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REVIEW

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REVIEW

Proteomics: Concepts and applications in human medicine

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Abstract

Proteomics is the complete evaluation of the function and structure of proteins to understand an organism's nature. Mass spectrometry is an essential tool that is used for profiling proteins in the cell. However, biomarker discovery remains the major challenge of proteomics because of their complexity and dynamicity. Therefore, combining the proteomics approach with genomics and bioinformatics will provide an understanding of the information of biological systems and their disease alteration. However, most studies have investigated a small part of the proteins in the blood. This review highlights the types of proteomics, the available proteomic techniques, and their applica-tions in different research fields.

Key Words: Proteomics; Biomarker; Mass spectrometry; Two-dimensional electrophoresis; Drug discovery

Core Tip: Proteomics is the complete evaluation of the structure and function of proteins to understand an organism's nature. Mass spectrometry is an essential tool that is used to profile proteins in the cell. However, biomarker discovery remains the major challenge of proteomics because of the complexity and dynamicity. This review highlights the types of proteomics, the available proteomic techniques, and their applications in different research fields.

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INTRODUCTION

Proteomics is a new type of 'omics' that has rapidly developed, especially in the therapeutics field. The word proteome was created by Marc Wilkins in 1995[1]. Proteomics is the study of the interactions, function, composition, and structures of proteins and their cellular activities[2]. Proteomics provides a better understanding of the structure and function of the organism than genomics. However, it is much more complicated than genomics because the protein expression is altered according to time and environmental conditions[3]. It is estimated that there are almost one million human proteins, many of which contain some modifications such as post-translational modifications (PTMs). However, it is also estimated that the human genome codes for about 26000-31000 proteins[4]. There are a variety of proteomics techniques including one-dimensional (1D) and two-dimensional (2D) gel electrophoresis (2-DE)[5], as well as gel-free high-throughput screening technologies such as multidimensional protein identification technology[6], stable isotope labeling with amino acids in cell culture[7], isotope-coded affinity tag, and isobaric tagging for relative and absolute quantitation [8]. Shotgun proteomics[9], 2D difference gel electrophoresis (2D-DIGE)[10], and protein microarrays[11] can be used in tissues, organelles, and cells. Large-scale western blot assays[12], multiple reaction monitoring assays[13], and label-free quantification of high mass resolution liquid chromatography (LC)-tandem mass spectrometry (MS) are commonly used for high-throughput processing. In the last decade, proteomics has been classified into protein expression mapping and protein interaction mapping[14]. The former method uses 2-DE combined with MS for quantitative proteome expression in cells, body fluids, or tissues. Protein expression mapping can provide an understanding of the PTMs of expressed proteins under different environmental conditions or disease states[14]. Protein-protein interaction mapping uses the yeast two-hybrid system coupled with MS to determine the interaction partners for each cell's encoded proteins and the proteome-wide scale[15].

Proteomics is a multi-step technique in which every step should be very well controlled to avoid non-biological factors interfering with protein expression and interaction. Sample preparation is the most important step because it solubilizes all proteins in the sample and eliminates all interfering inhibitory compounds such as lipids. Adequate sample preparation is crucial to obtain reliable, accurate, and reproducible results[16]. PAGE is the most widely used method for protein separation and isolation[17]. High-performance LC (HPLC)[18], 1-DE, and 2-DE are the methods used to separate proteins[19]. Proteins are isolated using 1-DE based on their molecular mass. Protein solubility is rarely an issue since proteins are solubilized in sodium dodecyl sulfate (SDS).

Furthermore, 1-DE is easy to use, repeatable, and capable of resolving proteins with molecular masses ranging from 10 kDa to 300 kDa[17]. As 1-DE gel has minimal resolving power, it is most commonly used to characterize proteins after being purified. However, in more complex protein mixtures, such as a crude cell lysate, 2-DE may be used. In 2-DE, proteins are determined by their net charge and their molecular mass[17].

Proteomics can analyze the expression of a protein at different levels allowing the assessment of specific quantitative and qualitative cellular responses related to that protein[20]. Qualitative and quantitative proteomes are measured at post-transcriptional, transcriptomic, and genomic levels[21]. According to the conditions, qualitative proteomics utilizes microarrays, 2-DE, and 2D-LC to monitor protein mixture composition and protein expression changes[20]. In addition, it can provide information on the molecular mechanisms of diseases and compare two groups such as patients with healthy controls[20]. Quantitative proteomics can also provide deep insights into disease mechanisms, cellular functions[22], and biomarker discovery[23]. Several new strategies are used in quantitative proteomics, such as post-extraction or metabolic stable-isotope labeling alone or in combination with affinity labeling[24,25]. MS identifies compounds by sorting cations according to their mass-to-charge ratio[26].

The study of proteomics has many applications in different fields such as medicine, oncology, food microbiology, and agriculture. This review will shed light on proteomics, their techniques, some of its applications, and the challenges currently faced in this field.

TYPES OF PROTEOMICS

Proteomics has three main types: expression proteomics, functional proteomics, and structural proteomics[27].

Expression proteomics

Expression proteomics is a novel approach that studies the quantitative and qualitative expression of proteins. It aims to specify the difference in protein expression between two conditions such as patients and controls[28]. In addition, it can identify disease-specific proteins and new proteins in signal transduction[17]. Expression proteomics experiments are usually used to study the patterns of protein expression in different cells. For example, a tumor tissue sample is compared to a normal tissue sample to identify differences in the levels of proteins[26]. Variations in protein expression, which are present or missing in tumor tissue compared to normal tissue, are detected using 2-DE and MS techniques[29].

Structural proteomics

Nuclear magnetic resonance spectroscopy and X-ray crystallography are used in structural proteomics to determine the three-dimensional structure and structural complexities of functional proteins. It specifies all protein interactions such as membranes, cell organelles, and ribosomes in the mixture[30]. The study of the nuclear pore complex is an example of structural proteomics[31].

Functional proteomics

This type of proteomics studies the protein functions and molecular mechanisms in the cell and determines the protein partner's interactions. In particular, it investigates the interaction of an unknown protein with partners from a specific protein complex involved in a particular process. This may indicate the biological role of the protein [32]. In addition, the elucidation of protein-protein interactions *in vivo* can lead to comprehensive descriptions of cellular signaling pathways[33].

PROTEOMICS WORKFLOW

Two methods can be used in proteomics: top-down and bottom-up workflows. The bottom-up method is sometimes called peptide-based proteomics. Here, the protein is digested by trypsin and separated by a specific column, followed by analysis of the peptides by MS[15]. The bottom-up approach can be classified into two groups according to the fractionation step. The first approach uses 2-DE to isolate the proteins from the gel. Then the proteins are digested into peptides that MS can identify. The second approach is called "shotgun" proteomics. Here, the digestion of protein occurs without fractionation, and LC is used to separate the peptides identified by MS[34]. In top-down proteomics, whole proteins or polypeptides are immediately assessed by MS. The molecular mass of proteins is sometimes calculated by using electrospray ionization (ESI) followed by matrix-assisted laser desorption/ionization (MALDI) MS [35]. Top-down proteomics can identify proteins with a molecular mass of > 200 kDa [36]. Both approaches have various advantages and limitations. In the bottom-up approach, there is low percentage coverage of the protein sequence, because the recovered sample includes small and inconsistent fractions of total peptides. This results in missing a large proportion of alternative splice variants and PTMs. However, in top-down proteomics, all characteristics of proteins are protected, and almost all existing modifications and correlations can also be recovered. Moreover, in top-down proteomics, the results of the exclusion of protein digestion with time are preserved [37]. The major challenge in top-down proteomics is the poor solubility of proteins compared to small peptides. Some proteins in the membrane have high solubility but need to be washed with SDS; however, SDS cannot be used in ESI[38]. Proteomics workflows involve sample preparation and analytical flow. The latter include separation of proteins, protein identification, and validation.

Sample preparation

Proteomics experiments highly depend on the accuracy of sample preparation, in addition to a well-designed pre-analytical workflow. There is no standard technique for sample preparation in proteomics. Each method depends on the number of proteins in the sample, the sample's complexity, and the study's objectives. Extraction of proteins from the mixture is the most vital step in the preparation of samples. To maximize protein extraction and solubilization, the extraction should include organic solvents and detergents followed by a tissue disruption technique. The organic solvents and detergents can be removed by lyophilization[39]. In previous detergent-based methods, the extraction of 2,2,2-trifluoroethanol (TFE) macro-scale (> 100 μg) materials and nano-scale (30 μg)-based lysis have provided comparable protein detection rates[40].

Separation and isolation of protein

Gel-based and chromatography-based approaches are used for the separation and isolation of proteins from the mixture.

Gel-based approach

The best technique for protein isolation and detection is PAGE[41]. For separation, 1-DE and 2-DE can be used. Furthermore, 2D-DIGE and SDS-PAGE are examples of 2D variations used in gel electrophoresis[42].

1-DE

1-DE, can isolate proteins with a molecular weight of 10 kDa to 300 kDa. It uses SDS, a detergent that denatures secondary and non-disulfide-linked tertiary structures, and combines them with a negative charge proportional to their volume. This allows the calculation of molecular weights[43]. SDS-PAGE can be used to verify the purity of samples, test protein purification, and calculate molecular weights for unknown proteins[44].

2-DE

2-DE differentiates proteins better than 1-DE due to the variation in molecular weight and isoelectric point of protein molecules[43]. It also has a better resolution than 1-DE because the protein is separated into two different dimensions. In 1-DE, the protein is separated based on net charge, but in 2-DE, protein separation is based on the molecular mass and isoelectric point. Thus, this method can detect different forms of proteins such as PTMs and phosphorylation. Some proteins that arise from different proteolysis processes and splicing of alternative mRNA can be resolved by 2-DE[45]. There are many applications of 2-DE, including protein expression profiling and cell map proteomics. Protein expression profiling can be used for comparing normal and diseased tissues. Mapping proteins in 2-DE can be used in cellular organelles[46], protein complexes[19], and microorganisms[47]. 2-DE can help catalog proteins, and the database can be created on the World Wide Web[48]. However, 2-DE cannot detect proteins at a low molecular weight and the limits of separation by isoelectric point and size[49].

Chromatography-based approach

Chromatography of affinity, size exclusion chromatography (SEC), and ion-exchange chromatography (IEC) techniques can be used to purify protein-based chromatography. In addition, western blotting and the enzyme-linked immunosorbent assay are used to identify selective proteins[50].

IEC

IEC is used to purify proteins according to their charges. This technique allows separating proteins according to their charge nature, which is not possible by other approaches. The charge accepted by the molecule of interest can be readily used by altering the pH of the buffer. The IEC technique is low cost and can persist in variable buffer conditions[30].

SEC

SEC can be used to separate different compounds according to their size (hydrodynamic volume) measured by how efficiently they enter the stationary phase's pores. However, this technique is not as useful as other proteomics techniques[51]. Two basic versions of SEC are utilized: gel permeation chromatography (GPC) using organic

solvents, which is used for polymer analysis; and gel filtration, which is performed using aqueous solvents.

Affinity chromatography

Affinity chromatography is the process of protein separation according to its interaction with an immobilized ligand. In 2-DE and non-2-DE, affinity chromatography helps decrease the protein complexity[52]. There are three types of affinity chromatography: separation of protein before 2-DE, affinity chromatography of protein before MS, and affinity chromatography of peptides before MS.

LC

LC is a powerful technique that can separate proteins from a complex mixture and can analyze large and fragile biomolecules. When combined with MS, it can be used for determining the peptides in the mixture[53]. LC can help researchers discover novel biomarkers and understand the mechanisms of carcinogenesis according to the modification of proteins. For example, some researchers use LC-MS/MS to rapidly monitor congenital adrenal hyperplasia from dried filter-paper blood samples[54].

Protein identification and characterization

The identification of proteins is a critical step in proteomics. MS can be used after the separation of the proteins by chromatography or electrophoresis[55]. Other techniques can also identify proteins such as Edman sequencing and protein microarray[17].

Edman sequencing

Edman sequencing has been used to detect the sequence of amino acids in peptides or proteins. This technique includes the reaction of chemicals, which remove and determine amino acid residues present at the N-terminus of the polypeptide chain. Thus, it plays a significant role in assessing biopharmaceutical quality and therapeutic proteins[17].

MS

MS is the best analytical tool for rapidly facilitating the sequencing of proteins[56]. It can also be used to detect the molecular weight of proteins. In this technique, protein molecules are ionized, and their mass is calculated according to mass-to-charge ratios. The mass spectrometer has three main components: an analyzer, an ion source, and a detector. The methods used for ionization are ESI and MALDI[57]. In MALDI, a chemical matrix is mixed with the peptides, and spotted onto a metal multiwall microliter plate to make a crystal lattice. The matrix chemicals pass the energy to the samples after absorbing it. Then peptide ions are detected by a mass analyzer. MALDI creates mostly singly charged ions that help to determine the m/z value[58]. In ESI, the power is activated in the protein sample to create charged droplets that increase gaseous ion production, which then are analyzed with a mass analyzer[59]. The advantages of ESI are its high reproducibility and high elasticity to combine many categories of MS. Furthermore, ESI can be fixed to time-of-flight (TOF)-MS, quadruple, ion traps, and fourier transform ion cyclotron resonance. On the other hand, the disadvantages of ESI are that it cannot be applied for molecular imaging, it requires a large quantity of samples, and multiple peaks are produced due to the many charged ions that result in the complexity of MS/MS spectra[60].

Protein identification and validation

Sequent, Mascot, Comet, and Tandem are instruments currently available for database searching[61]. However, most search devices do not produce matching data as they operate on differentiation algorithms and recording functions, creation integration, and data comparison from many studies and experiments. As a result, the identification of peptides by data search needs additional time[62]. High-quality data makes the data search more effective and less time consuming. Moreover, using accurate mass to measure ion fragments can shorten database explorations and produce more accurate results[63].

BIOINFORMATICS IN PROTEOMICS

Bioinformatics analyses use novel proteomics algorithms to manage the large and varied data in the process of marker discovery[64]. Controlling this massive quantity

of data and finding the association between other omics technologies (*e.g.*, metabolomics and genomics) remain difficult. The analyses of proteomics data is challenging because of the parameters used in processing, quality valuation, and shortage of standards for data formats. The big challenge is how to analyze massive data and create real biological understanding[65]. Protein pathways are a collection of internal cell reactions that have a specific biological impact. For protein pathways, a variety of tools and databases are available[66]. The Kyoto Encyclopedia of Genes and Genomes, BioCarta, Pathway Knowledge Base Reactome and Ingenuity pathway databases have extensive information on metabolism, signaling, and interactions[67,68]. Unique databases for signal transduction pathways, such as GenMAPP or protein analysis through evolutionary relationships (PANTHER), have been created[69,70]. Furthermore, databases such as Netpath, which include cancer-related pathways, have been created to detect proteins unique to a specific cancer type[71]. Details about protein interactions in complexes can be found in databases including BioGRID, IntAct, MINT, and HRPD [72-74]. The STRING database links to various other databases for literature mining and is commonly used for protein interaction. Furthermore, using the STRING database, protein networks can be drawn based on the list of genes given and the available interactions[75,76].

APPLICATIONS OF PROTEOMICS IN MEDICINE

Proteomics is a revolutionary technique that has been used in medicine, including drug and biomarker discovery. Proteomics can identify and monitor biomarkers by analyzing the proteins in the body fluids such as urine, serum, exhaled breath and spinal fluid. Proteomics can also facilitate drug development by providing a comprehensive map of protein interactions associated with disease pathways[77].

Biomarker discovery

A biomarker is an assessable pointer of a normal or abnormal biological state in the body[78]. In clinical settings, cancer development and its response to therapy are measured by cancer biomarkers[79]. 2D-PAGE is used for the discovery of biomarkers. It can also compare the profiles of proteins in normal and diseased cells such as tumor tissues and body fluids[80]. Cancer biomarkers are divided into three classes, predictive, prognostic and diagnostic, based on their uses. Predictive biomarkers can predict the response to therapy. For instance, in breast cancer, the activation and the positivity of human epidermal growth factor receptor 2 can predict the response to trastuzumab[81]. In addition, in colorectal cancer, mutation of Kirsten rat sarcoma virus gene can predict resistance to treatment with epidermal growth factor receptor inhibitors (*e.g.*, cetuximab)[82].

On the other hand, prognostic biomarkers can provide physicians with a prediction of the clinical outcomes. For example, the 21-gene repetition mark predicts breast cancer relapse and complete survival in node-negative, tamoxifen-treated breast cancer[83]. The third group of biomarkers is the diagnostic biomarker, which indicates if a patient has a specific disease condition. For example, in colorectal cancer, a stool DNA test is used as a diagnostic biomarker[84]. These biomarkers can be found in tissues, serum, blood, and urine. The body-fluid sampling for proteomics is thus less invasive and low cost. The discovery of biomarkers has progressed in many diseases such as acquired immune deficiency syndrome, cardiovascular diseases, diabetes, cancer, and renal diseases[85,86]. However, the highly complex mixtures of proteins and the high range of protein dynamics are examples of challenges in fluid sampling for proteomics. Each type of sample has a different usage according to the disease conditions. For instance, in kidney disease, the urine sample is used to assess urine proteins, reflecting changes in kidney functions[87]. In other human diseases, blood is also used for biomarker discovery. There are some challenges for using the plasma in biomarker discovery, such as protein dynamicity, the variation of the patient[87], and the low abundance of biomarkers in plasma. These challenges in biomarker discovery have yet to be addressed[88]. Most biomarker discovery studies are focused on cancer-related diseases due to their clinical importance. For instance, many biomarkers are associated with tumors that can be used to follow up with the patients[89].

Drug discovery

Drug discovery is a complex process with many different stages including chemical, functional, and clinical proteomics-based approaches. The application of proteomics in drug discovery has been developed to include patients' treatment and care[90]. 2-DE

cannot be used in drug discovery because it fails to separate the membrane proteins that characterized about 50% of important drug targets[91]. Moreover, 2-DE cannot detect low-abundance proteins[90]. In drug discovery proteomics, understanding the function of proteins and their interactions in the mixture is very important. Also, the methods should be able to detect low-abundance proteins and their activity. Therefore, many technologies such as MS and protein-chip have been used to identify and separate phage proteins. In addition, other techniques such as activity-based assays and two-hybrid assays can be used for the same purpose[92]. Using 2D-PAGE-MALDI-TOF/TOF, Lavandula angustifolia was used as a drug to treat Alzheimer's disease in rats[93].

Oncology

The application of proteomics in cancer is called oncoproteomics. Oncoproteomics can be used to identify anticancer drugs and the personalization of cancer management [94]. Microarrays and laser capture microdissection (LCM) of the tumor tissue can classify proteins in cancer. Oncoproteomics applications are used in many tissues such as the colon, breast, rectum, prostate, and brain. In addition, proteomics can be used to diagnose cancer and discover novel therapies[95]. Many proteomics techniques can be used to detect biomarkers in cancer such as aptamer-based molecular probes, cancer immunomics, tissue microarrays, nano-proteomics (to isolate signatures of autoantibodies), and antibody microarrays[94].

Two approaches can be used in tumor proteomics, LCM and MS imaging (MSI)[96]. LCM can separate the target proteins from the areas within the tumor before analysis with MS. In addition, this approach can help to determine proteins that correlate with tumor progression in the early and late stages of the disease using the proteinChip SELDI system®[97]. However, fewer studies use tumor tissues than serum due to the technical difficulties and low throughput using tumor tissues.

The second approach is using MSI. This direct tissue technique allows placing a small amount of MALDI matrix mixed directly with a fresh piece of the tumor[98]. This approach can help to map small molecules and proteins in a 3D view. This approach was to map eight normal lung tissues with 42 lung tumors[99]. Additionally, MSI can predict diagnosis, categorize lung cancer histology, and organize 85% of the nodal connections[96].

Leukemia

Proteomics was used to discover many leukemia biomarkers that could determine types of leukemia. Examples of these biomarkers include catalase, annexin 1, alpha-enolase, annexin A10, tropomyosin, tropomyosin 3, peroxiredoxin 2, and RhoGDI2. These biomarkers help to predict the diagnosis and outcome of the disease[100]. In addition, the proteomics approach can help developing new treatment pathways for leukemias using their proteomics profiles[101]. However, a major limitation of this approach is that important proteins controlling key cellular elements are present in low abundance and may not be readily detected.

Acute myeloid leukemia and proteomics

Acute myeloid leukemia (AML) is an aggressive blood cancer. Patients reach complete remission after intensive chemotherapy given as induction and consolidation[102]. However, relapsed AML may acquire at least one specific mutation such as FLT3, RUNX1, or ASXL1. Mutations in signaling genes such as KIT, NRAS, PTPN11, and NPM1 are less frequent[103]. The use of proteomics in AML may guide the post-induction strategy of either chemotherapy or allogeneic stem cell transplantation. Moreover, proteomics can help discover new or modified therapy options for AML patients[104]. Since the 1980s, many studies have focused on finding biomarkers in AML. For example, Hanash *et al*[105] used 2-DE to identify the cell of origin in acute leukemia.

While the prognosis of AML patients has improved through the years, especially in younger patients, mortality remains the highest among all other cancers[106]. Proteomics can assist the development of personalized therapy in AML[104]. Kwak *et al* [107] used 2-DE and MS to identify eight differentially expressed proteins between 12 healthy people and 12 patients with AML. Proteasome 26S ATPase subunit, immunoglobulin heavy-chain variant, and haptoglobin-1 were upregulated, while five proteins (unknown protein, lipoprotein C-III, RBP4 gene product, SP-40 and α-2-HS-glycoprotein) were downregulated[107]. Another study identified seven other proteins. These proteins were annexin A10, alpha-enolase, tropomyosin 3, lipocortin 1 (annexin 1), peroxiredoxin 2, RhoGDI2, and catalase[108].

In a recent study, BCL11A expression was found to play a role in AML. The study included 292 AML patients. The study found a significant association between the laboratory variables and the levels of BCL11A. However, BCL11A was not associated with survival and complete remission[109].

Most studies in proteomics in AML were performed on peripheral blood cells and bone marrow samples at an early stage. However, one study compared AML at diagnosis, remission, and relapse. It concluded that the proteome expression at diagnosis and relapse is similar at a high protein concentration[79,110]. Another study that focused on AML (subtypes M1 and M2) compared patients with healthy individuals. Twenty-five proteins were characterized in the peripheral blood and bone marrow samples. The study found that 6-phosphogluconate dehydrogenase, Annexin III and L-plastin were only found in the M2 subtype. The annexin I and actin gamma 1 levels were found to correlate with drug resistance at relapse[111].

CHALLENGES OF PROTEOMICS

There are many challenges in proteomics. The major challenge is the broad change in protein expression with the environment and cell type[112]. In addition, there is no comparable proteomics method, unlike genomics, that uses polymerase chain reaction [113]. Moreover, protein activities are highly regulated post-transnationally, which adds difficulty is proteomics[114]. Finally, the type of samples and sample preparation techniques are other challenges in proteomics that can significantly change the quality of MS data. For example, the protein and phosphoprotein levels in breast cancer tumor samples were affected by the sample manipulation technique and bio-specimen type [115].

CONCLUSION

Proteomics is a fast, sensitive technology that provides high proteome coverage. Expression proteomics, functional proteomics, and structural proteomics are the three major types of proteomics. There are two different workflows in proteomics: top-down and bottom-up proteomics. In addition, there are increasing uses of proteomics in the majority of biological sciences. Finally, proteomics can assist in finding new biomarkers in different diseases and discover new therapies.

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REVIEW

Culprits or consequences: Understanding the metabolic dysregulation of muscle in diabetes

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Abstract

The prevalence of type 2 diabetes (T2D) continues to rise despite the amount of research dedicated to finding the culprits of this debilitating disease. Skeletal muscle is arguably the most important contributor to glucose disposal making it a clear target in insulin resistance and T2D research. Within skeletal muscle there is a clear link to metabolic dysregulation during the progression of T2D but the determination of culprits *vs* consequences of the disease has been elusive. Emerging evidence in skeletal muscle implicates influential cross talk between a key anabolic regulatory protein, the mammalian target of rapamycin (mTOR) and its associated complexes (mTORC1 and mTORC2), and the well-described canonical signaling for insulin-stimulated glucose uptake. This new understanding of cellular signaling crosstalk has blurred the lines of what is a culprit and what is a consequence with regard to insulin resistance. Here, we briefly review the most recent understanding of insulin signaling in skeletal muscle, and how anabolic responses favoring anabolism directly impact cellular glucose disposal. This review highlights key cross-over interactions between protein and glucose regulatory pathways and the implications this may have for the design of new therapeutic targets for the control of glucoregulatory function in skeletal muscle.

Key Words: Insulin resistance; Skeletal muscle; Mammalian target of rapamycin; Glucose uptake; Glucose regulation; Insulin signaling

Core Tip: The prevalence of type 2 diabetes (T2D) continues to rise despite the amount of research dedicated to finding the culprits of this debilitating disease. Within skeletal muscle there is a clear link to metabolic dysregulation during the progression of T2D but the determination of culprits *vs* consequences of the disease has been elusive. Emerging evidence in skeletal muscle implicates influential cross talk between the

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mammalian target of rapamycin (mTOR) complexes (mTORC1 and mTORC2) during insulin stimulated glucose uptake. This review highlights interactions between protein and glucose regulatory pathways and the implications this may have for the control of glucoregulatory function in skeletal muscle.

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INTRODUCTION

Globally, 462 million individuals are affected by type 2 diabetes (T2D) and it is ranked as the 9th leading cause of mortality[1]. The prevalence of diabetes over the past few decades has continued to rise with no sign of this changing[1]. T2D is characterized by insulin resistance and hyperglycemia and can lead to various other outcomes and comorbidities reducing quality of life in those effected. While the pathogenesis and progression of T2D is still widely debated, it is clear that a complex interplay between the pancreas and peripheral tissues is dependent for maintenance of glucose homeostasis. Peripheral tissues account for 80%-90% of glucose disposal[2,3] and of those tissues skeletal muscle is a large contributor to glucose disposal[4,5] and arguably the most important for glucose clearance[6,7]. Within skeletal muscle there is clear link to metabolic dysregulation during the progression of T2D, but the definition of causes *vs* consequences within the development of this disease is difficult. Identifying clear relationships, interactions and feedback loops within the insulin signaling cascade and other metabolic pathways in skeletal muscle is imperative to our understanding for the development, its progression and ultimately a cure for this disease. To that end, this review will present the canonical understanding of insulin signaling, the influential connections between mammalian target of rapamycin (mTOR) complexes (mTORC1 and mTORC2) and the current intertwined implications of these signaling paradigms in skeletal muscle metabolic dysregulation.

INSULIN SIGNALING

The insulin signaling cascade involves both glucoregulatory and anabolic processes which is outlined in Figure 1. Insulin responsive tissues have insulin receptors (IR) on the cell surface plasma membrane. These IR contain subunits where insulin can bind as well as residues that provide docking sites for downstream signaling molecules including the IR substrates (IRS). The two predominant insulin receptor substrates are IRS1 and IRS2 with similar sequences but specific signaling roles[8,9]. IRS1 appears to be the insulin receptor substrate protein whose primary responsibility is glucose regulation, including glucose transporter 4 (GLUT-4) translocation[8] with speculation that IRS2 is more involved with fatty acid metabolism, currently known to occur in adipose tissue[9]. IRS1 is a clear mediator of insulin signaling through a specific intermediate phosphatidylinositol 3 kinase (PI3K). Interaction of PI3K to IRS produces membrane phosphatidylinositol 3,4,5-triphosphates (PIP3) which is necessary for the recruitment and localization of Protein Kinase B, also known as AKT[10].

Upstream glucose related substrates

This serine/threonine kinase is part of the AGC protein family and is known for its diverse function in growth, survival, proliferation and most importantly substrate metabolism[11-13] AKT is often referred to as one molecule but actually comprises of three distinct isoforms (AKT1, AKT2, AKT3) , while all isoforms are present in skeletal muscle, AKT2 is the most prevalent[12], but varies from low to immeasurable amounts in skeletal muscle[14,15]. While defining the variation and overlap between the AKT isoforms is important and needed, it is beyond the scope of this review but what is known currently can be found in these reviews[12,16] It is important to note that AKT2 is expressed primarily in insulin responsive tissues like fat and skeletal muscle and is

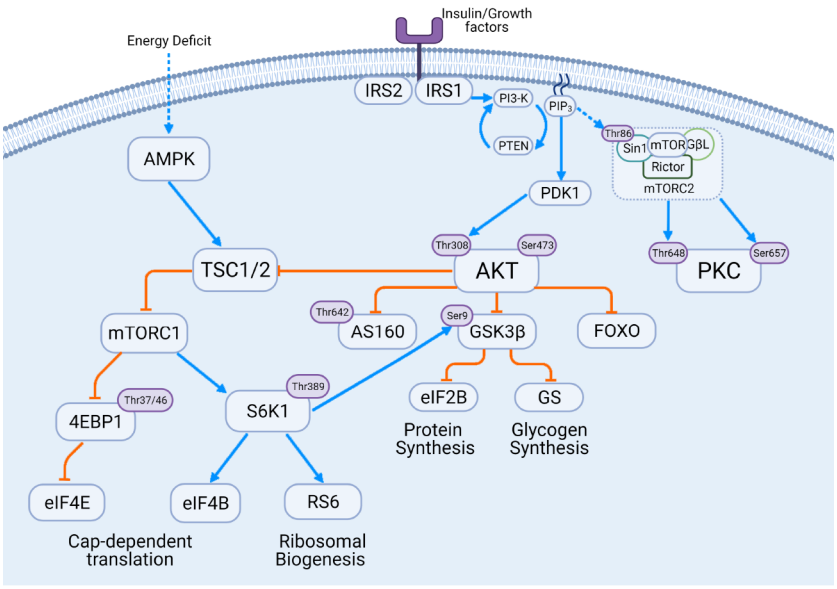

Figure 1 Insulin signaling cascade involving both glucoregulatory and anabolic pathways. Phosphorylation sites of interest indicated on figure. Blue arrows (→) indicate activation of the substrate, orange bars (⊥) indicate inhibitory action on the substrate. Figure created with BioRender.com. mTOR: Mammalian target of rapamycin; mTORC: mTOR complex; S6K1: S6 kinase beta-1; IRS: Insulin receptor substrates; PKC: Protein kinase C; AMPK: AMP-activated protein kinase; TSC: Tuberous sclerosis complex; GSK-3β: Glycogen synthase kinase 3β; PIP3: phosphatidylinositol 3,4,5-triphosphates.

the most abundant isoform in skeletal muscle[14,15,17,18]. AKT is as a critical regulator of insulin sensitive glucose uptake as well as anabolic signaling through mTORC1 making it a prime target in understanding metabolic dysregulation.

The upstream regulation of AKT, in its most simple iteration, appears to be very similar across isoforms. The two common phosphorylation sites of AKT are Ser473 (Ser474 in AKT2) and Thr308. The insulin receptors IRS1 and IRS2 will activate the PI3K-dependent conversion of PIP2 to PIP3, and PIP3 will recruit Pyruvate Dehydrogenase Kinase 1 (PDK1) and AKT to the membrane where colocalization will allow for phosphorylation at the Thr308 by PDK1[12,13]. Further, some evidence suggests that mitogen-activated protein kinase-associated protein 1 (mSin1) of the mTORC2 complex is brought to the membrane by PIP3 (binding with the pH domain) that promotes colocalization of mTORC2 to the membrane[19,20], which is the major kinase for the Ser473 phosphorylation site of AKT.

The regulation of mTORC2 activity by mSin1 phosphorylation is controversial. It has been proposed that PIP3) promotes mTORC2 activity directly[21,22]. Recent work has indicated a positive feedback loop between AKT and mTORC2 *via* phosphorylation of mSin1[23,24]. Those studies in adipocytes and Hela cells indicated that phosphorylation of mSin1 at Thr86 by AKT (*via* Thr308) increased mTORC2 activity and phosphorylation of AKT on Ser473[20,23]. This positive feedback loop provides an avenue for mTORC2 control *via* growth factors; however, the total impact of this feedback loop on mTORC2 activity and downstream substrates like AKT Ser473 is currently unknown. It is well established that PDK1 and mTORC2 are the major kinases involved upstream of AKT and that AKT is involved in a large scale, insulin sensitive pathway, but the distinct actions of these two phosphorylation sites are still not well understood.

There is also considerable debate over what the phosphorylation of specific AKT sites implicates for AKT activity and substrate specificity. Much of the early work in AKT reported a requirement of phosphorylation at Ser473 for full activation[25-28]. However, more recent work in platelets[29], HEK cells[27,30], and skeletal muscle[31,32] demonstrated that not all downstream substrates are impacted by Ser473 phosphorylation. There is some evidence to support that these changes in activity and substrate *via* phosphorylation site may be isoform specific[33,34] but more work needs to be done in this area.

The implications of Ser473 phosphorylation *via* mTORC2 has been studied in various tissues. In mSin1 knockout mouse embryonic fibroblasts, a regulator of mTORC2 complex formation and stability, Forkhead box 01/03 (FOX01/3a) phosphorylation was inhibited but tuberous sclerosis complex 2 (TSC2) and glycogen synthase kinase 3 (GSK-3) phosphorylation was unaffected[35]. In adipose tissue[36] and liver [37], rapamycin insensitive companion of mTOR (RICTOR) knockouts demonstrated

tissue specific differences in mTORC2 substrate specificity. When mTORC2 inhibitors were applied in skeletal muscle, phosphorylation of AKT at Thr308 was unaffected and the downstream phosphorylation of TSC1/2, S6 kinase beta-1 (S6K1) and GSK-3β, all associated with protein synthesis and growth, were also unaffected by the reduction of Ser473 phosphorylation[32]. However AKT substrate of 160 kDa (AS160), an enzyme associated with GLUT-4 translocation and glucose disposal as well as proteins in the FOXO family associated with apoptosis were negatively affected by Ser473 reduction[32]. That work demonstrated that there is some demarcation of substrate specificity within AKT of skeletal muscle. It may also indicate phosphorylation of Thr308 focuses AKT kinase activity towards substrates involved with growth and phosphorylation of Ser473 focuses on substrates involved in glucose regulation and cell survival. Alternatively, substrates unaffected by inhibition or downregulation of mTORC2 phosphorylation of AKT at Ser473 may be phosphorylated by other proteins. For example GSK-3 can be phosphorylated at the same phosphorylation site that AKT does Ser9 by S6K[38] and protein kinase C (PKC)[39]. Despite the alternative theory there is evidence for at least some context-dependent substrate specificity towards AKT's downstream targets. As for whether the activity of AKT is dependent on Ser473 for full activation, a recent study in adipose tissue purports that AKT2 activity is reduced by about 50% for its substrates TSC2, PRAS40, FOX01/3a and AS160[40]. Taken together, there may be argument for some combination of Ser473 impacting substrate specificity and activity, but to our knowledge this has not been validated in skeletal muscle and would need more systematic study in both AKT1 and AKT2 to truly define this regulatory mechanism.

Downstream glucose related substrates

As previously mentioned AKT has various downstream substrates that make the action of this kinase quite diverse in cell function. These substrates include members of the mTOR complexes Pras40 and Sin1, Glucose uptake proteins AS160 and GSK-3, Protein synthesis related Tuberous sclerosis 2, and apoptotic signaling through the FOX0 family. This section will focus on signal transduction related to glucose uptake.

GLUT-4 is the predominant isoform of the GLUT family found in skeletal muscle, and one of insulin's primary metabolic roles is to promote the translocation of GLUT-4 to the surface membrane. AKT has been linked to downstream substrates that impact insulin-dependent GLUT-4 translocation including GSK-3[41] as well as AKT Substrate of 160kd (AS160)[31,42,43] making it a prime target for understanding glucose uptake. GSK-3β is a well-known inhibitor of glycogen synthase, but is also an inhibitor of eiF2B which is a potent regulator of protein synthesis. When GSK-3β is phosphorylated at Ser79 its activity is inhibited, which allows for the activation of both glycogen synthase and eiF2B. Interestingly GSK-3 has been linked to mTORC2 regulation *via* RICTOR phosphorylation at Ser1235 which interferes with mTORC2 binding to AKT[44] and Ser1695[45] which marks RICTOR for degradation. Also been linked to AS160 is a substrate of AKT that contains a Rab-GTPase activating protein and has been associated with regulating glucose transport. In basal conditions AS160 maintains GLUT-4 containing vesicles in the cytosol (intracellular) through its gap domain[46, 47], when insulin is applied AS160 is rapidly phosphorylated which disengages AS160 from the vesicles allowing them to move to the membrane for exocytosis. In skeletal muscle, like fat[43,48], AS160 is phosphorylated in response to insulin in a dose dependent manner[49] and insulin stimulation of GLUT-4 exocytosis is dependent on AS160 phosphorylation[48]. AS160 can be phosphorylated by other proteins including AMP-activated protein kinase (AMPK) making it part of both insulin dependent and insulin independent translocation of GLUT-4[31,50].

Anabolic signaling

AKT phosphorylates TSC2 at Thr1462 which regulates the tuberin-hamartin complex and it's activity[51-53]. Phosphorylation at this site releases the tuberin-hamartin complex inhibition of the mTORC1 complex and allows for downstream targets to be phosphorylated[51]. mTORC1 is a prolific kinase with multiple downstream substrates, but Ribosomal protein S6K1 and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) are arguably the most well-known downstream targets. 4E-BP1 is known as a translation repressor protein because it inhibits cap-dependent mRNA translation by binding to peptide-chain initiation factor eIF4E. Phosphorylation of 4E-BP1 disrupts the interaction of 4E-BP1 and eIF4E, releasing it so that it may participate in translation by chaperoning specific cap-dependent transcripts to the translation apparatus[54]. S6K1 is best known for its action on ribosomal protein S6 (S6) which is involved in the translational control of 5′ oligopyrimidine tract (5′-TOP) mRNAs[55]. Phosphorylation of S6K1 at Thr389 is known to be critical for function of

the protein[55], as well as correlated with kinase activity *in vivo*[56]. The subsequent phosphorylation of S6 ribosomal protein correlates with increases in translation of cap-dependent proteins, that are necessary for the manufacture of ribosomal machinery and peptide-chain elongation factors necessary for mRNA translation[57,58]. The regulation of S6K1 activity is diverse but S6K1 activation has been shown to be elevated by hyperglycemia[59], hyperinsulinemia[60], and high fat diet in muscle and adipose tissue[61].

INSULIN SIGNALING AND DIABETES

It is generally agreed that glucose transport is the rate limiting step of glucose uptake, and the step most impacted by the progression of T2D. The consensus in diabetes research at large is that the translocation or trafficking of glucose transport molecules in skeletal muscle is impaired in T2D[43,62] but the culprit behind this impairment is still widely debated. In skeletal muscle GLUT-4 is the predominantly expressed isoform[63,64] and the localization of GLUT-4 has been confirmed with insulin[65], exercise[65,66] and hypoxia[67]. The first important finding with diabetes is that the limitation in glucose transport cannot be explained by production or maintenance of the GLUT itself, because total GLUT-4 protein is largely unchanged with diabetes[68-70]. This implies that the issue is not related to GLUT-4 expression, *per se*, but within the signaling cascades that assist in the translocation of GLUT-4 to the surface membranes.

As the initial step in the insulin signaling cascade, the insulin receptor was a primary target of research related to the breakdown of the glucoregulatory signals. While current data are conflicting on IR activity with some reporting impairment[62,71,72] and others reporting normal activity[73-77], it appears that the important signaling 'defects' of T2D are further down the signal cascade. Signaling defects in IRS1 phosphorylation[73,77-79] and PI3K[73,77,78,80,81], activity are consistently found in the diabetic model. More controversial is the activity of AKT with studies reporting significant reductions of insulin stimulated AKT phosphorylation on Ser473 or Thr308[69,75,82,83]. While others report not impact of T2D on insulin dependent phosphorylation[80,81]. Downstream substrates of AKT have also been presented in the diabetic model with reduced glycogen synthase activity with protein levels of GSK-3 reported as being elevated which would inhibit GS activity[84]. Additionally, insulin dependent phosphorylation of AS160 has also been reported to be higher in T2D[42], despite the fact that AKT phosphorylation was not different in the same study.

Despite the continued exploration and detailed understanding of what the signaling cascades are doing during diabetes, there is still no consensus on where these dysfunctions are originating. Molecular mechanisms that underlie this dysfunction of glucoregulatory processes associated with T2D as outlined above have been studied extensively, but the interaction of glucoregulatory processes with those of protein metabolism (protein turnover) are still lacking, despite the evidence that the two processes may be dependent on one another.

It is well documented that muscle mass and strength decline with T2D[85,86] and contribute to a decline in quality life over time. Interestingly despite a loss in muscle mass, there appears to be an upregulation of protein synthesis and the anabolic signal cascade in diabetic muscle[87,88]. Previously, studies assessing anabolic responses [fractional synthesis rate (FSR)] in diabetic skeletal muscle have been inconsistent, ranging from decreased[89,90], to normal[91,92] but more recently increased FSR has been confirmed by our lab[87,88,93,94] and others[95,96]. In Fatty Zucker rats, a well-documented model for T2D, upregulated protein synthesis in specific muscle fractions and increased phosphorylation of S6K1 were observed despite an overall decrease in muscle mass. This upregulation of S6K1 appears to be linked to a loss of control of upstream mTOR activation. While the hyperactive mTOR activity may be a result of the maintained state of hyperinsulinemia with glucose intolerance, we suspect something much more sinister for the progression of diabetes.

Our recent studies have demonstrated that the constitutive activation of mTOR may be a result of suppressed DEPTOR expression in the diabetic state. DEPTOR is one of the mTOR associated binding partners that can be a part of either mTORC1 or mTORC2 and is a negative regulator of mTOR activity. Similar to several lines of cancer[97]. DEPTOR is substantially lower in obese subjects[87,88]. Since DEPTOR is still a fairly new discovery in the mTOR signaling cascade, the implications of low DEPTOR and the regulation of mTORC1 are still speculative but the low DEPTOR

appears to allow the downstream anabolic signals to go unchecked[98] which has implications for mRNA translation[99], as well as glucoregulatory signaling cascades. This is unbridled mTORC1 activity without concomitant muscle mass accretion is indicative of high protein turnover[88], where it may not be warranted or wanted. It is also an important bridge between mTORC1 and mTORC2 which will be discussed in a later section.

CONNECTING ANABOLISM TO INSULIN RESISTANCE

A relatively recent but important discovery in the connection of anabolic and glucoregulatory signaling paths is an inhibitory pathway that directly links S6K1 to IRS1. IRS1 can be serine phosphorylated through many pathways including c-Jun NH2-terminal kinase, IkB kinase, PKC, and S6K1[100,101]. It is now known that the insulin receptor contains multiple phosphorylation sites[102] and even in a basal state it is highly phosphorylated[103]. Ser/Thr phosphorylation of IRS-1 has been linked to the degradation of IRS1 itself and the downstream signaling needed for glucose uptake. While the patterns and requirements of these phosphorylation's for the downstream signal disruption are still undefined it has been clearly demonstrated that chronic exposure of cells to insulin results in degradation of IRS-1 protein[104-106]. It was later found that AKT mediated the Ser/Thr phosphorylation of IRS-1 and that this was inhibited by rapamycin[107]. More specifically IRS1 phosphorylation at Ser307 and Ser636/639 were observed in moments of increased mTORC1 activation and this increase was absent in mice that were S6K1 deficient[61]. In support of this constitutive activation of S6K1 lead to IRS1 phosphorylation and degradation as well as inhibition of IRS-1 transcription[108,109]. It is now a well-supported conclusion that IRS1 phosphorylation by S6K1 (Figure 2), decreases insulin signaling through the insulin receptor substrate[61,100,103,110,111]. This critical role is highlighted in the elevated levels of activation in liver adipose and muscle of obese animals[61,87,88,112] and is further supported by S6K1 deficient mice being protected against diet-induced obesity and insulin resistance[61]. This clearly links mTORC1 and more specifically S6K1 to the general insulin signaling cascade making it a target molecule for alteration of insulin signaling.

While we are gaining perspective in the current literature about the interaction between mTORC1 signaling for protein synthesis and the disruption of insulin signaling for glucose disposal in skeletal muscle, far less is known about how the two mTOR complexes interact in this process. While the S6K1 connection to IRS1 is now fairly accepted, S6K1 also appears to have a role in the cross-talk between the two mTOR complexes that is not yet well defined but thought to play a role in insulin resistance. To date, very little is known about the regulation of mTORC2[113] despite its role in phosphorylation of AKT at Ser473. The role of AKT and its regulation through Ser473, both upstream and downstream is still quite controversial in the literature as discussed earlier in section 2.1 AKT/protein kinase B (PKB), despite its being a widely used marker of AKT activity[25-27]. The downstream targets of AKT include various substrates involved in glucose uptake so the choice of this important intermediate as a marker seems obvious; however, the interpretation of what phosphorylation of AKT at Ser473 truly implies remains ambiguous.

The mTORC2 complex is best known for its involvement in cell survival but is known to phosphorylate AKT through Ser473[25,114-117] as well as the PKC family [40,116-119]. This complex is composed of binding partners mSin1, DEPTOR, Protor1, mLST8 and RICTOR. While all of these binding partners play roles in mTORC2 activity, the RICTOR has currently demarcated mTORC2's role in signal transduction [25]. RICTOR aids in localization of mTOR to the plasma membrane as well as the binding of mSin1 to the mTORC2 complex[19], making it an important binding partner worthy of the interest it has received. While mTORC2 has been established as the kinase responsible for phosphorylation of AKT at Ser473 the mechanism behind this phosphorylation is controversial. Two binding partners, RICTOR and Sin1, have been established as important regulators of mTORC2 complex activity, and of interest is that both of these binding partners appear to be regulated by S6K1. RICTOR is prone to phosphorylation[114,120,121] and that phosphorylation may impact downstream targets like AKT, as indicated by phosphorylation at Ser473[115,122].

Work by others indicated that the muscle-specific deletion of RICTOR led to decreased Ser473 phosphorylation of AKT and was accompanied by reduced phosphorylation of AS160 at Thr642 and overall glucose intolerance[123]. That work lead to speculation that regulation of RICTOR through phosphorylation was responsible for

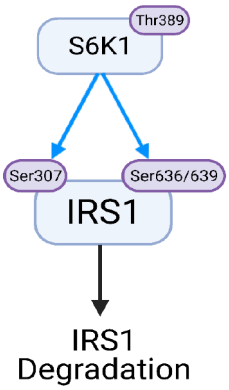

Figure 2 Downstream mammalian target of rapamycin complex 1 substrate S6 kinase beta-1 phosphorylation of insulin receptor substrates 1 at Ser307 and Ser636/639 leads to insulin receptor substrates 1 degradation. Blue arrows (→) indicate activation of the substrate, black arrow (→) indicates degradative pathway. Figure created with BioRender.com. S6K1: S6 kinase beta-1; IRS: Insulin receptor substrates.

the increases or decreases in Ser473 phosphorylation[115,122], and the concomitant responses of insulin-stimulated glucose homeostasis. Others determined that the phosphorylation of RICTOR at thr1135 (Figure 3) was responsible for inhibition of kinase activity toward AKT at Ser473[119,122,124,125]. Phosphorylation of RICTOR at Thr1135 was sensitive to both growth factors and rapamycin[124] and was the direct target, established through silencing and pharmacology, of S6K1[119]. Although the evidence connecting S6K1 to RICTOR regulation is compelling, the functional consequences of this phosphorylation are controversial. Some studies have indicated that this phosphorylation is a direct regulator of mTORC2 activity towards AKT[119,122], while others report no alteration in mTORC2 activity[124,125]. It must be noted that different experimental models were used across these studies, so it is possible that some of the differences observed were due to the differences in genetic models used to arrive at those conclusions. Despite those discrepancies, the S6K1-RICTOR interaction further supports the concept of crosstalk between the insulin glucoregulatory and protein synthesis pathways, as implicated by data demonstrating that mTORC1 regulation is important for Ser473 regulation. With mTORC1 and S6K1 activity being upregulated with diabetes, this connection to the insulin signaling pathways and the direct control mTORC1 may be critically important for further understanding of the metabolic dysregulation of T2D.

RESISTANCE EXERCISE

Exercise and physical activity are effective, low cost interventions for insulin resistance and T2D[126,127]. The benefits of aerobic exercise on glucose tolerance are well established[128-132] and the improvements are independent of improvements in general condition[132]. However many people with T2D are overweight and/or obese, have mobility issues and other neuropathies making aerobic-type exercises difficult to accomplish[133,134]. Resistance exercise has been proposed as a more feasible activity when aerobic exercise is inaccessible and there is a growing body of evidence to support that this form of exercise can be beneficial with regard to glucose tolerance [135,136]. Much of this work attributes the glucoregulatory improvements following resistance training are due to increased muscle mass[2,137,138] which may or may not be applicable to T2D. Additionally, acute resistance exercise appears to increase insulin clearance without a change in glucose tolerance[139], which was originally attributed to increases in insulin sensitivity *via* receptor number or a greater liver or tissue clearance following exercise.

It is often speculated that insulin-resistant skeletal muscle is desensitized or 'resistant' to the anabolic actions of exercise[88,140,141], making it difficult to achieve gains in muscle mass. Given the aforementioned hyperactivation of mTOR with insulin resistance, the current theory is that the 'anabolic resistance' observed with diabetes/obesity may really be due to an "anabolic ceiling" in skeletal muscle that has been achieved in the hyper-insulinemic state. In healthy tissue. resistance exercise is a potent stimulator of rates of protein synthesis in muscle and repeated bouts of resistance exercise lead to skeletal muscle hypertrophy[142]. It has also been established that insulin is a necessary component in elevated protein synthesis rates after re-

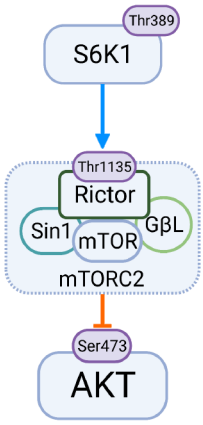

Figure 3 Downstream mammalian target of rapamycin complex 1 substrate S6 kinase beta-1 is the primary kinase responsible for phosphorylation of the mammalian target of rapamycin complex 2 component Rictor at Thr1135 which has been implicated in phosphorylation of AKT at Ser473. Blue arrow (→) indicates activation of the substrate, orange bar (⊥) indicates inhibitory action on the substrate. Figure created with BioRender.com. mTOR: Mammalian target of rapamycin; mTORC: mTOR complex; S6K1: S6 kinase beta-1.

sistance exercise and it is the combination of resistance exercise and insulin that causes this modulation[143,144]. This effect of insulin appears to be through a rapamycin sensitive pathway[145-148] at least in healthy unperturbed tissue, but en-gaging in a moderate to high intensity exercise bouts involving eccentric muscle actions lead to a transiently-reduced capacity of insulin to elevate glucose uptake[149,150]. The mechanisms behind this alteration are still not well defined, but speculation includes a diminished capacity for glycogen synthesis and reductions in GLUT-4 protein which may be fiber type specific[150]. Further, as noted above, there are circumstances where the activation of protein anabolism requires S6K1 activation, which may feedback on upstream signals that impair glucose uptake by insulin[61,87,88,111]. More work is warranted to better define these mechanisms.

Aside from insulin sensitivity, there are benefits to regular exercise, whether it is of an aerobic or anaerobic nature. It is important to note here that there are insulin independent pathways that trigger glucose uptake that are directly related to skeletal muscle contraction. This pathway is triggered by muscle contraction and involves a distinct subset of GLUT-4[66,151-153]. These pathways can involve nitric oxide[154] and activation of AMPK[155,156] as well as cytosolic calcium[130] but these effects are distinct and additive to those of insulin mediated glucose uptake[2,157-159]. Probably most important for T2D research is that these contraction mediated glucose pathways are not only present in T2D but are fully functional[160,161].

Interestingly, in insulin resistant muscle there seems to be a difference in the control of muscle protein synthesis. It appears that in tissue where the upstream activators of the mTORC1 pathway are impaired there are alterations to the use in protein synthesis. Unlike their lean counterparts obese Zucker rats administered insulin had augmented rates of muscle protein synthesis and that these actions persisted in the presence of rapamycin[94]. This suggest that the rapamycin sensitive mTORC1 pathway is not responsible for the increased muscle protein synthesis rates observed.

One key player that may have an impact on muscle protein synthesis in response to insulin is a serine/threonine kinase called PKC. PKC has long been considered as a regulatory contributor during mRNA translation in a number of tissues[162,163] but more recently specific isoforms of PKC have been implicated in the regulation of glucose uptake. Specifically, the conventional family of PKCs (α, β, γ) lead to attenuated insulin receptor tyrosine kinase and PI3K activity[164,165] which leads to reduced glucose disposal. It has been discovered that in diabetic tissue, when insulin complexes with its receptor PKC is activated which then impairs downstream insulin signal[93]. This phenomenon is not observed in muscle from lean humans who have normal glucose response, mirroring the observed changes in insulin induced protein synthesis not present in lean counterparts[94]. Additionally inhibition of PKC activity through pharmacology has been demonstrated to partially restore signal transduction and glucose disposal in otherwise insulin resistant muscle[164,166]

The regulation of PKC, like many of the enzymes related to insulin signal transduction and glucose uptake is complex. It is known that PKCα is a downstream substrate of mTORC2 at both its turn motif (Thr638) and is hydrophobic motif (Ser657) both of which are required for PKCα stability[40,116-119]. Deletion of RICTOR, abo-

lishes phosphorylation of the hydrophobic motif of PKCα[114,115] and deletion of either RICTOR or Sin1 dramatically reduces PKCα protein content[117], implicating that RICTOR, a component of mTORC2, plays a role in PKC activation much like it does for the activation of AKT at Ser473. This draws mTORC2 further into the complex crosstalk that impacts insulin signaling and provides a feasible opportunity for mTORC2 to assist in the bypass of normal insulin signaling with the upregulation of PKC. It is important to note that PKC activation does not rely on mTORC2 however because it can also be activated by Diacylglycerol[117] which would be high in the obese state.

CONCLUSION

Dysregulation of mTOR signaling is a key player in the development of many disease states including diabetes. While decades of research have been dedicated to understanding the insulin signaling cascade, many aspects of its regulation and control remain elusive. It is becoming clear that crosstalk between the two mTOR complexes is adding considerable complexity by impacting both hormone-mediated glucose uptake and the underlying pathogenesis of this disease. This emerging evidence now blurs their roles and responsibilities of fixtures in protein homeostasis. Research in this area has focused on specific culprits in the glucoregulatory pathway that are thought to cause the manifestation of the disease, but with all of the newly emerging anabolic/glucoregulatory cross talk that are involved with the manifestation of this disease, it is possible that the factors once viewed as culprits for this disease may actually be the consequence of anabolic/glucoregulatory cross talk. These recent findings offer exciting new targets for the control of insulin resistance.

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REVIEW

Alcoholic liver disease: Current insights into cellular mechanisms

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Abstract

Alcoholic liver disease (ALD) due to chronic alcohol consumption is a significant global disease burden and a leading cause of mortality. Alcohol abuse induces a myriad of aberrant changes in hepatocytes at both the cellular and molecular level. Although the disease spectrum of ALD is widely recognized, the precise triggers for disease progression are still to be fully elucidated. Oxidative stress, mitochondrial dysfunction, gut dysbiosis and altered immune system response plays an important role in disease pathogenesis, triggering the activation of inflammatory pathways and apoptosis. Despite many recent clinical studies treatment options for ALD are limited, especially at the alcoholic hepatitis stage. We have therefore reviewed some of the key pathways involved in the pathogenesis of ALD and highlighted current trials for treating patients.

Key Words: Liver; Alcohol; Oxidative stress; Inflammation; Gut microbiome; Mitochondria

Core Tip: Alcoholic liver disease (ALD) causes significant global disease burden inducing both cellular and molecular modifications in hepatocytes. Although the spectrum of disease is widely recognized, the precise disease pathogenesis is yet to be fully elucidated. In this review we summarize some of the key pathways responsible for the pathogenesis of ALD and highlight current available treatments.

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the most prevalent chronic liver diseases and causes significant mortality globally. Chronic alcohol consumption has been implicated in multiple medical conditions including cancer, diabetes, cardiovascular disease, liver and pancreatic disorders. However, no new treatment options have been developed for many years. At present, abstinence remains the most important treatment for ALD, however, there is a need to develop effective treatment options associated with alcohol misuse.

The spectrum of ALD is widely recognized ranging from simple liver steatosis; which can occur in up to 90% of heavy drinkers[1] to alcoholic hepatitis (AH), which develops in 10% to 35% of heavy drinkers[2]. AH can ultimately progress to fibrosis, where hepatic stellate cell (HSC) activation, collagen synthesis and accumulation of extracellular matrix proteins occurs due to the formation of protein adducts. Cirrhosis and lastly hepatocellular cancer are the final stages. Fibrosis/cirrhosis can cause hepatocyte inactivation, and is associated with abnormal DNA repair, damage to mitochondrial function and oxygen utilization disorders[3]. This can lead to hepatocellular failure and portal hypertension which ultimately requires a liver transplantation.

The molecular and biochemical mechanisms underlying the pathogenesis of ALD as well as the precise triggers for disease progression are not completely understood. Recent evidence suggests disease progression is thought to involve several pathological stages such as mitochondrial dysfunction, oxidative stress, altered methionine metabolism, iron dysregulation, gut dysbiosis, activation of inflammatory pathways and decreased production of antioxidants (Figure 1)[4]. Currently, no effective treatment for ALD exists due to the incomplete understanding of hepatic biochemical alterations and pathogenic mechanisms responsible for disease progression.

EPIDEMIOLOGY AND PREVALENCE

Alcohol misuse is a leading cause of liver disease worldwide. ALD is a global disease burden and results in approximately 3 million deaths *per* year[5]. In 2016, 5.3% of all deaths were caused by the harmful effects of alcohol worldwide[5]. Alcohol accounts for 5.1% of total disease burden worldwide, measured in disability-adjusted life years [5]. Total alcohol consumption *per* capita increased in 2005 from 5.5 L to 6.4 L in 2010 and was sustained at 6.4 L in 2016[5]. Europe has the highest *per* capita alcohol consumption and disability-adjusted life years[5], as well as binge drinking particularly, in France and England[6].

Although the overall prevalence of ALD has remained stable from 2001-2016 at between 8.1%-8.8%, the proportion of ALD with stage 3 fibrosis and above has increased from 2.2%-6.6%[7], such that in the United States chronic liver disease and cirrhosis is the 12th leading cause of death[7]. In the United States, the number of adults listed as waiting for liver transplants also increased by 63% from 2007 to 2017[7], where approximately one third of all liver transplants are due to alcohol-related disease[8]. Due to the high-risk drinking rates, the number of deaths due to alcohol-related liver disease in the United States is projected to increase by 84% from 2019-2040 [9]. In the United Kingdom, the number of deaths from ALD was reported at 5964 deaths in 2020 compared to 4954 deaths in 2019, increasing by 20%[10]. From 2001 the number of deaths due to ALD has increased by 72% from 2001-2020[10].

There are several factors which effect the development of ALD. There are significant differences in the amounts of alcohol consumed between males and females. It is well documented that men consume more alcohol than women, leading to a higher prevalence of alcohol related liver disease[11]. It is estimated 237 million men and 46 million women have alcohol use disorders[5]. Although men often consume more alcohol, women are more susceptible to the toxic effects of alcohol and have a higher risk of advanced liver disease[11], due to higher blood alcohol concentrations despite equal dosing between genders[11]. Therefore, female gender is an important risk factor for progression of ALD. Genomic data has also discovered that a variation in the *PNPLA3* gene is associated with increased hepatic fat content, increasing the risk of both ALD and non-alcoholic fatty liver disease, which has the highest frequency in Hispanics[12].

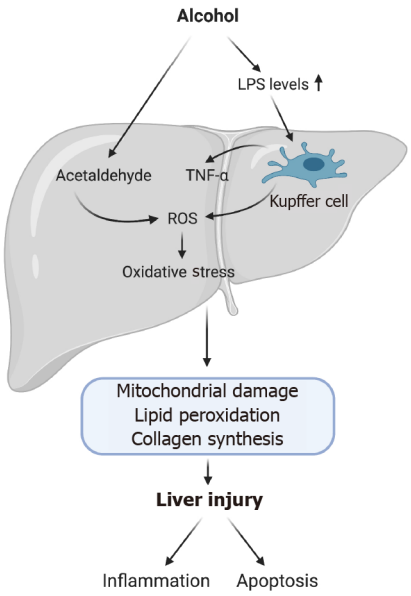

Figure 1 Alcohol-related induction of oxidative stress and liver injury. Alcohol misuse leads to loss of tight junctions in the gut increasing its permeability. This causes translocation of lipopolysaccharide into the liver activating toll-like receptor 4 on Kupffer cells (KCs). Activation of KCs can cause reactive oxygen species (ROS) production and pro-inflammatory cytokines such as tumor necrosis factor-α. ROS production also occurs due to the metabolism of alcohol. ROS production and inflammatory cytokines leads to inflammation and recruitment of inflammatory cells as well as activation of apoptotic pathways. (Figure created with BioRender.com). LPS: Lipopolysaccharide; ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-α.

DISEASE SPECTRUM

Sustained excessive alcohol consumption produces a vast range of hepatic lesions. The first stage is liver steatosis or alcoholic fatty liver, which can occur in up to 90% of heavy drinkers, emerging as early as 3 to 7 d after initial excessive alcohol consumption[1]. Steatosis is often asymptomatic with normal or only slightly increased liver enzymes levels. The deposition of microscopic fat droplets occurs initially in the centrilobular zone then spreads towards the periportal region of hepatocytes[13]. Steatosis, although reversible and originally thought to be a benign state, is a now a priming phase for AH, which develops in 10% to 35% of heavy drinkers and is a more severe stage of ALD. AH is characterized by hepatocyte ballooning, the formation of Mallory-Denk bodies, infiltration of white blood cells, Kupffer cell (KC) activation, and collagen deposition *via* once dormant HSCs[14], the latter playing an important role in fibrosis leading to cirrhosis. At this stage hepatocyte inactivation, abnormal DNA repair, damage to mitochondrial structures, oxygen utilization disorders, and the accumulation of extracellular matrix proteins occurs[3]. Continuation of hepatic scarring and the spread of collagen (bridging fibrosis) throughout the liver can lead to cirrhosis and in some cases hepatocellular cancer.

Although the pathogenesis of ALD is yet to be fully understood, it is thought to include multiple interplaying factors and pathways including the production of toxic ethanol metabolites, oxidative stress, innate and adaptive immune activation, fibrogenesis and cell death. Upon activation of these pathways tissue damage can occur leading to the progression of the disease. This review will focus on mechanisms involved in inflammation that predominantly occur at the AH stage.

ALCOHOL METABOLISM

Within the liver, alcohol dehydrogenase and cytochrome p450 2E1 (CYP2E1) are the main oxidative pathways of alcohol metabolism. Another minor pathway of alcohol metabolism in the liver is *via* the peroxisomal enzyme catalase[15]. A small proportion of ethanol may also be metabolized by non-oxidative pathways such as by interaction with fatty acids, generating fatty acid ethyl esters[16]. Alcohol dehydrogenase oxidatively metabolizes alcohol to acetaldehyde, a highly reactive and toxic by product that contributes to tissue damage. This conversion reaction requires the cofactor nicotinamide adenine dinucleotide (NAD^+), creating reduced NAD^+ in the process. Due to the toxic nature of acetaldehyde, it is further oxidized to acetate, catalyzed by the

enzyme mitochondrial aldehyde dehydrogenase-2[17]. Increased conversion of NAD^+ results in leakage of electrons and reactive oxygen species (ROS) production. The toxic metabolites produced during alcohol metabolism as well as increased ROS can also trigger endoplasmic reticulum (ER) stress. The second major pathway is *via* the microsomal ethanol-oxidizing system and involves CYP2E1, which is involved in ethanol oxidation to acetaldehyde[17]. The activity of CYP2E1 is induced by alcohol, increasing its hepatocellular content causing accumulation of CYP2E1[18]. Electron leakage from the CYP2E1 pathway, leads to ROS generation, including hydroxyethyl, superoxide anion and hydroxyl radicals[19]. ROS can also form lipid peroxides and DNA adducts such as N2-ethyldeoxyguanosine, which has been detected both in the livers of alcohol-fed rats as well as leukocytes in ALD patients[20].

AUTOPHAGY, MITOPHAGY AND INFLAMMASOMES

Alcohol metabolism increases ROS production and ER stress leading to calcium depletion, glycosylation and lipid overloading, triggering the unfolded protein response (UPR)[21]. The UPR can restore ER homeostasis by attenuating translation of proteins, increasing folding capacity and degrading unfolded proteins. However, a prolonged UPR causes inflammation, fat accumulation, mitochondrial stress and apoptosis *via* direct activation apoptosis signal-regulating kinase 1[22], nuclear factor-κB (NF-κB), c-Jun N-terminal kinases and P38[23]. Alcohol induced ER stress involving an impaired UPR was first identified in a model of intragastric alcohol fed mice [24].

Alcohol can induce autophagy, a self-degradative process which occurs by the action of lysosomes and can be selective only for damaged mitochondria (mitophagy). Evidence suggests that autophagy in ALD can have inhibitory effects on inflammation and steatosis as well as the ability to remove lipid droplets, Mallory-Denk bodies and damaged mitochondria[25]. Whilst tumor necrosis factor (TNF)-α induces autophagy, generation of ROS can lead to inhibition of TNF-α induced autophagy through activation of NF-κB[26]. This suggests dysfunction of autophagy is associated with ALD pathogenesis[27,28]. Multiple animal models have observed autophagy as a protective response in ALD, as well as confirming ameliorative effects in ALD. Acute ethanol feeding (6 g/kg bodyweight) increased autophagy as measured by autophagosome numbers, however, chronic ethanol feeding (5.2% ethanol by volume) inhibited hepatic autophagy[29], suggesting that this protective mechanism is lost with longer term alcohol consumption.

Mitophagy can also be induced as a protective response to both acute and chronic alcohol consumption due to accumulation of ROS or loss of mitochondrial membrane potential *via* elimination of dysfunctional mitochondria. The process of mitophagy depends on induction of autophagy and priming of damaged mitochondria for recognition and is mediated by phosphatase and tensin homolog-induced putative kinase 1-Parkin signalling pathway or Nip3-like protein X[30]. The duration of alcohol exposure can affect the mitophagy process[30]. In rats, binge-models of alcohol consumption have been shown to increase mitophagy, decreasing alcohol-induced hepatoxicity[30,31]. Acute ethanol consumption also increases transcription factor EB, a master regulator of lysosomal biogenesis, however, chronic ethanol exposure decreased transcription factor EB[29]. Accumulation of damaged mitochondria occurs in chronic ethanol models which releases mitochondrial damage-associated molecular patterns (DAMPs), which in turn promote inflammation and fibrogenesis contributing to accelerated disease state[30]. Mitochondrial DNA (a mitochondrial DAMP), can bind to toll-like receptor (TLR)-9 activating HSCs and fibrogenesis[31]. Therefore, targeting mitophagy may be a potential therapeutic for ALD.

Inflammasomes

Oxidative stress in response to alcohol metabolism can damage hepatocytes, releasing endogenous DAMPs. Recognition of DAMPs can induce inflammation by release of proinflammatory cytokines, immune cell localization and stimulation of the inflammasome[32]. Inflammasomes are expressed in hepatic cells and are multi-protein complex's containing a nucleotide-binding oligomerization domain-like receptor (NLR). Inflammasome activation is thought to be a two-step process. Inflammasome sensor molecules can trigger the assembly of inflammasomes, including NLR molecules, for example NOD-, LRR- and pyrin domain-containing 3 (NLRP3)[33]. Assembly is initiated by TLR and pathogen-associated molecular pattern (PAMP)/DAMP signaling which results in the NLR forming complexes with pro-caspase 1 with or

without an adaptor molecule, apoptosis-associated speck like CARD-domain containing protein (ASC)[4,33]. Inflammasome assembly initiates cleavage of procaspase-1 to its active form caspase-1. Activated caspase-1 then promotes the secretion and activation of pro-inflammatory cytokines *via* cleavage of pro-interleukin (IL)-1β and pro-IL-18 into their active forms IL-1β and IL-18[33]. IL-1β plays an important role in the infiltration of immune cells and IL-18 is important for the production interferon-gamma (IFN-γ)[33]. Inflammasome activation also leads to a pathway of cell death called pyroptosis.

NLRP3 can be activated by a variety of stimuli including bacterial toxins, mitochondrial dysfunction and production of ROS. Interestingly, increased levels of IL-1β, ASC and NLRP3 have been documented in the livers of ethanol fed mice, whereas elevated mRNA expression of IL-1β, IL-18 and caspase-1 has been documented in the liver of ALD patients which correlated with liver lesions[34] and has also been associated with the development of liver fibrosis. Blocking IL-1β activity strongly decreases liver inflammation and damage[35]. Higher levels of serum IL-1 has also been documented in patients with AH in comparison to healthy controls[36]. Caspase-1 knockout mice have also been shown to be protected from fibrosis as well as treatment with IL-1 receptor antagonist has been shown to attenuate steatosis and liver injury when administered 2 wk post-ethanol feeding[36]. Decreased inflammation, steatosis and IL-1β expression has been associated in NLRP3 deficiency. Research has also shown mice deficient in NLRP3 have protection against ethanol-induced inflammation including attenuation of steatosis and liver injury[37]. Previous research has also shown inflammasome components such as NLRP3 and ASC are present in HSCs and are required for the development of liver fibrosis by inducing changes including upregulation of transforming growth factor-β and collagen[38]. Therefore, this demonstrates the importance of IL-1β signaling, inflammasome components and activation in ALD.

INNATE AND ADAPTIVE IMMUNITY

Gut permeability

Increases in gut permeability due to alcohol has been confirmed in both clinical and experimental studies[39,40]. Increased gut permeability enables the entrance of PAMPs, such as lipopolysaccharide (LPS) in the portal circulation[41]. LPS is one of the exogenous ligands for TLR4, a pattern recognition receptor found on KCs. LPS interaction with TLR4 initiates downstream signaling *via* TIR-domain-containing adapter-inducing IFN-β and IFN regulatory factor 3, leading to production of proinflammatory cytokines proinflammatory cytokines such as TNF-α and IL-1β (Figure 2), the former can activate the extrinsic pathway of apoptosis *via* the TNF receptor 1 and TNF receptor 2 signaling[42]. High levels of these death receptors, including Fas, are expressed in all liver cells, therefore, the extrinsic pathway is the main apoptotic pathway in hepatocytes, such that hepatocyte apoptosis has been correlated with severity of disease in AH[43]. Serum TNF-α and IL-6 are also increased after exposure to alcohol and LPS[44], with TNF-α correlating with liver injury and mortality. However, chronic ethanol exposure to TNF-α knockout mice does not cause alcohol-associated inflammation and liver injury[45], therefore, mechanisms to reduce TNF-α may be an important tool in preventing inflammation.

Natural killer cells

LPS and HSCs can directly interact with immune cells such as natural killer (NK) cells, natural killer T (NKT) cells and T cells leading to disease progression[46]. NK cells can kill activated HSCs *via* TNF-α related apoptosis, however, HSCs isolated from ethanol-fed mice showed reduced sensitivity to NK cell killing[46]. Alcohol consumption also enhances splenic NK cell apoptosis and blocks NK cell release from the bone marrow, as well as accelerating progression to fibrosis due to reduced NK cell activity[46]. Gut-derived bacteria such as LPS may influence the activation of hepatic NKT cells leading to induction of HSCs and apoptosis, further exacerbating liver injury. Patients with ALD have shown a decreased number of circulating NK cells along with reduced cytotoxic activity resulting in decreased anti-viral, anti-fibrotic, and anti-tumor effects which can contribute to accelerated progression of disease state[47]. However, NK/NKT cells may inhibit fibrosis through deletion of activated HSCs and production of IFN-γ[48]. On the other hand, activation of NKT cells also promotes liver fibrosis *via* enhancing hepatocellular damage and promoting HSC activation[48]. Therefore, a balance is required between inhibitory and stimulatory effects for liver health.

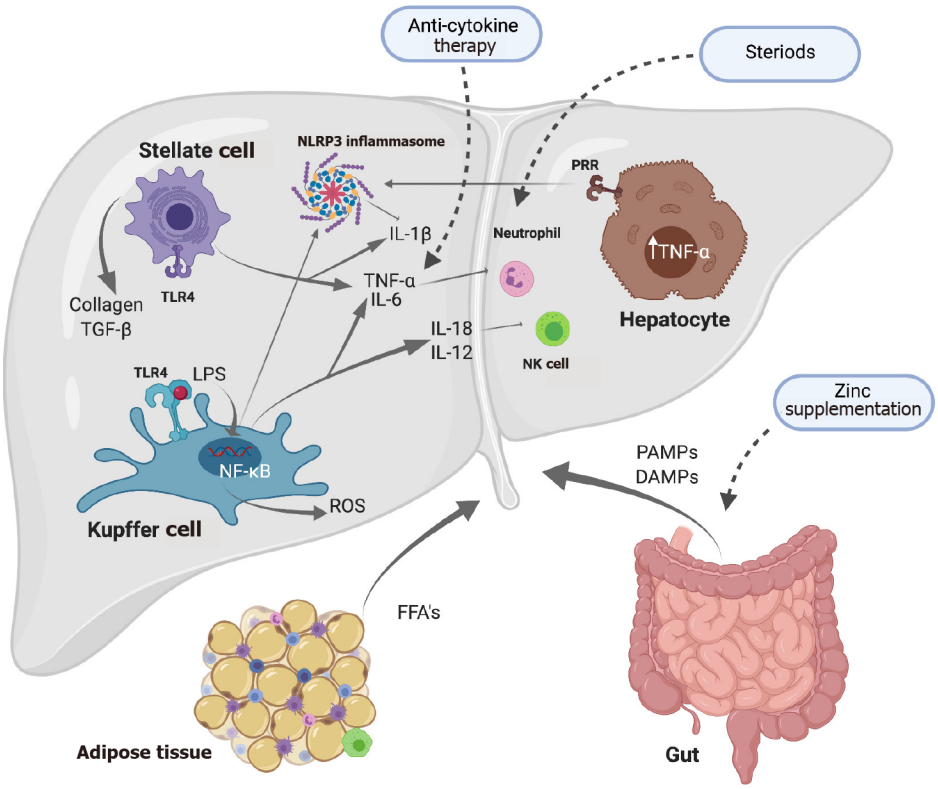

Figure 2 The inflammatory response during alcoholic liver disease. Excessive consumption of alcohol causes lipopolysaccharide release from the gut activating toll-like receptor 4 on Kupffer cells (KCs). Pattern recognition receptors become activated by pathogen-associated molecular patterns/damage-associated molecular patterns which induces inflammation *via* release of proinflammatory cytokines and inflammasome activation. Interleukin (IL)-18 production from KCs causes activation of natural killer cells. Toll-like receptor stimulation in hepatic stellate cells results in the expression of IL-6, transforming growth factor-β1 and tumor necrosis factor-α. (Figure created with BioRender.com). DAMPs: Damage-associated molecular patterns; FFA: Free fatty acids; PAMPs: Pathogen-associated molecular patterns; IL: Interleukin; LPS: Lipopolysaccharide; NF-κB: Nuclear factor-κB; NK: Natural killer; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; PRR: Pattern recognition receptor; ROS: Reactive oxygen species; TGF-β: Transforming growth factor β; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α.

Neutrophil activation

Neutrophils can be recruited in response to liver injury to form neutrophil extracellular traps (NETs), which generate ROS and undertake phagocytosis. Acute alcohol consumption leads to neutrophil imbalance in the liver releasing spontaneous NETs [49]. The scavenging ability of macrophages to eliminate NETs diminishes resulting in persistent inflammation *via* hepatocyte damage[49]. During AH, the infiltration of neutrophils is believed to occur *via* the activation of KCs, which recruit cytokines and chemokines including IL-8 and IL-17. In mouse models, blockade of inflammatory mediators such as IL-8 and IL-17, which are necessary for neutrophil infiltration, can ameliorate liver disease[50,51], which supports neutrophil dysfunction in disease progression. Patients with ALD have a decreased baseline function of neutrophils in the liver[14], which may provide an explanation for high rates of bacterial, fungal and viral infection as well as organ failure and mortality. Neutrophil dysfunction has been shown to be reversed in patients with AH following endotoxin removal[52]. In AH patients, extensive modification of albumin occurs, further activating neutrophil infiltration causing inflammation and oxidative stress[46,49]. Monocyte chemoattractant protein-1 also known as C–C motif chemokine ligand 2 is involved in proinflammatory cytokine activation and its levels have been found to be correlated with neutrophil infiltration and disease severity[46]. Therefore, neutrophils have been implicated in disease pathogenesis and a balance between anti-bacterial and anti-inflammatory functions is important for ALD patients.

Adaptive immunity

The adaptive immune response has also been implicated in pathogenesis of ALD (Figure 3). Early studies in both animals and humans have shown excessive alcohol consumption reduces peripheral T cell numbers, disrupts the balance between phenotypes, impairs function and promotes apoptosis[53]. Alcohol consumption can cause lymphopenia as well as disrupt the balance between T cell phenotypes, causing a shift from naïve populations to memory cells, experimentally and clinically[54-56]. Cy-

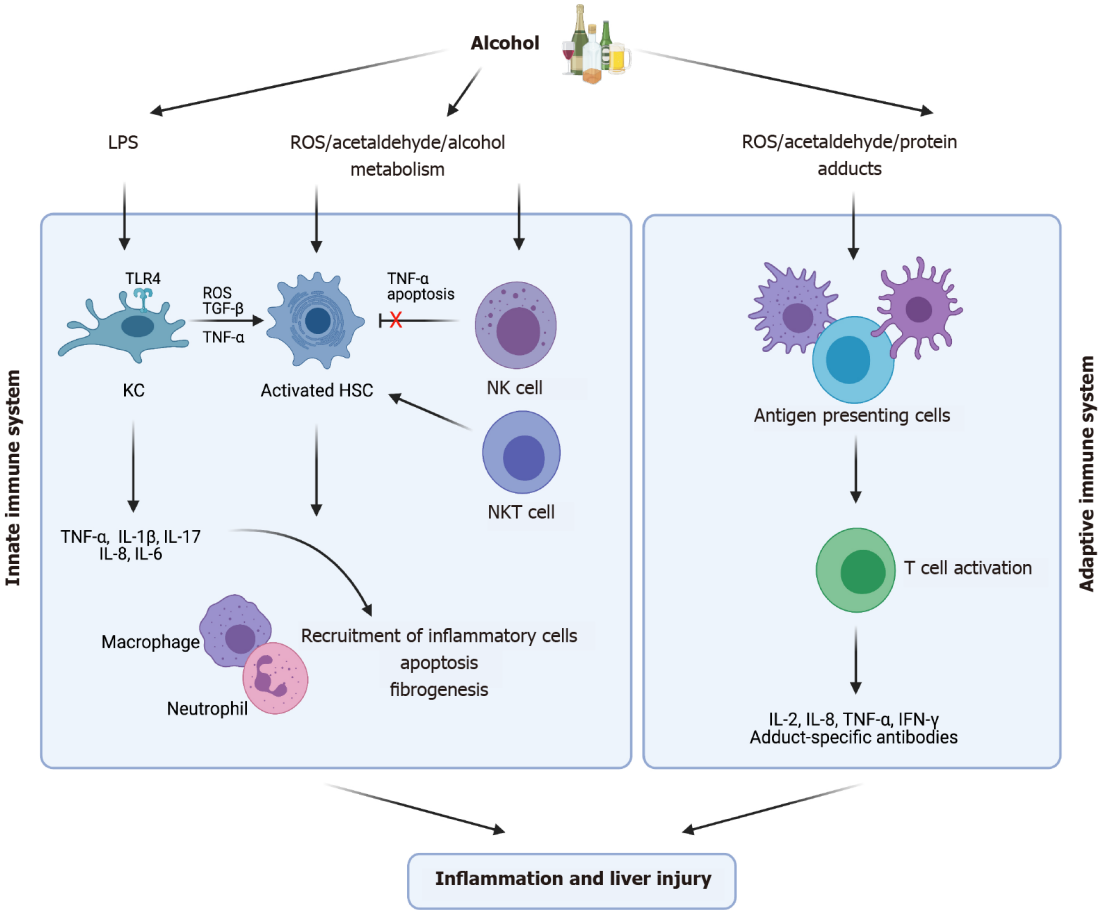

Figure 3 Innate and adaptive immune response to alcohol exposure. Kupffer cells, hepatic stellate cells and natural killer cells are components of the innate immune system which becomes activated following chronic alcohol consumption. This leads to the release of inflammatory cytokines, causing further recruitment of inflammatory cells. The adaptive immune system also becomes activated releasing inflammatory mediators as well as antibody generation to protein and Malondialdehyde-acetaldehyde adducts. Both immune response mechanisms eventually become dysregulated over time with alcohol consumption. (Figure created with BioRender.com). HSC: Hepatic stellate cell; IFN-γ: Interferon-γ; IL: Interleukin; KC: Kupffer cell; LPS: Lipopolysaccharide; NK: Natural killer; NKT: Natural killer T; ROS: Reactive oxygen species; TGF-β: Transforming growth factor β; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α.

totoxic CD8$^+$ T cells are greatly reduced, and this reduction was shown to correlate with stage of fibrosis and Child-Pugh (CTP) score, impairing cytotoxic functions leading to immune incompetence[56]. Decreased numbers of regulatory T cells are also associated with immune activation and increased inflammatory cytokines in AH[57].

More recently increasing phenotypes of T cells have been implicated in ALD. Chemokines such as CCL5, a chemoattractant for immune cells such as T lymphocytes, has been found to be upregulated in the liver[58]. Various proteins are expressed on the cell surface such as T-cell receptor, which recognize antigens and elicit a response. Infiltration and activation of both CD4$^+$ and CD8$^+$ T cells have been found to be increased in the livers of patients with ALD[58,59]. Until recently it had not been defined whether the increased activation of T cells in the livers of ALD patients were caused by bystander activation or due to an antigen-specific response[58,59]. Protein adducts derived from alcohol metabolism and lipid peroxidation have been identified in the liver of patients which act as neoantigens. These neoantigens are presented to CD4$^+$ T cells by antigen presenting cells inducing proliferation[58,59]. As well as antigen-specific activation, bystander activation of T cells can occur induced in the absence of *via* cytokines, DAMPs and PAMPs[59]. This infiltration of T cells has been found to be correlated with inflammation and necrosis in ALD as well as regeneration. Therefore, both antigen-specific and bystander activation may contribute to the progression of ALD but also provide a beneficial role, however, this requires further research.

Alterations in regulatory cells may also provide an explanation for disease pathogenesis, as oxidative stress is known to lower regulatory T cell populations in the liver[60]. Th17 cells have been identified in the livers of ALD patients and are critical for defense of bacterial infections[46]. These cells produce IL-17 and promote neutrophil infiltration. Mucosa-associated invariant T cells (MAIT), an innate-like subset

of T cells which inhibit bacterial infection, have been found to be reduced in ALD patients, consequentially increasing bacterial infection. Transcription factors (RORC/RORγt, ZBTB16/PLZF and Eomes) which control the differentiation of MAIT cells were lower in AH patients compared to heathy controls[61]. Therefore, MAIT cell function may provide an important therapeutic approach for the treatment of ALD.

During ALD a loss of peripheral B cells also occurs, as well as increased amounts of circulating immunoglobulin[53]. B cell numbers are documented to be lower in heavy drinkers (90 to 249 drinks/mo) compared to moderate (30 to 89 drinks/mo) to light drinkers (< 9 drinks/mo) as well as a loss or circulating B cells in patients consuming 164.9 to 400 g of alcohol/day on average. The differentiation of progenitor B cells can be affected by ethanol exposure *via* down-regulation of transcription factors (early B cell factor and Pax5) and cytokine receptors (IL-7R α)[62] and thus, alcohol use can affect subpopulations of B cells (B-1a, B-1b, B2-B)[53]. Exposure to 100 mmol/L of alcohol *in vitro* blocks the expression of transcription factors which has been shown to impair B cell differentiation[62]. Alcoholics cannot respond adequately to antigens which is likely due to a reduction in high-affinity antibody-producing B-2B subset[53]. Further, this decrease in B-2B subsets is typically associated with a decrease in the number of B-1a cells as well as a relative increase in the percentage of B-1b cells, important for T cell independent responses[53]. Although B cells numbers appear to be reduced in alcoholics, during cirrhosis, circulating levels of immunoglobulins (IgA, IgG, and IgE) may be increased against liver antigens. It has also been reported that IgG antibodies against CYP2E1 have developed in both alcohol fed rats and patients with advanced ALD[63]. The role of B cells in ALD requires further research.

DIAGNOSIS

Diagnosis of ALD is challenging as many patients present as asymptomatic. An ALD diagnosis is commonly made on a combination of clinical laboratory abnormalities, imaging and a history of alcohol abuse. Laboratory blood tests are used to identify abnormal aspartate aminotransferase (AST), alanine aminotransferase (ALT) level, gamma-glutamyl transpeptidase (GGT), mean corpuscular volume (MCV), carbohydrate-deficient transferrin (CDT) levels, albumin, prothrombin time (PT) (international normalized ratio), bilirubin and platelet counts. These blood tests are useful to suggest alcohol misuse but are inadequate at predicting alcohol use on their own or the disease severity[64]. Historically GGT was used alone as a marker for ALD, although elevated GGT alone has low sensitivity and specificity for alcohol abuse and may be limited by a high rate for false positives[64]. An AST/ALT ration above 2 is regarded as an indicator of ALD, although it is used less frequently to predict chronic alcohol abuse due to low sensitivity[64]. CDT is used as a biomarker for chronic ethanol intake (> 60 g ethanol/d) and has a higher specificity (sensitivity 46%-73%, specificity 70%) than conventional markers such as GGT and MCV. However, no individual biomarker alone provides suitable sensitivity and specificity for ALD diagnosis, therefore, a combination of these biomarkers, imaging and in some cases a biopsy can provide an improved diagnosis.

CLINICAL STAGING OF DISEASE SEVERITY

There are various algorithms used to assess the severity of liver disease as well as predicating survival and treatment options (Table 1). The first models developed were the CTP score and the model for end-stage liver disease (MELD) score. The CTP score identifies patients as class A, B or C determined by serum levels of bilirubin and albumin, prothrombin time, ascites, and encephalopathy[65]. These measures are scored 1-3, with 3 being the most severe. A CTP defined as Class A (5-6 points) indicates a 100% 1-year survival and 85% 2-year survival. Class B (7-9 points) has an 80% 1-year survival and 60% 2-year survival. Class C (10-15 points) has a 45% 1-year survival and a 35% 2-year survival[66]. The MELD score is determined by total bilirubin, creatinine, and international normalized ratio (INR) levels, and is a widely useful tool for evaluation of liver transplantation in patients[67]. MELD is calculated by the formula 9.57 × loge (creatinine) + 3.78 × loge (total bilirubin) + 11.2 × loge (INR) + 6.43[66]. Although these scores are useful in predicting mortality, they are less useful in assessing prognosis and treatment options. Research by Sheth *et al*[68] has shown that 30-d mortality predictions from the MELD scoring were 86% sensitive and 82% specific for MELD scores greater than 11[68].

Table 1 Models for clinical staging in alcoholic liver disease

Model	Stratification	Bilirubin	Albumin	Prothrombin Time	Ascites	Encephalopathy	INR	Creatinine	White blood cell count	Serum urea	Age
Child-Pugh[65, 66]	Severe: ≥ 10	+	+	+	+	+					
Model for End-Stage Liver disease[66]	Severe: ≥ 21	+					+	+			
Maddrey Discriminant Function score [66]	Severe: ≥ 32	+		+							
The Glasgow Alcoholic Hepatitis Score [66]	Poor prognosis: ≥ 9	+		+					+	+	+
Lille Model[66, 69]	≥ 0.45: Nonresponse. < 0.45: Response	+	+	+				+			+

INR: International normalized ratio.

The Maddrey Discriminant Function score (MDF) and The Glasgow AH Score (GAHS) were developed to determine disease severity and treatment options in AH patients. The MDF score assesses serum bilirubin and prothrombin time *via* the equation DF = {4.6 x [PT (sec) - control PT (sec)]} + (serum bilirubin) and classifies disease as either severe (MDF > 32) or non-severe (MDF < 32) and patients who fall in the severe category are most likely to benefit from steroid treatment[66]. The diagnostic sensitivity and specificity of the MDF scoring system was 86% and 48% when the DF was greater than 32[68]. The GAHS was developed to predict outcomes and to initiate therapy in AH patients. The GAHS includes age, white blood cell count, serum urea, bilirubin, and PT. Each variable is given a score and a final combined score between 5-12 is obtained. Patients with a GAHS above 9 have a poorer prognostic outcome. A study has shown patients with an MDF > 32 and a GAHS > 9 who were treated with steroids has a higher survival rate than those without treatment (59% compared to 38%)[67]. The Lille model is another prognostic model developed to identify response to corticosteroids in severe AH patients after 7 d of treatment[69]. Lille Model Score is defined as [exp(-R)]/[1 + exp(-R)]. Where r = 3.19 – 0.101*(age, years) + 0.147*(albumin day 0, g/L) + 0.0165* (evolution in bilirubin level, μmol/L) - 0.206*(renal insufficiency) - 0.0065*(bilirubin day 0, μmol/L) - 0.0096*(PT, seconds) [66]. It is useful for predicting short-term survival due to its high sensitivity and specificity and is able to identify patients at high risk of death at 6 mo[69].

THERAPEUTIC INTERVENTION

The consequences of excessive alcohol consumption causes significant morbidity and mortality with 704300 projected deaths due to alcohol-related liver disease in the United States between 2019-2040[9]. However, with a lack of new therapeutic options, abstinence is still regarded as the most important treatment, as well as treatments such as nutritional therapy, pharmacological therapy, combination therapy and transplantation (Table 2).

Abstinence

Abstinence is the most important treatment for patients with ALD[70]. Abstinence from alcohol improves overall survival and prognosis as well as preventing further disease progression[71]. A reduction in portal pressure, decreased progression to cirrhosis and an improvement in survival has been shown after a period of abstinence [70,72]. However, relapse rates are as high as 67%-81% in alcoholics[73]. Several drugs such as disulfiram, naltrexone and acamprosate have been trialed to sustain abstinence

Table 2 Current available treatments for alcoholic liver disease

Intervention	Objective	Treatment method	Treatment effects
Abstinence	Stop drinking	Abstinence combined with disulfiram, naltrexone or acamprosate	Improve overall survival at all stages[70]. Acamprosate has been shown to be effective in reducing withdrawal symptoms[77]
Nutritional Therapy	Replenish nutrition	1.5 g protein and 35 to 49 kcal *per* kg of body weight. Supplementation with vitamins	Nutritional support showed improved hepatic encephalopathy and reduced infections in AH patients[80]
Corticosteroid	Anti-inflammatory	40 mg daily for 28 d, then 20 mg daily for 7 d, and 10 mg daily for 7 d	Short-term histological improvement has been documented, however, no improvement in long term survival[83]
Pentoxifylline	Anti-cytokine	400 mg orally three times a day for 4 wk	Reduction in the levels of cytokines and lower mortality rate[86]
Infliximab	Anti-cytokine	Not confirmed. 5 mg/kg studied	Further studies required. Treatment has shown to predispose patients to higher rate of infections as well as higher likelihood of mortality[93]
Liver transplantation	Surgery	Healthy 'donor' liver transplanted. 6 mo abstinence required	Transplantation has been shown to improve in quality of life[95,96]
N-acetylcysteine	Antioxidant	Not confirmed	In animal models NAC has been shown to prevent relapse and improve injury[111]. Further research is required
SAM	Antioxidant	Not confirmed	SAM therapy has improved survival and delayed the need for transplants however other studies have not found evidence to support or refute its use[102,103]
Silymarin	Herbal	Eurosil 85® 420 mg a day	Treatment with Silymarin reduced mortality and improved 4-yr overall survival in cirrhotic patients as well as improving liver function[119]
Betaine	Nutrient	Not confirmed	Animal models have shown betaine supplementation can attenuate alcoholic fatty liver[106-108,120,121]

AH: Alcoholic hepatitis; NAC: N-acetylcysteine; SAM: S-adenosyl methionine.

and treat alcohol addiction. However, disulfiram has little evidence at improving abstinence[74]. Naltrexone, an opioid antagonist aimed to control alcohol cravings lowers the risk of relapse, although, it also has been shown to cause hepatocellular injury[71,75]. Acamprosate is used to minimize withdrawal symptoms when abstaining from alcohol[76]. It has been shown to reduce withdrawal in 15 controlled trials [77].

Nutritional therapy

Malnutrition is often correlated with disease severity in ALD patients[78]. Alcoholics suffer from deficiencies in several vitamins and minerals, including vitamin A, vitamin D, thiamine, folate, pyridoxine, and zinc[78]. Supplementation with zinc has shown to improve and prevent liver disease, as well as block mechanisms of liver injury including 'leaky gut', oxidative stress and apoptosis in animal models[79]. The recommended amount for ALD patients is 1.5 g of protein/kg body weight[2]. Supplementation with micronutrients may be necessary if an individual develops deficiencies. Nutritional support in AH has been reported to improve hepatic encephalopathy and reduce infections[80]. A reverse in both energy and protein deficits has been shown reduce morbidity and mortality in patients with acute AH and cirrhosis[81].

Steroid and anti-cytokine therapy

Steroids serve as the primary treatment for severe AH[82]. Treatment with glucocorticoids have decreased proinflammatory cytokines as well as inhibiting neutrophil activation[83]. Glucocorticoid therapy in AH patients showed short-term histological improvement and 28-d survival, however, long-term survival (beyond 1 year) was not improved[83].

Pentoxifylline, a phosphodiesterase inhibitor, is an anti-TNF-α agent. Pentoxifylline has been trialed in 101 patients with severe AH[84,85]. In-hospital mortality was 40% lower in those patients who were treated with pentoxifylline as well as reducing the likelihood of hepatorenal syndrome (HRS). 50% of deaths in the pentoxifylline treatment group were due to HRS, compared to 92% in the placebo group. Pentoxifylline also exhibited a higher 6-mo survival and a reduced incidence of HRS in patients with severe AH[86].

The Steroids or Pentoxifylline for AH trial (double-blind, randomized control trial) has evaluated the effects of both treatment with prednisolone and/or pentoxifylline

[87-89]. 1103 patients underwent randomization with 1053 suitable for primary end point analysis. The primary endpoint was mortality at 28 d[89]. Results showed primary end point mortality at 28 d was 17% in the placebo–placebo group, 14% in the prednisolone–placebo group, 19% in the pentoxifylline–placebo group, and 13% in the prednisolone–pentoxifylline group, showing that pentoxifylline did not improve patients overall survival[89]. Although not significant, the steroid group showed a trend toward reduced 28-d mortality[89]. However, in those patients who received steroid treatment the rate of serious infection was nearly doubled[89,90].

Another anti-cytokine therapy used in the treatment of ALD is infliximab, a monoclonal chimeric anti-TNF antibody. In a primary randomized study using infliximab, 20 AH patients were given either 5 mg/kg of infliximab as well as 40 mg/d of prednisone or prednisone alone[91]. The results indicated there was no change in overall mortality, however, combination therapy decreased cytokine levels[84]. In France, a clinical trial studied prednisolone (40 mg/d for 4 wk) treatment compared to prednisolone with infliximab (10 mg/kg, at study entry, 2 wk and 4 wk after entry) in 36 patients[92]. Unfortunately the trial was stopped early due to mortality and infection[84], and therefore, this study has received criticism for the dose of infliximab in the trial as this predisposed patients to infections[93]. These trials suggest that anti-cytokine treatment in ALD is associated with an increase likelihood of severe infections and mortality. Canakinumab, a licensed monoclonal antibody inhibitor of IL-1 is currently being studied to treat ALD, as IL-1 has the ability to mediate disease progression in ALD[58].

Liver transplantation

Liver transplantation is a common treatment for end stage chronic liver disease; however, it remains controversial due to the increasing demand for donor organs as well as concerns of relapse from abstinence. Prior to surgery patients must abstain from alcohol for a fixed period of 6 mo[94]. Studies have shown patients who receive transplants have a better quality of life[95,96], however, less than 20% of patients whom have an end-stage liver disease receive surgery[97].

Antioxidants

Oxidative stress is a major contributor to the pathogenesis of chronic liver disease; therefore, antioxidant therapy has been considered to be beneficial in the treatment of ALD. Antioxidant agents able to mediate ROS include vitamins E and C, N-acetylcysteine (NAC) as well as S-adenosyl methionine (SAM), and betaine.

SAM operates to synthesize glutathione, the primary cellular antioxidant[98,99]. Patients with AH and cirrhosis have decreased hepatic SAM levels[100]. In animal models, SAM supplementation can reverse liver injury and mitochondrial damage caused by alcohol[101]. However, no significant difference between SAM supplementation and placebo groups has been reported[102,103]. Betaine, a nutrient involved in the formation of glutathione, is effective in protecting against damage from chronic alcohol consumption[104,105]. Supplementation with dietary betaine in animal studies has shown to ameliorate effects of oxidative stress[106-109]. In rats, NAC was able to reduce ethanol seeking behavior by 77%[110] as well as inhibiting ethanol intake by up to 70%[111]. In 174 patients with severe AH, combination therapy with NAC and prednisolone, compared to prednisolone only increased 1-mo survival in patients with AH, although 6-mo survival did not improve[112]. There are several trials underway investigating treatment options for ALD. A current clinical trial is assessing the effects of SAM and choline treatment for 24 wk against a placebo (trial number NCT 03938662). Choline can help the liver undergo glucose metabolism as well as repairing the cell membrane[113]. As damaged livers cannot produce SAM sufficiently, administration of choline and SAM may be a beneficial treatment in patients with ALD.

Fecal bacteria transplants

Chronic alcohol consumption leads to bacterial overgrowth promoting gut dysbiosis which correlates to disease severity. In cirrhosis patients Bacteroidetes and Firmicutes phyla were found to be decreased[114], however, Proteobacteria, Fusobacteria and Actinobacteria phyla were increased[114]. Cirrhosis is also characterized by reduced beneficial autochthonous bacteria such as Lachnospiraceae, Ruminococcaceae, and Clostridiales XIV as well increased pathogenic bacteria such as *Enterococcaeae, Staphylococcaceae and Enterobacteriaceae*[115]. The significance of bacteria in liver disease has been demonstrated when ethanol-fed, germ-free mice developed severe inflammation and necrosis when supplemented with gut microbiota from AH patients[116]. Furthermore, subsequent transfer of gut microbiota from patients without ALD, led to less

inflammation and liver injury[117] implicating the importance of healthy bacteria. More recently administration of probiotics containing beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* to patients with ALD has improved liver damage and function, including reduction of ALT, AST and bilirubin[118]. Modulation of the gut microbiota with Profermin®, a disease specific food for special medical purposes, has been hypothesized to reduce disease progression (trial number NCT03863730). These studies provide strong evidence that fecal bacteria transplants/probiotic administration may prove an effective mode for the treatment of ALD.

CONCLUSION

Previous research has uncovered many elements in the pathogenesis of ALD, however, the precise triggers and biochemical alterations are yet to be fully understood. Oxidative stress can impair proliferation and alter the immune system leading to bacterial overgrowth and an increased risk of infection. Poor treatments options are available for patients with ALD which have not transformed for several years. Treatment options rely on abstinence, steroids, nutritional therapy and lastly liver transplantation. Current new therapies are aimed at reducing pro-inflammatory signals as well as treating the gut-liver axis. This highlights a need for new therapeutic intervention and advancements in the understanding of the mechanisms involved in disease pathogenesis.

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