature of my study. All the patients are recruited from single centre and only from single ethnicity (white British), but the recruited patients have clinical characteristics that are consistent with large randomized clinical trials e.g. STOPAH. I acknowledge that all of my work has been performed with peripheral bloods (rather than infiltrating liver immune cells or liver tissue) as they are the easiest to access and also appropriate to use for testing of systemic immune function.

In this context, further investigation into the specific effects of HDAC/HAT using ChIP-seq/ ATAC-seq will be useful. There is global reduction in HDAC/HAT/ PI3Kδ activities in AH non-survivors, which suggests a profound global immune dysfunction. This suggests that epigenetics, in particular histone modification may not be amenable as sole therapeutic target.

6.5 Potential therapeutic options to reverse cellular GC resistance

Individual response to GC predicts clinical outcome as GC remains the mainstay of treatment in many inflammatory diseases including AH. Excessive inflammation driven by oxidative stress partly explains disease severity. However, my research highlights that oxidative stress does not follow a single pathway/ process. Therefore, even if oxidative stress remains the mastermind behind GC resistance, it can be orchestrated by a myriad of intracellular processes.

GC sensitivity is usually quantified in vitro by measuring the GC's ability to inhibit PHA stimulated proliferation of diluted whole blood or isolated PBMC [197-201].

In the past, Dexamethasone Inhibition of Lymphocyte Proliferation Assay (DILPA) has been used to measure GC sensitivity [197-201]. However, the technique necessitates highly skilled laboratory workers (dividing cells with tritiated thymidine) and radiation (measuring beta radiation emission with beta counter) [197-201]. Therefore, the clinical translation of this method is limited.

In addition, chemiluminescence and flow cytometry methods for cell measurement proliferated by BrdU has also been used [197-201]. However, these methods again require appropriate expertise and equipment which will struggle to deal with large sample volume [197-201].

As a result, I have opted for BLISS assay which is an easy, reproducible, non-radioactive in vitro bioassay [197-201]. This assay has been standardized with laboratory protocol and accurately determines intracellular GC sensitivity by measuring absorbance of samples in an ELISA plate reader [197-201]. Details of the protocol has been described in the methodology chapter.

Firstly from my data, AH patients exhibit significantly higher level of oxidative stress. This signifies that antioxidants, which serve to counteract the effects of oxidants could theoretically be beneficial.

There are evidence from pre-clinical and clinical studies that antioxidants like NAC can improve survival in AH [162, 163]. Along the line of antioxidants, Zinc supplementation was also experimented with in patients with ArLD as these patients commonly suffer from malnutrition [166, 246-248, 272]. No similar work was done in AH patients. From my cell culture work, neither NAC nor Zinc has produced any significant effect on the cellular GC sensitivity (i.e. no difference between baseline IMAX values and post treatment IMAX values).

On the other hand, there is evidence that patients with ArLD have altered gut microbiome and good gut bacteria composition including butyrate, which is an important factor for gut barrier function [38-42]. An impaired gut barrier (leaky gut) prompts bacterial dysbiosis and translocation of endotoxin, subsequently leading to persistent chronic inflammation and damage to hepatocytes [38-42]. However, there is a mismatch between the concept and data yielded from my cell culture work. Sodium butyrate has shown deleterious effects on the PBMC cells of my recruited severe AH patients, although the heterogeneity makes the result difficult to interpret. I have chosen to work with peripheral bloods of severe AH patients who represent only one end of the inflammatory spectrum. Therefore it would be appropriate to experiment with non-severe AH patients.

The role of class 1 HDAC inhibitors has been validated and approved for the treatment of certain haematological malignancies [253-255]. The reversibility of epigenetic changes during cancer development makes epigenetic therapies attractive [253-255]. However, when testing on PBMCs of AH patients, the established class 1 HDAC inhibitors (Vorinostat) does not seem to demonstrate beneficial effects on the cellular glucocorticoid responsiveness.

In fact, Vorinostat has further desensitized the cells and worsened glucocorticoid resistance. This is understandable as class 1 HDAC activities including that of HDAC2 are already significantly downregulated in patients with severe AH. Further inhibition with vorinostat is likely to have negatively impacted the cellular resistance to GC. The lack of HDAC agonists makes it challenging to test my hypothesis that by improving HDAC activity, the cellular glucocorticoid resistance can be improved/ reversed.

There is a clear paucity of cell culture work in the field of AH. A likely reason for this is patients with AH often present a heterogeneous phenotype, and this effects the quality of experiments. Consequently, I have limited references to go by when it comes to pharmacokinetics of each therapy in AH. Larger in vitro studies are obligated to investigate the potentials of each therapy.

The data is insufficient to reach significance with any of the therapies I trialled attributable to the limited number of patient samples I experiment with. Oxidative stress can affect GC responsiveness via downstream modulators. However, the clinical effects will need to be tested in a bigger cohort of patients and with primary human liver tissues.

With consideration of above limitations, I would strongly advocate for my data as they provide valuable insights to potential liver disease indices. My data supports the hypotheses that oxidative stress predicts outcome and that altering HDAC expression may change glucocorticoid resistance. Further validation with primary human cell/ tissue material is justified to decide wheather the quantification of oxidative stress could be utilised as predictor of an ongoing process of liver damage and global immune dysfunction.

Chapter 7: Future directions

The work presented in this thesis has highlighted several new avenues that could be explored in future studies. The experiments carried out in this thesis have demonstrated that oxidative stress can be measured in peripheral blood. The data highlighted that oxidative stress is upregulated in AH patients, especially more pronounced in the non-survivors when compared to survivors.

The goal is to facilitate clinical use of measurement of intracellular oxidative stress with the intent of advancing the prediction of prognosis and individualising treatments. Therefore, it is pertinent to validate any differences in oxidative stress in patients with mild, moderate, or severe AH and whether peripherally measurement of oxidative stress is an accurate representation of the "true" oxidative stress status of the tissue.

This can be achieved by prospective analysis of MDA level and DCFH-DA fluorescence on liver tissues of AH patients. Currently, there are no tissue based *in vitro* studies on oxidative stress in AH patients. Two potential challenges in the conduct of such experiments would include the availability of liver tissue and the clinical stratification of AH disease to fully appreciate the heterogeneity of levels of oxidative stress in cells. Additionally, the volatile nature of intracellular ROS often lead to inaccurate measurement. By recognizing the potential limitations of these, we can focus on how to interpret the data obtained from these techniques.

It has been previously demonstrated that in vitro glucocorticoid resistance can be appraised with the BLISS assay, which quantifies the inhibition of proliferation of lymphocytes by dexamethasone. This assay has been further examined in my cell culture experiments. Further, previous literature demonstrated that glucocorticoid resistance is regulated by the Th17 subset of CD4 cells which could be caused by underlying histone modifications. I have demonstrated that severe AH patients have differential histone activities and histone gene expression. To further assess if there is a differentially accessible chromatin region due to the histone modifications, I have collaborated with Imperial College London on a project using ATAC-Seq [273, 274]. The methodology ATAC-Seq (Assay for transposase-accessible chromatin) with high throughput sequencing can reliably locate the changes in chromatin accessibility regardless of the underlying mechanism [273, 274]. This method uses NGS (Next generation sequencing) adaptors and transposase Tn5 [273-275]. The adaptors integrate into the open chromatin site whilst the transposase simultaneously fragment the chromatin [273-275]. The reconstruction sequenced by this combination then generates bioinformatics data which could be analysed on a genome wide scale [273-275].

My director of study and myself have collaborated with the genetics and genomics group from Imperial College London to conduct these experiments using ATAC-Seq. First, we identified twelve patients with severe AH (six of which were responsive to glucocorticoids and six were resistant to glucocorticoids). Their responsiveness to glucocorticoids was determined by their BLISS IMAX values. Then, we sorted their PBMCs into $CD4+CD25^{+}$ cells prior to undergoing steps of transposition.

From our preliminary results of twelve patients, we identified fewer than 10 significantly differentially accessible areas of DNA comparing glucocorticoid sensitive and resistant patients. The most differentially accessible area is close to the gene SLCO4C1 which is an organic anion which facilitates the conveyance of bile acids and conjugated steroids [275-277]. This family of transporter is implicated in the pharmacokinetics of cardiac glycosides, thyroid hormones, and cAMP methotrexate [275-277]. SLCO6A1 is a murine paralog of this gene but has no detectable expression in immune cells. Further analysis of this finding is currently underway [275-277].

This piece of novel data will be validated with a wider population of AH patients as the finding has raised the possibility of a physiological genotype variant that permits chromatin accessibility rather than a true epigenetic phenomenon. The functional relevance of this on tissue-based model is not yet appreciated.

Suggestions for Future Research

The following are my suggestions for additional research/data specifically for AH patients:

1. Studies providing the assessments of oxidative stress in mild, moderate and severe AH phenotypes to expand the potential use of oxidative stress as non-invasive biomarkers and assessment scoring tool.
2. Measurements of oxidative stress at different time point in the course of AH disease progression to determine intracellular recovery potential from maximal oxidative stress.
3. Further experiments on functional effects of HDAC/HAT with ChIP-seq.
4. Further experiments with *ex vivo* liver tissue or liver infiltrating immune cells to examine the effects of each proposed therapy to GC sensitivity.

Chapter 8: Personal reflection

I always knew a doctorate degree in hepatology is something I wanted to achieve at some stage of my life. What I was less clear on was the topic of my research and the institution where I wanted to study.

After finishing my 4th year of hepatology specialist training, I was introduced to the hepatology research group at Derriford and Professor Cramp who later became one of my supervisors. Dr Dhanda then came into light with a research idea of investigating glucocorticoids resistance in alcoholic hepatitis. I was charmed by the prospect of this idea as novel data from this research can potentially make a difference to the wider family of inflammatory diseases. One point to make before I proceed, throughout this journey, both my supervisors gave their usual stalwart support. I am grateful to them.

The first year of my PhD journey was nothing but a colossal and unquantifiable learning curve. Swiftly after I enrolled onto the programme, I was made aware just how unprepared I was for my research, which is largely laboratory-based sciences. Unlike undergraduate education, doctoral study necessitates so much personal academic initiative which to me, is a cultural shock. No syllabuses were set and certainly no allocated teaching sessions to acquire laboratory skills. I was required to do 'dogsbody' work in the laboratory and a lot of self-learning on the computer.

Towards the end of first year, I realized that research requires a different style of operation and there is no time and space for sitting around waiting to be told what to do.

The real test came in second year (year 2020) which was also one of the most transformative years for many lives around the world as the pandemic hits. The pandemic results in closure of research laboratory and my relocation back to clinical activities means a halt to my research. The situation got better towards the second half of the year when I was given the permission to continue working in the lab. At this point, I was merging overwhelming laboratory work with a clinical job which is simply something I wasn't prepared for. It required me to transition between different hats, typically within the same day. It was a delicate balance at the best of times and required me to work both hard and smart.

The circumstances surrounding my third year was interesting as opportunities were opening for me to take initiative in discussing my research data to the wider audience. Presenting research data is a craft. From the presenter perspective, it reinvigorates the feelings of exploration, excitement, and involvement. From a period of self-doubt, I emerge with a new identity as a 'young investigator' who is competent to present and argue my viewpoints on both national and international platforms. (Note to self: researchers younger than 35 years of age is categorized as young investigators). I can still remember

the internal scream of joy and excitement when I won third place in the 3-minute thesis presentation competition and best poster presentation on a national liver meeting.

I spent the next six months immersing myself in thesis write-up. This period was a test for grit and perseverance as the more I write, the more I know about the disease, and then the more I realise how little I know. It was a steep upward climb, not helped by the mental state of ‘get it and forget it’ as I was approaching the start of my final year of specialist training. The best thing that came out of this period was that when my data was drawn together, I could see further directions and ideas for research applications on clinical grounds.

If you have made it this far, you would have noticed how much I have progressed on a professional and personal level. In the last four years, I have journeyed through uncertainties and technical incapability, to eventually taking autonomy of my own research, presenting and publishing papers. To me, this has been an evolution.

A reflection on my PhD journey is no less than a tale of sweat, tears, joy, and success. Now that I have had a feel of what the research world is like, I have decided a full-time academic career is not for me. However, the skills I acquired remain firmly on me and will be transferable to the clinical grounds. I regard this as a perfectly appropriate outcome.

Chapter 9: Supplementary material

Patient	Gender	Age	DF	MELD	Baseline Parameters						Outcome		
					Bilirubin	Albumin	INR	WCC	Lympho	Neut	28 days	90 days	1 year
PL32	F	57	90.5	25	400	30	1.8	9	1.6	6.3	A	D	D
PL44	M	49	35.1	18	199	28	1.2	8	3.1	4.2	A	D	D
PL54	F	58	35.9	21	134	27	1.4	8	0.8	6.8	A	D	D
PL58	M	56	76.8	30	472	26	1.6	7	1.1	5.2	A	A	A
PL60	M	57	41.8	15	86	35	1.3	6	0.3	5.3	A	A	A
PL68	M	50	63.9	22	243	30	1.6	13	1	10	A	A	A
PL69	F	35	41.8	20	102	32	1.4	12	3.9	0.8	A	A	A
PL73	M	62	26.9	27	67	24	1.2	16	1.3	12.6	D	D	D
PL75	M	46	47.3	22	211	21	1.4	6	1	3.9	A	A	D
PL80	M	41	106.6	25	203	30	2.2	11	3.2	6.5	A	A	A
PL86	M	75	181.8	32	175	18	3.2	13	0.5	11.3	A	A	A
PL88	F	46	420.7	40	351	29	6.9	10	0.9	8.6	D	D	D
PL89	F	35	32.4	17	122	28	1.2	5	1.1	3.3	A	A	A
PL90	M	62	56.8	22	239	27	1.5	14	1.3	10.9	D	D	D
PL91	F	48	27.8	20	50	33	1.2	8	0.7	6.5	A	A	A
PL92	M	69	36.9	19	270	33	1.2	7	2	4.1	A	A	A
PL94	M	42	64.1	26	443	30	1.4	13	0.8	11	D	D	D
PL95	M	52	51.8	20	99	24	1.6	5	1.1	2.5	A	A	D
PL96	F	56	17.9	13	86	34	1	4	1.2	2.5	A	A	A
PL97	M	48	76.6	24	255	29	1.7	15	1.5	12.9	A	A	A
PL98	M	46	37	19	66	28	1.3	13	1.5	9.6	A	A	A
PL99	F	65	42.1	18	90	21	1.4	7	1.4	5	A	A	A
PL100	F	64	36.9	12	81	35	1.3	6	1.6	3.3	A	A	A
PL101	F	64	59.1	26	264	27	1.5	19	2.3	16.3	A	D	D
PL102	M	35	17.5	16	119	31	1	18	1.1	14.5	A	A	A
PL103	F	45	125.8	30	444	26	2.2	7	0.9	4.4	D	D	D
PL104	F	67	60.7	17	62	30	1.7	7	1.4	4.4	D	D	D
M0M02-001	M	61	38.1	27	204	27	1.2	14	1.1	12.4	A	A	A
M0M02-002	M	48	80.9	27	77	24	1.9	4	0.6	3	A	A	A
M0M02-003	M	60	350.7	40	223	28	5.5	11	0.3	9.9	D	D	D
M0M02-004	M	68	85.5	25	94	31	2	11	1.1	9.2	A	A	A
M0M02-005	F	60	156.5	37	639	29	2.5	4	0.9	2.7	A	D	D
M0M02-006	F	47	48	20	184	39	1.4	19	0.9	16.7	A	A	A
M0M02-007	M	49	26.8	15	88	29	1.2	7	0.8	5	A	A	A
M0M02-008	F	51	38	22	76	32	1.3	6	0.8	4.7	A	A	
M0M02-009	F	60	34.9	15	70	27	1.3	11	2	7.5	A	A	
M0M02-0010	M	53	81.6	25	428	36	1.7	7	0.2	4.9	A	A	
M0M02-0011	F	48	33.4	19	163	25	1.2	14	0.7	12.1	A	A	
M0M02-0012	F	53	98.8	27	203	21	2.1	11	0.8	9	A	A	
M0M02-0013	M	49	158.6	38	85	23	3	20	0.6	17.5	A	D	D
M0M02-0014	M	59	71.3	24	276	32	1.6	6	0.8	4.5	D	D	D
M0M02-0015	M	48	113.1	33	519	23	2	31	1.4	26.5	A	D	D
M0M02-0016	M	62	47.7	23	273	31	1.3	9	1.3	5.9	A	A	
M0M02-0017	M	48	24.9	16	159	35	1.1	11	1.3	8.5	A	A	
M0M02-0018	M	56	44.8	20	121	32	1.4	6	1.9	2.6	A	A	
M0M02-0020	M	47	55.7	19	126	23	1.5	15	1.5	10.4	A	A	
M0M02-0021	M	35	62	22	265	31	1.5	10	0.6	8.7	A	A	
M0M02-0022	F	42	66.8	21	214	24	1.6	8	0.8	6.4	A	A	
M0M02-0023	M	57	29.6	16	89	31	1.3	4	0.3	2.4	A	A	
M0M02-0024	F	59	39.3	24	215	30	1.4	13	1.5	9.8	A	A	
M0M02-0025	M	54	20.5	13	52	30	1.2	3	0.7	1.7	A	A	
M0M02-0026	M	55	41.9	24	362	22	1.3	14	1.8	10.6	D	D	D
M0M02-0028	M	55	43.2	25	235	25	1.4	12	1.4	7.7	A	A	
M0M02-0029	F	59	28	15	86	28	1.3	8	1.1	5.7	A	A	
M0M02-0030	M	61	29.3	21	194	26	1.2	14	2.9	9.6	A	A	

Table above tabulates the characteristics of all the patients recruited to this research. DF- Maddrey's discriminant function score; MELD- Model for end-stage liver disease score; INR- International normalised ratio; WCC- White blood cell count, Lympo- Lymphocyte, Neut- neutrophils; A- Alive; D- Dead.

Chapter 10: References

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