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Mass Spectrometry for the Discovery of Biomarkers of Sepsis

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Abstract

Sepsis is a serious medical condition that occurs in 30% of patients in intensive care units (ICUs). Early detection of sepsis is key to prevent its progression to severe sepsis and septic shock, which can cause organ failure and death. Diagnostic criteria for sepsis are nonspecific and hinder a timely diagnosis in patients. Therefore, there is currently a large effort to detect biomarkers that can aid physicians in the diagnosis and prognosis of sepsis. Mass spectrometry is often the method of choice to detect metabolomic and proteomic changes that occur during sepsis progression. These “omics” strategies allow for untargeted profiling of thousands of metabolites and proteins from human biological samples obtained from septic patients. Differential expression of or modifications to these metabolites and proteins can provide a more reliable source of diagnostic biomarkers for sepsis. Here, we focus on the current knowledge of biomarkers of sepsis and discuss the various mass spectrometric technologies used in their detection. We consider studies of the metabolome and proteome and summarize information regarding potential biomarkers in both general and neonatal sepsis.

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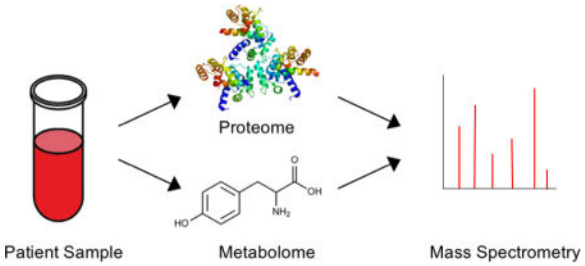

1. Sepsis Progression, Diagnosis, and Treatment

Sepsis is a potentially life-threatening medical condition characterized by systemic inflammation from infection[1,2,3]. The term “sepsis” often describes a progression of infection through a number of stages, from systemic inflammatory response syndrome (SIRS) to septic shock, which can result in multiple organ dysfunction syndrome (MODS) and death[4]. SIRS is characterized by the presence of two or more specific symptoms, including fever, hyperventilation, and leukocytosis[5] (Table 1). When patients exhibit

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symptoms of SIRS in the presence of infection and systemic inflammatory response, it is termed sepsis. This condition can progress to severe sepsis with the onset of organ dysfunction. In the final stages of sepsis, the patient is diagnosed with septic shock, which presents as severe sepsis with hypotension that does not respond to fluid resuscitation[6]. Mortality rates for patients with sepsis and severe sepsis are between 25–30%, and increase to 40–50% for septic shock[7]. Sepsis can occur in up to 30% of intensive care unit (ICU) patients, and approximately 2% of all hospitalized patients in developed countries present with sepsis[8]. The incidence of sepsis is steadily increasing across populations in the United States and Europe, corresponding to a larger aging population and increased antibiotic resistance[9].

Sepsis commonly manifests after an infection from, viruses, fungi, or Gram-negative or Gram-positive bacteria. Bacterial causes of sepsis have steadily increased since the 1970's, while the incidence of fungal sepsis has escalated rapidly, likely due to more successful treatments for bacterial sepsis[11]. Sepsis is commonly derived from urinary tract infections, community-acquired pneumonia, and abdominal infections[12, 13]. There are a number of risk factors that increase the chance of a patient becoming septic, including gender, age, and ethnicity. Males reportedly have a higher risk of developing sepsis than females, though the mechanism behind this fact is not known. Elderly and neonatal populations are at the greatest risk of developing sepsis. Elderly populations are more likely to have simultaneous chronic diseases, increasing their sepsis risk, while the immune systems of newborn children are immature and cannot readily fight off infections. Furthermore, the incidence of severe sepsis is reportedly higher in patients of African descent than those of Caucasian descent. This higher incidence has been attributed to an increased likelihood for African-American patients to be hospitalized with infection, although the underlying mechanisms are not well understood[14]. The presence of other diseases, such as HIV, cancer, and other conditions affecting the immune system, also increase the likelihood of sepsis development.

Early diagnosis of sepsis is critical to halt progression to septic shock. Blood cultures are currently the gold standard to determine the presence of microbial species in the body, though it is estimated that only 30–40% of severe sepsis or septic shock patients yield positive test results[6,7]. Serum lactate levels and white blood cell counts are also used to aid diagnosis. Patients who present with sepsis are treated by physical removal of the infection source, as well as antimicrobial therapy. Those patients who present with severe sepsis or septic shock undergo several other treatments depending upon the progression of the syndrome, usually including cardiorespiratory resuscitation with intravenous fluids[6, 15]. Although numerous advances have been made in the fundamental understanding of sepsis pathophysiology, few successful therapies have been developed over the last three decades[16]. Human recombinant activated protein C (APC) has been the only drug to pass through Phase 3 clinical trials in the last 25 years, but APC was removed from the market due to its failure to prove clinically effective[17]. Therefore, it is essential for researchers to reevaluate current research methods regarding sepsis to produce more successful outcomes in the future.

I. Molecular Mechanisms of Sepsis Progression

The interactions between infectious agents and the host are highly dynamic and complex in nature. Early studies based on animal models proposed a massive inflammatory response in hosts due to the presence of pathogens that result in damage or death of the host. The most common mouse models were based on treatment with endotoxin, which initiated an immense cytokine cascade and inflammatory response[2]. Based on these findings, researchers and physicians believed the best course of action was to curb the immune response as early as possible. However, these animal models did not accurately recapitulate human sepsis pathophysiology, and the measured levels of proinflammatory cytokines in mice were much greater than their human counterparts[18].

Currently, researchers and physicians believe a number of pro-inflammatory and anti-inflammatory pathways are activated during sepsis progression that may vary depending on the responsible pathogen and individual host[15]. During sepsis progression, the normal homeostasis of the body is disrupted upon the release of damage-associated molecular patterns (DAMPs) from the invading pathogen. DAMPs are recognized by pattern-recognition receptors on immune cells, which then release pro-inflammatory mediators, including cytokines. This development is often referred to as the “cytokine storm” in sepsis[19]. This process activates the host complement system, coagulation system, and adrenergic pathways in an attempt to clear invading pathogens from the body. Over-activation of these processes is responsible for tissue damage during sepsis progression.

Patients who survive this period of intense inflammatory response eventually give in to a period of immunosuppression[20, 21]. Neuroendocrine regulation releases acetylcholine which targets macrophages to suppress their release of inflammatory cytokines[22]. In addition, extensive apoptosis of leukocytes and dendritic cells contribute to immunoparalysis in septic patients. When surviving macrophages uptake apoptotic cells, they release anti-inflammatory molecules including interleukin-10 (IL-10) and Transforming Growth Factor-beta (TGFβ). The surviving antigen-presenting dendritic cells may also deactivate helper-T cells. The combination of these processes leads to a steep decline in the immune system. This interval is a dangerous, and often fatal period for patients with severe sepsis and septic shock.

II. Traditional Biomarkers

Biomarkers are biologically relevant molecules that indicate the presence, progression, or possible outcome of disease conditions. For sepsis, biomarkers have the potential to diagnose the responsible pathogen, stage of the disease, and possible response to treatment. An ideal biomarker is able to differentiate bacterial SIRS from other causes in a quick and sensitive manner. Over 178 protein biomarkers have been proposed for sepsis detection, including procalcitonin[23], C-reactive protein[24], interleukin(IL)-6, and soluble urokinase-type plasminogen activator receptor (suPAR). However, their clinical utility faces a number of limitations. These biomarkers are limited in their specificity and are not able to adequately distinguish sepsis from other inflammatory processes, and no single biomarker has been approved for absolute diagnosis of sepsis.

The only biomarker to achieve clinical implementation for sepsis is procalcitonin. High concentrations of procalcitonin, a precursor to the thyroid hormone calcitonin, in the serum of septic patients were first described in 1993[23]. In healthy patients, procalcitonin levels are measured at <1 ng/mL, while levels can increase to 4–45ng/mL in patients with septic shock[25,26]. Procalcitonin levels increase within 4–12 hours of infection and return to normal levels within 2–3 days[27, 28]. Recent studies calculated sensitivity of 0.77 and specificity of 0.79, and an area under the receiver operating characteristic curve (AUROC) of 85% for procalcitonin as a biomarker in sepsis[29]. However, increased concentrations of procalcitonin are known to occur after surgery, trauma, and systemic viral infections[30]. The current guidelines in the Surviving Sepsis campaign conclude that current research does not demonstrate the ability of procalcitonin to distinguish sepsis from other causes of inflammation. Instead, procalcitonin levels help establish when physicians should end antibiotic treatment[6].

C-reactive protein (CRP) has also been used as a marker for the diagnosis of infection. CRP is an acute-phase protein that is secreted from the liver during inflammation. Detection of CRP has a wider window than procalcitonin, with a peak in secretion at 36 hours post-infection. Increased levels of CRP correlate with organ failure and death, and are often used by physicians to differentiate infectious and non-infectious causes of abdominal pain[26, 31]. The sensitivity and specificity of CRP for the diagnosis of sepsis were reported as 0.75 and 0.67, respectively. CRP has been reported to be inferior to procalcitonin in its ability to diagnose sepsis, and was not able to adequately predict the outcome of blood cultures or patient prognosis[32]. The lack of specificity and slow kinetic profile of CRP have hindered its implementation for the diagnosis of sepsis.

Furthermore, IL-6 has been described as a marker of sepsis for a number of decades[33]. IL-6 is a proinflammatory cytokine with normal serum concentrations of less than 5pg/mL. The concentration increases 100-fold within two hours of sepsis onset, and decreases within six hours[34]. The fast kinetic profile of IL-6 make it a biomarker of interest for rapid sepsis diagnosis. A number of studies have reached mixed conclusions regarding IL-6's ability to diagnose sepsis. Early studies found that IL-6 had favorable abilities to discriminate septic patients from those who had non-infectious causes of SIRS, with an AUROC of 0.837[35]. A recent meta-analysis affirmed the utility of IL-6, with a calculated sensitivity of 80%, specificity of 75%, and AUROC of 0.868 for the detection of early sepsis[36]. Other studies comparing the value of procalcitonin, IL-6, and IL-8 found that procalcitonin was superior for sepsis diagnosis[37]. Although these studies have been promising, IL-6 has not been incorporated into the current guidelines regarding sepsis diagnosis[6]. The discrepancies between studies of sepsis biomarkers and their clinical implementation are likely due to variation in statistical parameters and criteria used across laboratories.

Soluble urokinase-type plasminogen activator receptor (SuPAR) is a membrane glycoprotein that is released into the bloodstream after inflammatory stimulation[38]. Like the other diagnostic markers of sepsis, researchers have found that suPAR is a general marker of infection, and is not specific enough for sepsis diagnosis. However, suPAR levels do correlate well with prognosis of septic patients. Systemic levels of suPAR were much greater in patients who eventually died than those who recovered from their illness[39]. SuPAR is a

more favorable biomarker than procalcitonin or CRP for prognosis due to its relatively stable levels. This stability is advantageous for a biomarker because it eliminates patient sample collection time as a variable in suPAR's efficacy as a biomarker[40]. However, the relatively constant levels of suPAR during sepsis preclude its use as a biomarker to direct treatment options.

Hundreds of studies have addressed the need for a biomarker to diagnose septic patients quickly and efficiently. However, no single biomarker is likely to meet this need in a specific and sensitive manner. Currently, it is thought that generating panels of biomarkers may increase their sensitivity and accuracy for sepsis diagnosis. One study of patients presenting with SIRS analyzed a number of biomarkers, including procalcitonin, CRP, suPAR, and others, individually and in concert. They found the combined AUROC of these biomarkers was significantly higher than any biomarker individually[40]. Additional studies analyzed the ability of procalcitonin, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), and neutrophil CD64 expression for sepsis diagnosis. This group developed expression thresholds and sample collection protocols to correctly diagnose 80% of the patient cohort. However, the facilities and equipment necessary for the analysis of these three biomarkers are not necessarily practical for all clinical settings[41]. Despite the advances with these established biomarkers, researchers have turned to methods to perform unbiased studies identifying new biomarkers of sepsis progression. In particular, the use of mass spectrometry for biomarker detection has become critical for disease research.

III. Mass Spectrometry for Biological Studies

Throughout the past two decades, the combination of "omics" fields have advanced the understanding of many biological systems. Analysis of the proteins (proteomics) and metabolites (metabolomics) in a biological system provide new sources of information that cannot be obtained through purely genomic investigations. Development of mass spectrometry-based techniques has been essential for the advancement of these fields. The advent of proteomics and metabolomics resulted in successful applications for the analysis of peptides[42], intact proteins[43], protein complexes[44], metabolites[45], and other biologically relevant molecules using mass spectrometry.

4.1 Instrumentation

A number of biological samples can be used for the study of biomarkers. Cell and tissue lysates from animal models provide important information that can often be translated to humans. Furthermore, specimens from humans including tumor samples, plasma, urine, and blood are crucial to understand disease progression. Each of these samples is incredibly complex and contains hundreds of thousands of analytes that may be of interest to researchers. Untargeted mass spectrometry experiments stochastically measure the most abundant species in a sample. Without proper separations and instrument parameters, high-abundance species will outcompete low-abundance species for ionization. This phenomenon is referred to as "ion suppression." To combat this issue, one or more dimensions of separation are performed on complex samples. These separations can include high-pH reversed phase-liquid chromatography (RPLC), low-pH RPLC, strong cation exchange

chromatography (SCX), strong anion exchange chromatography (SAX), hydrophilic interaction liquid chromatography (HILIC), capillary zone electrophoresis (CZE), and many others. Advancements in RPLC column technology allow ultraperformance liquid chromatography (UPLC) systems to run at nanoflow rates in columns packed with 1.7μm C18 particles, which greatly enhances the resolution and sensitivity of the separation[46, 47]. CZE is an increasingly popular method that provides complementary identification of analytes to UPLC[48, 49]. This method separates molecules based on their mass-to-charge ratio is often able to detect hydrophilic molecules that are weakly retained on reversed-phase columns. Advancements in CZE technology have increased the loading capacity substantially. The combination of orthogonal separation methods increases the number of analytes detected by the mass spectrometer, which is critical for biomarker discovery.

Mass spectrometry analyzes ions in the gas phase according to their mass-to-charge ratio (*m/z*). All instruments are composed of an ionization source, a mass analyzer, and a detector. The two most common ionization mechanisms used for biological molecules are electrospray ionization (ESI)[50] and matrix-assisted laser desorption ionization (MALDI)[51]. Both of these techniques employ “soft” ionization mechanisms that result in minimal fragmentation during the ionization process[52]. Ionization can be performed in negative or positive ion mode to enhance the coverage of the metabolome.

Modern mass spectrometers are assembled from a number of mass analyzers to identify analytes in space or time. The most common setups for instruments used in proteomics and metabolomics research include Orbitraps, ion traps, quadrupoles, Fourier transform ion cyclotron resonance (FT-ICR), and time-of-flight mass analyzers (TOFs). The advent of the Orbitrap mass analyzer was critical for biomarker discovery studies due to its increased resolution compared to ion trap and TOF mass analyzers[53]. This allows for high-throughput studies of thousands of molecules with high-resolution parent and fragment spectra. Detailed discussions regarding the characteristics of each mass analyzer and their various configurations can be found elsewhere[54, 55]. Triple quadrupole mass spectrometers are ideal to perform targeted mass spectrometry experiments due to their unique capability to filter specific precursor and product ion *m/z* values. Ion counts for particular parent ion/fragment ion pairs are monitored over time to produce a chromatographic trace that can be used for quantification[56]. Discovery-based mass spectrometry experiments are often conducted on hybrid instruments containing combinations including a quadrupole-TOF (QTOF), quadrupole-Orbitrap, or a linear ion trap-Orbitrap configurations. These combinations have proved to be favorable for the detection of peptides and metabolites in solutions.

IV. Metabolomics

Metabolomics is the study of physiologically relevant small molecules that are responsible for metabolic processes in organisms. The presence or quantity of these molecules can reflect the state of the cells, tissues, and organisms from which they are derived[45]. Metabolomic experiments focus on the analysis of species less than 1000 Daltons (Da). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry are the methods of choice to analyze the metabolome. Both can perform unbiased screens of the metabolites in a given system and require database spectral comparison for identification. NMR is a non-

destructive technique that requires minimal sample handing prior to analysis. Mass spectrometry is a destructive technique that can require extensive sample preparation. However, the advantage of mass spectrometry lies in its sensitivity; it can detect analytes in the femtomolar to attomolar ranges. NMR spectroscopy does not allow for online separation of complex mixtures, and spectra show interference due to water resonance[57]. Therefore, mass spectrometry is often the method of choice for metabolomics experiments. Current mass spectrometers allow researchers to perform unbiased screens for thousands of metabolites in a single experiment. Metabolomics technologies can be applied to biological systems to analyze changes after physiological perturbations and search for useful biomarkers to identify disease states.

A number of studies have combined analyses from NMR spectroscopy and mass spectrometry to investigate biological systems. Characterization of the serum metabolome by Psychogios *et al.* combined data from NMR, GC-MS, and LC-MS methods to identify and quantify a set of metabolites in human serum[58]. The combination of methods increased the depth of the metabolome that was profiled, but the spectra gained from each method were not integrated into a single analysis pipeline. A hybrid NMR/MS method, termed the "NMR/MS Translator", was developed by Bingol *et al.*[59]. In this strategy, metabolites are identified based on their NMR spectral match in NMR metabolomics databases. The expected *m/z* ratios are then compared with the MS^1 spectra obtained from the sample sample to identify metabolites of interest and their various adducts. The NMR/MS Translator combines both techniques to increase the accuracy and efficiency of identification of metabolites that are recorded in metabolite databases. This group then expanded upon the NMR/MS Translator by developing a strategy called "SUMMIT MS/NMR." This technique uses high-resolution mass spectra to derive chemical formulas and predicts all feasible structures for the corresponding molecule. The NMR spectra of these potential metabolites are predicted and compared with experimentally derived NMR spectra[60]. SUMMIT MS/NMR can identify a range of metabolites in biological mixtures in a high-throughput fashion by combining the information gleaned from both techniques. The development and enhancement of strategies that combine NMR and MS are vital to advance the field of metabolomics and increase its utility in disease biomarker detection.

A typical MS metabolomics workflow begins with the extraction of small molecules from a sample of interest. Commonly, samples from a cohort of patients diagnosed with a specific condition are compared to healthy donor patients. Extracted molecules are then prepared for mass spectrometry analysis. The ability to couple chromatography directly to a mass spectrometer is a significant advantage of this method. RPLC and CZE are commonly used to separate samples because they utilize solvents that are compatible with mass spectrometry. Targeted metabolomics experiments are designed to analyze a subset of molecules of interest, which may fall into a specific class or are part of the same metabolic pathway. Triple quadrupole mass spectrometers can be used to target specific biomolecules in a particular sample. Global metabolomics studies use stochastic screening by mass spectrometry to identify as many metabolites as possible in a limited amount of time.

The metabolic changes that occur during sepsis are extensive and outside the scope of this review. Other reviews cover this information in detail[61, 62]. Briefly, the wave of

proinflammatory cytokines that are released during sepsis progression increases metabolism through the Warburg effect, with an increase in glycolysis and a decrease in oxidative phosphorylation. This phase results in mitochondrial dysfunction and increased production of reactive oxygen species (ROS). There is upregulated proteolysis, gluconeogenesis, hepatic glucose output, and lipolysis due to increased energy demands on the body. As sepsis progresses to septic shock, the acute phase gives way to a period of immunoparalysis, with a decrease in glycolysis, TCA cycle, and triglyceride metabolism[63]. These changes that occur during sepsis progression indicate the metabolome may contain biomarkers relevant for the diagnosis and treatment of sepsis.

5.1 Metabolomics for Biomarkers of Sepsis

Animal Studies—Animal studies play an important role in disease research. Like many other disease models, mice are the most common organism used to model sepsis. Mice are an ideal model organism due to their relative lab safety, accelerated lifespan, and physiological complexity[18]. A number of methods have been used to induce SIRS, sepsis, and septic shock in mice. Injection with lipopolysaccharide (LPS) results in systemic activation of the innate immune system, resulting in a phenotype similar to gram-negative sepsis in humans. However, the dose of LPS required to induce this phenotype in mice is much higher than humans, indicating this model may not be reflective of human sepsis pathophysiology. Other mouse models of sepsis, including infection by *S. aureus*, also suffer from this drawback. Infection with a single strain of pathogen does not accurately reflect the combinations of infectious organisms found in human sepsis[64]. Furthermore, mouse models are generally analyzed in young adult animals, while humans are generally at extreme ages when sepsis onset occurs[65]. For these reasons, mouse models of sepsis have inherent severe limitations.

Another popular mouse model for sepsis is the cecal ligation and puncture (CLP) model. This models bacterial peritonitis and most accurately recapitulates human sepsis. These mice exhibit polymicrobial infection, hyperdynamic circulatory systems, and acute lung injury. CLP mouse models exhibit the acute proinflammatory phase followed by an anti-inflammatory phase, as seen in humans[64]. Furthermore, a number of potential sepsis treatments that failed in human clinical trials also failed in the CLP model of sepsis[65]. Although there are distinct advantages to this model, it still suffers from a number of drawbacks. According to genetic studies, the mechanisms of systemic inflammatory responses appear to differ between humans and CLP mouse models. Furthermore, it is difficult to control the magnitude of sepsis in these models because it is highly dependent on the amount of ligation and induced necrosis[64]. This fact weakens the utility of this model for drug discovery studies for human sepsis.

Patients who suffer from severe burns are often encountered in the emergency room. These thermal injuries can lead to damage to organs distant from the original burn site, and patients may become septic. In 2010, work by Liu *et al.* examined the metabolic changes when thermally injured rats became septic using the CLP procedure. Using UPLC-QTOF-MS analysis, they identified nine metabolites that could differentiate septic and non-septic burn patients, including hypoxanthine, indoxyl sulfate, glucuronic acid, gluconic acid, proline,

uracil, nitrotyrosine, uric acid, and trihydroxy cholanoic acid. These biomarkers are mainly related to oxidative stress and tissue damage, which are pathways commonly found to be altered in metabolomics studies of sepsis[66]. Studies have also examined metabolomics of the CLP mouse model versus mice with *S. aureus* induced sepsis. Three potential metabolite biomarkers were found in each model. The first, glutathione, is an antioxidant that protects against reactive oxygen species[67]. Glutathione was found to be lower in expression in the septic rat models than the normal rats, which corresponds to the oxidative imbalance known to occur during sepsis[68]. 2-oxoarginine, an indicator of neural damage during sepsis, was increased in expression in both rat models. Interestingly, the acyl glycine 2-methylhippuric acid was found to be increased in the CLP model and decreased in the *S. aureus* model. A number of biomarkers specific to each model were also identified. This dichotomy illustrates the fact that these two animal models of sepsis induced complex metabolic processes that vary depending on the source of infection[67].

A number of studies have employed various mouse models to examine the metabolic changes to serum and urine samples during sepsis. However, translating findings from mice to humans is often hindered by differences in organismal complexity. To remedy this issue, Steelman *et al.* performed a metabolomics study on an equine gut-derived model of sepsis. In horses, acute equine laminitis occurs secondary to sepsis or SIRS, and can be identified by inflammation in the tissue of the foot. Serum and plasma concentrations of metabolites in these horses were measured using UPLC-TOF-MS and GC-MS in healthy horses and horses that became septic due to acute laminitis. A total of 1,177 metabolites were detected in equine serum. A total of 160 metabolites were found to be differentially expressed between septic and healthy horses, though only 17 could be unambiguously identified. Salicylic acid was upregulated threefold, while lipid palmitoyl lysolecithin and citrulline were decreased during sepsis. Citrulline had previously been identified as a marker for gastrointestinal dysfunction in critically ill human patients[69]. The authors validated the utility of this biomarker in a group of 19 horses admitted to veterinary hospitals and were diagnosed with sepsis. Citrulline concentrations were lower in horses that did not recover with standard treatment protocols, and had a calculated sensitivity and specificity of 83% and 62%, respectively. Extending the search for biomarkers to more physiologically complex species may increase the translational nature of metabolomics research.

Human Studies—LC-MS/MS based analysis of serum metabolomes have also been performed using human clinical samples. Serum, plasma, and urine are commonly profiled using “omics” techniques due to their noninvasive nature for patients. In 2014, Su *et al.* examined serum from 35 septic patients, 15 SIRS patients, and 15 healthy patients to detect metabolites that indicated disease progression. Researchers found that S-(3-methylbutanoyl)-dihydrolipoamide-E, lactitol dehydrate, N-nonanoyl-glycine, and S-phenyl-D-cysteine) were predictive of the presence of infection in these patients. S-(3-methylbutanoyl)-dihydrolipoamide is important for the degradation of valine, leucine, and isoleucine, which are known to have reduced levels during sepsis[70]. Upregulation of this metabolite may be important for this process. Further analysis revealed that 2-phenylacetamide, dimethyllysine, glyceryl-phosphoryl-ethanolamine and D-cysteine could predict the severity of sepsis. Glyceryl-phosphoryl-ethanolamine is necessary for

anticoagulation activity, and its altered expression level could be due to coagulation disorders and vascular endothelial dysfunction during severe sepsis[71]. It is necessary to validate these results in additional cohorts to determine their clinical utility. An additional untargeted metabolomics study was performed on 150 individuals with suspected sepsis from three US hospitals. These patients were profiled at the time of diagnosis, as well as 24 hours later and data was correlated with patient survival[72]. Global expression of metabolites and proteins in plasma were monitored at each stage of sepsis progression and compared to controls (Figure 1). A major finding of this study was that the plasma metabolome and proteome did not differ significantly between sepsis survivors, severe sepsis survivors, and septic shock survivors. There were also no major differences when the infectious pathogen was taken into account. However, large changes were found in sepsis survivors and non-survivors when compared to uninfected patients with SIRS. Defects in fatty acid oxidation were seen in sepsis non-survivors that were absent in survivors. Glycolysis, gluconeogenesis, and the TCA cycle were decreased in non-survivors. Lower body temperatures were recorded in these patients and could correlate with poor aerobic catabolism[73].

In addition to discovery-based studies in animal models and on human biological fluids, a number of targeted mass spectrometry studies have been performed. Schmerler *et al.* analyzed plasma samples from 74 SIRS patients and 69 sepsis patients and profiled 186 total metabolites using targeted mass spectrometry. The patient sample pool was a mixture of gram-positive and gram-negative bacterial sepsis. Two lipids (C10:1 and phosphatidylcholine 32:0) showed higher concentrations in septic patients. These markers demonstrated 80% and 70% correct classification of septic versus non-septic patients, indicating their potential as biomarkers[74]. Other studies have shown that lipemia in sepsis is integral to provide energy to the host organism. In addition, it is essential to neutralize bacterial toxins by embedding LPS into a phospholipid layer of lipoproteins. In particular, phosphatidylcholine (PC) is thought to bind the lipid-A moiety of LPS. Therefore, an increase in PC levels in sepsis indicates systemic inflammation and has the potential to be a useful biomarker for sepsis[74].

In 2013, Seymour *et al.* completed a large-scale study analyzing the global metabolic profile in surviving and non-surviving patients with community-acquired pneumonia and sepsis. Researchers compared patients who died after 90 days to those who survived, while matching for demographics, infection type, and procalcitonin levels. Both UPLC-MS/MS and GC-MS were used to analyze patient plasma samples. The levels of 17% of the 423 detected metabolites were found to differ between surviving and non-surviving patients, including a number of nucleic acid metabolites. These metabolites, including beta-pseudouridine, stimulated the release of TNFα and IL-1β in monocyte cell culture supernatants, indicating their potential role as damage-associated molecular patterns. Rather than focusing on disease-free versus septic patients, these researchers studied the pathways that may predict the mortality of patients who have sepsis. These pathways included regulation of hepatocyte bile acid transporters and structural and functional changes to hepatocyte tight junctions. They also found changes in stress response pathways and oxidative damage in non-surviving sepsis and pneumonia patients[75]. This study is limited by

its small sample size and lack of a validation cohort, but demonstrate the potential to find new DAMPs and biomarkers in sepsis.

Recently, work performed by Ferrario *et al.* focused on a number of metabolite classes known to change during sepsis progression, including glycerophospholipids, amino acids, biogenic amines and acylcarnitines. This work further supported the notion that changes in lipid homeostasis occur during sepsis progression. Patients with septic shock were profiled at the time of diagnosis, as well as 7 days later. The metabolome data was correlated with follow-up survival data at 28 and 90 days post-diagnosis. At day 7, patients who survived 90 days had a vastly different metabolic profile than non-survivors. Non-survivors had significantly higher levels of polyamines, which mediate interactions between pathogens and host immune complexes. Lysophosphatidylcholines (LPC) and PC were found at lower levels in non-survivors than survivors on day 1 and day 7. When all of their data was compiled and analyzed using regression models, lower levels of LPC at day 7 were the strongest predictor of 90-day mortality. Metabolite levels at day 1 were not found to be predictive of mortality. The correlation of lower levels of PC with increased mortality supports the notion that PC is essential to neutralize the effects of bacterial toxins. This study integrated metabolic profiles of septic shock patients with the clinical manifestations of septic shock to detect early biomarkers of the condition. The discoveries made in this study support the idea that lipid homeostasis may play a role in septic shock mortality[76].

Researchers have also performed studies using low-dose LPS in healthy human volunteers. Although this experimental set-up does not fully recapitulate the complexities of sepsis in humans, these experiments have allowed for reproducible systems to explore physiological responses to inflammatory stimuli. A study in 2013 examined plasma metabolic changes at five time points in subjects who were administered LPS or placebos. Researchers profiled 366 metabolites using discovery-based UPLC-MS or GC-MS. Upregulation of plasma long chain fatty acids (FA) was seen at 6 hours post-injection, which is consistent with lipolysis due to inflammation. Elevated levels of 2-hydroxybutyrate were detected and are indicative of oxidative stress. Amino acids and members of the urea cycle were decreased at 6 hours after LPS injection. These metabolites are used to synthesize acute phase proteins and are substrates for energy production[77]. However, LPS injections into healthy humans do not recapitulate the physiological state of septic patients. LPS stimulates systemic inflammation and many of the same pathways, but it is difficult to obtain clinically relevant biomarkers from this system.

5.2 Metabolomics of neonatal sepsis

Neonatal sepsis remains a large problem in neonatal intensive care units (NICU). In the United States, the incidence of neonatal sepsis is 0.76–0.77 of every 1,000 live births[78]. The fatality rate for infants who develop neonatal sepsis is as high as 24.4%. Neonatal sepsis accounts for 36% of the 4 million neonatal deaths worldwide[79]. This condition often results from *E. coli* or *S. aureus* infection during the first four weeks of life. “Early-onset” neonatal sepsis (EONS) occurs within the first 72 hours of life, while “late-onset” neonatal sepsis (LONS) occurs after the first week. Newborns are particularly susceptible to infection due to their immature immune systems. The signs and symptoms of sepsis in newborns are

similarly ambiguous to the symptoms in adults. Respiratory distress and altered eating habits are known signs of early-onset and late-onset neonatal sepsis[80]. Other symptoms include hypothermia, lethargy, apnea, diarrhea, vomiting and abdominal distension. Depending on the site of infection, pneumonia or meningitis may be present. However, these symptoms can be easily attributed to non-infectious conditions. A number of tests are used clinically to aid the diagnosis of neonatal sepsis. White blood cell (WBC) counts and blood cultures are commonly employed, as well as neutrophil counts to detect neutrophilia or neutropenia. CRP is commonly used for sepsis diagnosis, but measurements of the sensitivity and specificity of this biomarker have varied greatly[81]. These limitations indicate a need for further biomarker studies to identify a new biomarker, or panel of biomarkers, that can aid neonatal sepsis diagnosis.

In recent years, researchers have studied the metabolomics of neonatal sepsis to explore a new source of potential biomarkers. One study in 2014 by Fanos *et al.* examined the metabolic profile of urine from infants diagnosed with sepsis versus healthy control infants using ^{1}H-NMR and GC-MS. A number of distinct differences were detected in the quantity of metabolites between these two groups. Septic neonates were found to have increased levels of acetone ketone bodies, including hydroxybutyrate and acetoacetate, as well as glucose, lactate, and acetate. Members of the TCA cycle, such as citrate, were found to be decreased in septic infants. This data supports the notion that sepsis induces major changes in glucose metabolism and oxidative stress[79]. Sepsis is known to block the enzyme pyruvate dehydrogenase, which is responsible for the entrance of lactate into the TCA cycle. Overall, this alteration leads to an increase in fatty acid oxidation and alternative metabolic pathways, including the production of ketone bodies[82]. This study proposed that metabolic profiles may be useful to detect neonatal sepsis in infants, and possibly distinguish early and late-onset neonatal sepsis.

Another study examined biomarkers in fungal neonatal sepsis by analyzing the urine metabolome. Fungal sources of neonatal sepsis commonly include *C. albicans* and *C. parapsilosis*. Symptoms of fungal neonatal sepsis are ambiguous, as described above[83]. Using GC-MS, researchers found increased levels of N-glycine, D-serine, L-threonine, and D-glucose in septic urine, while citric acid, hexadecanoic acid, and octadecanoic acid were found at decreased levels. The increase in amino acid levels are a result of the increased energy demand on the body during sepsis, which results in proteolysis to create amino acid substrates for gluconeogenesis and other processes[84]. As in previous metabolomics studies, citric acid levels were decreased in septic patients, indicating a decrease in TCA cycle levels[79]. Both studies support the notion that sepsis onset causes major metabolic changes in infants and increases the energy demand from the body. However, the clinical relevance of the detected biomarkers requires further study and validation.

Although few studies regarding the metabolome of neonatal sepsis have been performed, it is a promising area of future research. The field of metabolomics has grown immensely in the past five years, and this improved technology will increase the depth of the metabolome that researchers are able to access.

V. Proteomics for Biomarkers of Sepsis

The field of proteomics encompasses the study of the quantity, modifications, and changes in proteins within a biological system. This field is dominated by mass spectrometry for global and targeted analyses of proteins and peptides in complex mixtures. Like metabolites, proteins can be indicative of the biological state of an organism or tissue and aide in disease diagnosis[42]. Extensive research has been performed using human blood, plasma, serum, urine, and other easily accessible fluids to determine their diagnostic ability through proteomics. Human blood is an ideal place to find disease biomarkers due to its noninvasive collection procedures for patients and ease of access for physicians. However, the dynamic range of the plasma exceeds 10 orders of magnitude, and 90% of the protein content of plasma is derived from only 10 proteins[85] (Figure 2). Therefore, analysis of the plasma proteome requires compression of the dynamic range to obtain meaningful results.

A number of approaches have been developed to aide analysis of the plasma proteome. Immunodepletion technologies can remove 1–20 of the most abundant proteins. Commonly, columns with a stationary phase that contains antibodies for the most abundant proteins in serum/plasma are used for depletion. With these systems, the low-abundance proteins are able to flow through the column. A 2006 study from Gong *et al.* profiled the flow-through and bound fractions from two immunodepletion columns and around 10 low-abundance proteins were only detectable in depleted samples[86]. Brand *et al.* further characterized the immunodepletion process using spiking/recovery experiments. This group reported five-fold enrichment of a potential biomarker based on ELISA measurements of 11 proteins[87]. The characterizations presented in these early studies do not demonstrate if the process enhances detection of many low-abundance species in plasma, and do not address the reproducibility of the methods. Further studies expanded on these questions and found high specificity and reproducibility of depletion[88, 89]. However, there are a number of limitations involved in the analysis of immunodepleted plasma. A comparison of protein identifications from equal amounts of human colon tumor cell line and immunodepleted plasma protein samples revealed a 10-fold greater number of detected proteins in the cell line samples. This is due to the fact that the most abundant proteins in plasma are sampled far more than the most abundant proteins in this cell lysate. Despite the abundance bias, immunodepletion enhanced detection of nontargeted proteins by 4-fold and increased the number of detected proteins by 25%[90]. This area of sample preparation has progressed over the last decade, but more work needs to be done to enhance detection of low-abundance species in plasma. The steep concentration difference between high-abundance and low-abundance analytes makes this an incredibly challenging biological fluid for in-depth proteomic analysis.

Analyses of post-translational modifications (PTMs) of proteins are also an area of interest for disease biomarkers. The most commonly studied PTMs include phosphorylation, glycosylation, methylation, ubiquitination, acetylation, oxidation, and nitration[91]. PTMs are commonly monitored in bottom-up experiments, but modified peptides are detected at substoichiometric levels compared to their unmodified counterparts. Therefore, robust detection of PTMs often requires enrichment from complex mixtures, and reproducibility is lower than general bottom-up proteomics experiments. Despite these challenges, new

sample preparation techniques and increasingly sensitive mass spectrometers make protein PTMs a promising area of research for biomarker discovery.

6.1 Proteomics for biomarkers of sepsis

Animal Studies—A number of studies over the last decade have examined proteomic changes in animal models of sepsis. Early studies of the blood proteome of CLP mice were analyzed using 2D-geLC-MS/MS to identify 62 proteins that changed significantly during sepsis. The proteins that were significantly altered were involved in immune modulation, nutrient transport, structural repair, metabolism, and leukocyte activity. In particular, they found α-2 HS glycoprotein to be reduced by 50% 24 hours after the onset of sepsis. This protein is important for calcium homeostasis, and modulates insulin sensitivity. Control of plasma glucose is known to correlate with patient survival. They also detected decreased levels of leucine-rich- α-glycoprotein, which is associated with granulocyte differentiation. This change corresponds to a decrease in peripheral leukocyte numbers during sepsis[92]. Another group pursued a similar study of CLP mouse plasma using 2D electrophoresis and MALDI-TOF/TOF MS[93]. The researchers confirmed upregulation of plasma proteins related to inflammatory processes that were reported by other investigators, including α-1-antitrypsin, hemopexin, kininogen, α1-acid glycoprotein, apolipoprotein A-1,and complement C3. An additional investigation of plasma from CLP rats found tubulin alpha 4A, vinculin, and tropomyosin to be upregulated, while complement components including C3, C6, and C9 were downregulated[94]. However, most of these proteins are highly abundant in plasma and are increased during inflammatory responses in the body. Therefore, they are unlikely to act as useful biomarkers of sepsis.

Additional mouse studies demonstrated that the redox state of proteins can be an important marker for disease. In an endotoxaemic mouse model of sepsis, mice were injected with LPS and monitored 6–18 hours after injection. Cardiac tissue was chosen for proteomic analysis due to the cardiovascular dysfunction from acute organ failure (AOF) that can be observed during sepsis. There was pronounced upregulation of pentraxin 3 (PTX3) in LPS-treated mice. Levels of octameric, tetrameric, and monomeric PTX3 were measured in septic patients without organ failure. PTX3 levels were monitored 1–6 and 11 days after admission to the ICU. Over the course of 11 days, transformation of octameric to monomeric PTX3 was associated with a greater 28-day survival rate. PTX3 oligomerization predicted survival as early as 2 days post-admission. The redox state of PTX3 could provide more information than total PTX3 regarding cardiac damage in sepsis, and PTX3 multimers could play a role in the host response in sepsis[95].

Animal models have also been used to study acute kidney injury (AKI), which is a frequent complication during sepsis. One group induced sepsis in young and old mice using uterine ligation followed by bacterial inoculation. Mouse urine from septic mice with and without AKI were compared, and high levels of the proteins acidic mammalian chitinase (CHIA), chitinase 3-like protein-1 (CHI3L1), and chitinase 3-like protein 3 (CHI3L3) were only detectable in mice with AKI. Both CHI3L1 and CHI3L3 are members of the chitinase-like protein family that are produced by activated macrophages and neutrophils during sepsis[96]. Their specific role in septic AKI requires further study. In urine samples from human septic

patients, CHI3L1 was markedly higher in those with AKI than those without. CHIA was less successful in discriminating sepsis with AKI from sepsis without AKI when analyzed in human samples[97]. Further studies of septic AKI have been pursued in larger mammals, including pigs. Analysis of protein alterations from swine kidney cortical biopsies at 12 and 22 hours of sepsis was performed using 2D gel-based LC-MS/MS. Twenty proteins involved in endoplasmic reticulum stress, oxidative stress, mitochondrial energy metabolism, immune signaling, and tubular transport were found to be altered due to sepsis with AKI[98]. Although mouse models have clear advantages in cost, safety, and time when compared to larger mammals, genomic data suggests mouse inflammatory models are not readily translatable to humans[99].

Since the advent of the field of proteomics, DDA has become a well-established and popular method for discovery-based experiments. However, sampling of peptides using DDA is limited to the most abundant species present, and extensive fractionation is required for sufficient depth into the proteome. A new acquisition method, termed “data-independent acquisition” (DIA) uses a user-defined precursor retention time and fragment ion spectral library to perform peptide and protein identifications. This strategy is termed “SWATH MS”[100]. Dynamics of the blood plasma proteome during severe sepsis were characterized using SWATH-MS in mice infected with *S. pyogenes*[101]. A spectral library of proteins was created from plasma and organs in healthy mice, and the library was used to measure how surrounding tissues, organs, blood vessels, and cells influence the plasma composition (Figure 3). 786 proteins were identified from <1 μL of non-depleted plasma, which is threefold higher than comparable DDA experiments. They observed a dose-dependent increase in acute phase proteins, coagulation proteins, and cytokines that have been previously established as biomarkers of sepsis. However, biomarkers involved in vasodilation did not perform successfully in this system. Using DIA allows for the measurement of several biomarker groups and supports the future discovery of new biomarkers in blood plasma[101].

Human Studies—Proteomics studies have examined human biological fluids, tissues, and cells, to discover new biomarkers for sepsis and other complications. The first quantitative study of human plasma protein changes after LPS injection was performed over a decade ago. A number of mediators of the inflammatory response, including LPS binding protein, CRP, and serum amyloid A, were found to be upregulated using label-free quantification[102]. This group further characterized plasma after LPS injections using $^{16}O/^{18}O$ labeling for protein quantification 9 hours after LPS injection. When both studies are combined, a number of proteins were found to be consistently upregulated. These include CRP, LBP, SAA, SAA2, and hepatocyte growth factor-like protein. Fewer proteins were found to be upregulated when oxygen metabolic labeling was employed for quantification[103].

A year later, Shen *et al.* examined plasma from 25 SIRS and 25 sepsis patients and used three dimensions of separation to maximize identifications in immunodepleted plasma. Seven proteins showed an increase in plasma from sepsis patients compared to SIRS, while three showed a decrease. Upregulated proteins included C4, CRP, plasminogen precursor, apolipoprotein A-II, plasma protease C1 inhibitor precursor, transthyretin precursor, and serum amyloid P-component precursor. Apolipoprotein A-I precursor, antithrombin-III

precursor, and serum amyloid A-4 protein precursor were found to be downregulated. The gene ontology categories of proteins in this data were similar to a number of studies previously discussed. The acute-phase response, complement activation, and blood coagulation were found to be overrepresented. Furthermore, KEGG analysis revealed the complement and coagulation cascades were the most relevant pathways found to be altered in these samples. Early studies of septic patient plasma confirmed a number of biomarkers, including CRP, and established changes that occur in major pathways of interest in patient plasma[104].

Studies of protein PTMs in human plasma have proven to be a useful source of potential biomarkers. N-linked glycosylation is the most common glycan modification on proteins in humans. They are often found on extracellular proteins and can act as therapeutic targets or biomarkers of disease. DeCoux *et al.* enriched the N-linked plasma glycoproteome from the plasma of septic patients. Plasma samples from survivors and non-survivors were analyzed and compared. Glycopeptides were isolated by solid-phase extraction using hydrazide chemistry and quantified using label-free mass spectrometry. 501 glycopeptides were identified corresponding to 234 distinct proteins. Researchers found a set of 66 glycopeptides that were unique to sepsis survivors, and 60 glycopeptides unique to non-survivors. Survivors relied on the extrinsic pathway of the complement and coagulation cascade, while non-survivors relied on the intrinsic pathway. Non-survivors had higher levels of total kininogen and decreased cathepsin-L1, periostin, and neutrophil gelatinase-associated lipocalin. Previously detected markers of sepsis, including NGAL and VCAM, were found to be downregulated in non-survivors according to their glycopeptide levels. Although these proteins are found to be upregulated in sepsis, they may be part of a beneficial immune response that contributes to survival[105].

The majority of septic patients can be classified as part of the neonatal or elderly population. However, all patients aged 65 and older are generally classified as members of the elderly population. This range represents a large span of ages for patients to fall into. The molecular mechanisms associated with age-related risk for sepsis are not fully elucidated. One study examined plasma samples from patients with community-acquired pneumonia who did and did not develop sepsis. These populations were further divided by age into two categories: patients aged 50–65 and patients aged 70–85. In this study, 18 proteins from the acute phase response, including CRP, LBP, and A1ACT, were found to be differentially expressed in younger adults. However, these proteins had lower concentrations in older adults who later developed severe sepsis. This data indicates that the hypoinflammatory response in older adults may increase the risk of severe sepsis. Younger adults with severe sepsis were found to have higher levels of members of the coagulation pathway, including fibrinogen, and von Willebrand factor, than older adults. This difference reveals that reduced coagulation activity may contribute to increased mortality in older adults with severe sepsis. Lipid metabolism was also found to differ between the younger adults and older adults. Increased levels of Apo M in older adults indicated a reduced inflammatory state, which could also lead to increased mortality. The findings from this thought-provoking study demonstrate the proteomic differences due to age in severe sepsis, and indicate that age may be an important variable that needs to be controlled during biomarker studies[106].

Urine is a biofluid of interest for sepsis research because of the kidney damage known to occur as sepsis progresses. A number of studies have used proteomics to examine human urine for biomarkers. A 2013 study used iTRAQ-based quantitative proteomics to monitor protein levels in SIRS and sepsis patients. Cadherin-1 (CDH1), Haptoglobin, C3, SERPINA, and CP were all found to be altered between SIRS and sepsis patients with normal patients in the early stages of ICU admission. Haptoglobin, C3, and CP all confirmed other studies of sepsis involving haptoglobin levels and the complement system as biomarkers of sepsis[104, 107, 108]. CDH1 is important in endothelial cell adhesion, and dysregulation of this protein can cause excess shedding of proximal tubule epithelial cells and the kidney. CDH1 was upregulated more in patients with sepsis than those with SIRS, and could play a role in the tissue damage found in sepsis[109]. A similar study using iTRAQ was performed by Su *et al.* comparing urine from SIRS patients with sepsis patients 24 hours after admission and 48 hours before death. For sepsis prognosis, selenium binding protein-1, heparin sulfate proteoglycan-2, alpha-1-B glycoprotein, haptoglobin, and lipocalin were found to be upregulated. Lysosome-associated membrane protein-1 (LAMP1) and dipeptidyl peptidase-4 were found to be downregulated. These findings require validation in a larger sample size of clinical specimens[110].

Blood can be separated to analyze the content of a number of immune cell types by proteomics. Neutrophils, platelets, and mast cells have all been examined for their utility in diagnosing sepsis. Neutrophils are classified as polymorphonuclear cells (PMNs) due to their lobed nuclei and are the most abundant phagocyte cell type in humans[111]. Malmström *et al.* used mass spectrometry to perform a discovery-based screen of neutrophil proteins followed by a targeted approach to detect secreted neutrophil proteins in the plasma. Neutrophils comprise nearly 60% of all leukocytes, and they secrete proteins into the plasma quickly and abundantly upon stimulation[112]. The presence of these cells allows for a new pool of biomarkers for discovery in an easily accessible patient sample. A number of proteins, including Defensin alpha-1, myeloperoxidase, resistin, Orosomucoid-1, and Haptoglobin were found to be upregulated, while CD44 antigen, Granulins, NGAL, Serotransferrin, Catalase, and others were found to be downregulated. Most of these proteins participate in the host defense against infection or oxidative stress.

Platelets from septic patients have also been explored by mass spectrometry to find biomarkers. Platelets play an important role in coagulation in the blood, which is known to be altered in sepsis[113]. Platelets accumulate in the vasculature of organs during sepsis as they progress to organ failure and play a role in inflammation and pathogenesis during infection[114]. The proteome of platelets isolated from six patients with severe sepsis or septic shock was compared to healthy donors using 2DE-MALDI-TOF-MS. Five proteins were detected with increased expression, including EFCAB7, actin, IL-1β, GPIX, and GPIIB and are involved in processes including calcium binding, the acute phase response, cell structure and integrity, platelet aggregation, and thrombosis[115]. The pathways altered in platelets are similar to other components of blood and changes in the major pathways of inflammation are readily detected.

The majority of biomarker studies search for a single marker to predict diagnosis or prognosis of sepsis. Researchers have expanded on this concept by searching for a panel of

biomarkers to differentiate the stages of sepsis or predict mortality. A 2010 study found that measurements of IL-1α, IP-10, sTNF-R2, and sFAS could indicate the progression from sepsis to septic shock. Furthermore, a combined measurement of MMP-3, IL-1α, IP-10, sIL-2R, sFas, sTNF-R1, sRAGE, GM-CSF, IL-1β and Eotaxin could differentiate survivors from non-survivors. However, this study only used 16 total patients[116]. Further studies combined pro- and anti-inflammatory markers to predict mortality in sepsis. Examination of 29 patients with severe sepsis or septic shock showed that levels of IL-6, IL-8, and MCP-1 were higher in non-surviving patients[117]. Like the previous studies with single biomarkers, these biomarker panels have not been validated in large-scale studies and prepared for clinical implementation. Recently, a study of 300 septic and non-septic patients showed that age, gender, CRP, and PCT levels could be combined to make a positive diagnosis of sepsis. Individually, these markers did not show diagnostic significance, but the combination improved diagnostic capabilities. Though this study is larger than most biomarker studies for sepsis, it still needs validation in more patients in multiple geographic areas[118]. This multi-marker approach may yield more useful diagnostic data with previously discovered biomarkers in the future.

6.2 Proteomics of neonatal sepsis

The search for biomarkers using proteomics has also been extended to neonatal sepsis. This field has primarily been pioneered over the last decade through work performed by Buhimschi *et al.*[107, 119–122]. They have examined amniotic fluid and cord blood from infants that were born prematurely to detect biomarkers of inflammation, infection, and neonatal sepsis. In their first study, they analyzed the proteome of amniotic fluid from 104 women using surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry. In this approach, target proteins adhere to a surface while contaminants and excess proteins are washed away. Proteins are then cocrystallized with matrix and ionized by a laser[123]. Using this method, researchers could detect the presence of proteins found in amniotic fluid. Patients with intra-amniotic inflammation and preterm births had altered levels of neutrophil defensins-1 and -2, and calgranulins A and C[119]. They used this information to create a “Mass Restricted” (MR) score, which when combined with Gram stain, was the best predictor of infection in amniotic fluid[120].

Researchers next explored the cord blood proteome of premature newborns for markers of EONS using 2D-gel based-LC-MS/MS. 19 proteins classified as transfer, carrier, immunity, defense, or extracellular matrix proteins were found to be differentially expressed in the cord blood of infants with EONS. There were also changes in proteins synthesized by hepatic parenchymal cells, including haptoglobin. The authors postulate that exposure to intra-amniotic infection (IAI) leads to a “switch-on” in haptoglobin and haptoglobin-related protein expression, representing a fetal adaptive response to inflammation *in utero*. Haptoglobin is nearly absent in healthy term newborns, which was termed the “switch-off” pattern. An optimal algorithm was created to combine haptoglobin, haptoglobin-related protein, interleukin-6 expression, and neonatal hematological indices to discriminate two newborn populations with low and high probability of IAI exposure. The agreement between this classification algorithm and clinical EONS was moderate, with an agreement in 70% of

cases[107]. This study represents a promising new step forward in the diagnosis of neonatal sepsis and the translational nature of proteomics for the clinic.

As discussed in the context of metabolomics earlier, a recent study by Stewart *et al.* examined the metabolomic and proteomic profile of serum from preterm infants with LONS. α-2-Macroglobulinha and α-1-antitrypsin were the most abundant proteins detected, but showed no association with poor health in neonates. α-fetoprotein levels decreased from week 14 of gestation, but likely just relates to the prematurity of neonatal patients. In their proteomic evaluation, the authors also found Haptoglobin expression levels to be associated with LONS. Other proteins associated with LONS included plasma membrane calcium-transporting ATPase 4, Transthyretin, and U5 small nuclear riboprotein 200 kDa helicase[124]. Like many biomarker studies, it is important to expand this work into a larger patient cohort to test the validity of their findings. It is ideal for studies to combine multiple “omic” data sets in this manner and form a panel of potential biomarkers for the most comprehensive analysis of disease systems. A summary of some of the biomarkers of sepsis found using metabolomics and proteomics studies can be seen in Table 2.

7. Conclusions and Future Directions

To date, the biomarkers found in metabolomics and proteomic studies of sepsis have not met the thresholds for clinical implementation. There are a number of limitations of metabolomics and proteomics studies that hinder biomarker progression into the clinic. Each study is performed with different sample preparation, data acquisition, and data analysis parameters that result in variable metabolic and proteomic signatures. In addition, the lack of concrete diagnostic criteria for sepsis makes it difficult to collect samples at the same point in sepsis progression. There are large variations in patient sample collection time, type, and storage that make it difficult to repeat experiments consistently in mulitiple locations. Investigations using multiple protein, metabolite, or gene markers may provide more successful routes for diagnosis. The most successful marker may not be found in the low-abundance areas of the metabolome or proteome. Future research in the field focusing on the changes that occur in high-abundance molecules due to massive physiological changes may have more use for diagnosis of SIRS, sepsis, severe sepsis, or septic shock.

The limited ability of animal models to accurately recapitulate the physiological changes of human sepsis adds another layer of difficulty to biomarker detection. As discussed above, LPS and CLP murine models show serious physiological discrepancies when compared to human septic patients. One suggested improvement is to use aged mice, which are more susceptible to CLP and LPS induction of sepsis[125]. “Humanized mice” are another alternative to increase the efficacy of murine models of sepsis. This process involves transplanting cord blood hematopoetic stem cells into nude mice, which allows them to develop a number of human immune cell types and a more realistic immune response to stimuli[126]. However, the expense involved in generating these mice precludes its widespread utility in most laboratories. Therefore, the most logical step forward for proteomics and metabolomics studies would involve more complex animal models, such as sheep, baboons, or monkeys. Without an appropriate model system, “omics” animal studies for biomarkers are unlikely to reach clinical implementation.

The use of metabolomics and proteomics in sepsis offers a wealth of new information regarding the physiological state of the patient and possible diagnostic and prognostic markers. The hyperinflammatory and hypoinflammatory responses in sepsis can be seen by monitoring patients with metabolomics or proteomics, and their findings regarding these fundamental pathways corroborate previously known pathways in sepsis progression[127]. Pathways including oxidative stress, fatty acid metabolism, the complement system, coagulation, and the acute-phase response were consistently found to be altered during sepsis by a large number of studies despite variation in sample preparation and acquisition. This consistency reveals important findings regarding the host response in sepsis and potential pathways to find robust biomarkers for the disease and offer hope for more precise clinical tests.

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Biography

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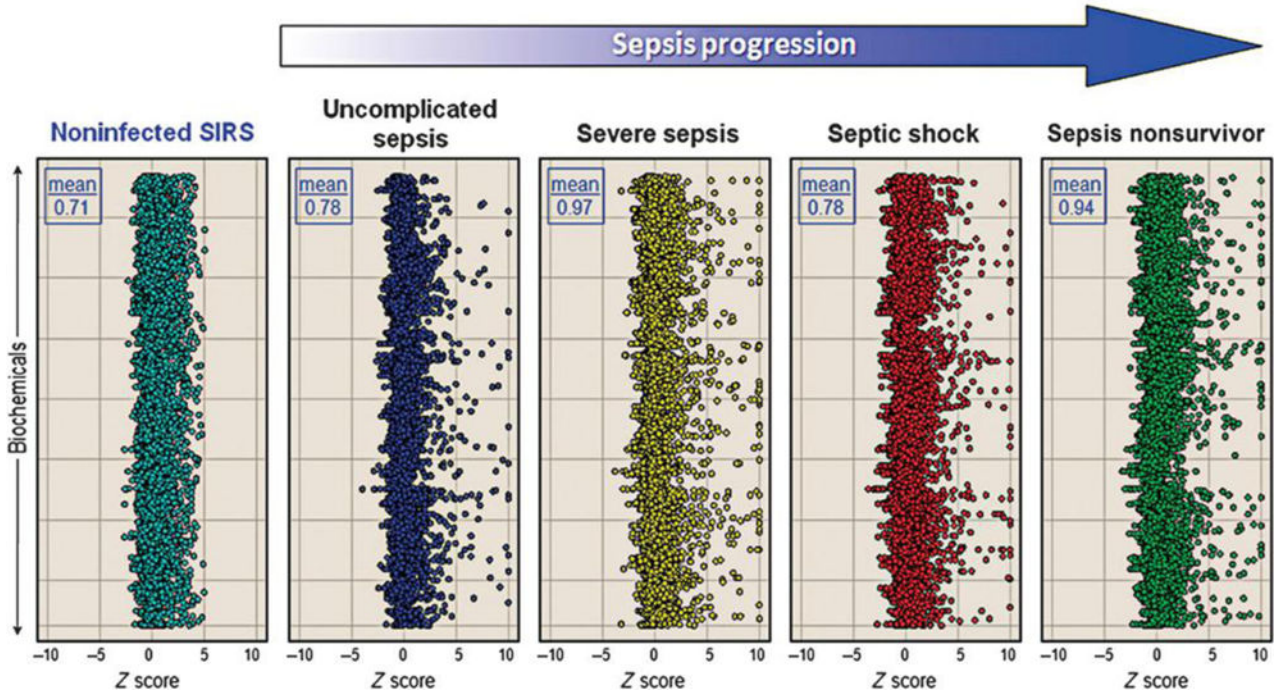

Figure 1.

Z-score distributions for metabolites profiled during sepsis progression. Zero score on the x-axis indicates the mean of the control group, and Z-scores represent the number of standard deviations from the mean of the controls. Figure from Langley *et al.* [63]. Reprinted with permission from AAAS.

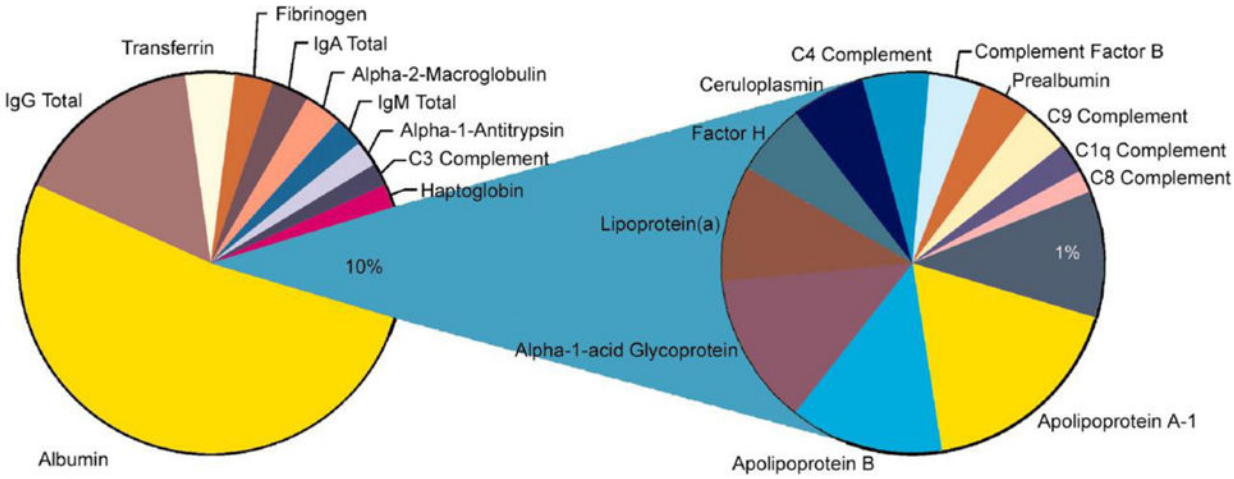

Figure 2.
Proteomic composition of serum. Figure adapted with permission from Tirumalai *et al.* [76]

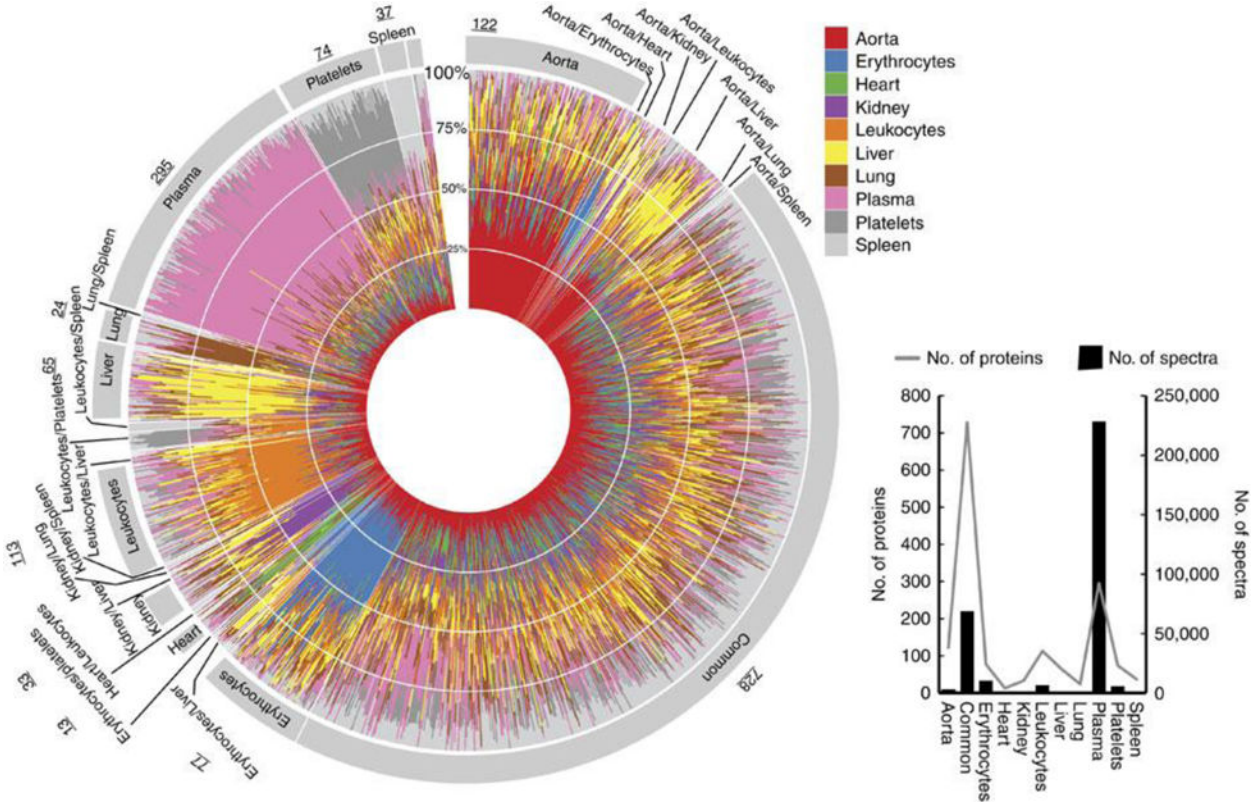

Figure 3.

Circular polar histogram showing the distribution of the protein intensity across organs and cells. Spectra were used to create a library for data-independent acquisition and SWATH-MS analysis. Figure adapted with permission from Malmstrom *et al.* [87]

Table 1

Diagnostic criteria of sepsis. Figure adapted with permission from Reinhart *et al.*[10]

I. Confirmation of infection Diagnosis of an infection on the basis of microbiological evidence or clinical criteria
II. Systemic inflammatory host response (SIRS) (at least two criteria) • Fever (≥38°C) or hypothermia (≤36°C) confirmed by rectal, intravascular, or intravesical measurement • Tachycardia: heart rate ≥90 bpm • Tachypnea (frequency ≥20/min) or hyperventilation (PCO_2 ≤4.3 kPa/<33 mm Hg) • Leukocytosis (≥1200/mm^3)or leukopenia (≤4000/mm^3) or ≥10% immature neutrophils in differential blood count
III. Acute organ dysfunction (at least 1 criterion) • Acute encephalopathy: reduced alertness, disorientation, agitation, delirium • Relative or absolute thrombocytopenia: decrease in platelet counts by more than 30% within 24 hours or a platelet count of less than 100,000/mm^3. Thrombocytopenia due to acute hemorrhage or immunological causes must be ruled out • Arterial hypoxemia: PaO_2 ≤ 10 kPa (≤75 mmHg) while breathing ambient air or a PaO_2/FiO_2 ratio of ≤33 kPa (≤250 mmHg) on oxygen administration. A clinically manifest heart or lung disease must be ruled out as a cause of hypoxemia. • Renal impairment: diuresis of ≤0.5 mL/kg/h for at least 2 hours despite adequate volume resuscitation and/or an increase in serum creatine level to > twice the upper limit of normal • Metabolic acidosis: Base excess of ≤ 5mmol/L or lactate concentration of > 1.5 × upper limit of normal
Sepsis: criteria I and II, **Severe Sepsis:** criteria I, II, and III **Septic Shock:** criteria I and II, as well as a systolic arterial blood pressure of ≤ 90 mmHg for at least 1 hour, or mean arterial pressure of ≤65 mmHg, or the necessity of vasopressor administration to maintain a target systolic arterial pressure of ≥90 mmHg or mean arterial pressure of ≥65 mmHg. Hypotension persists despite adequate fluid resuscitation and cannot be explained by other causes.

Table 2

Summary of biomarkers found in metabolomics and proteomics studies of sepsis.

Metabolomics:-				
Biomarker-	**Model/Sample-Type-**	**Altered-Pathways-**	**Method-**	**Reference-**
Hypoxanthine,-indoxyl-sulfate,-gluconic-acid-	Thermally-injured-CLP-mouse-model-	Oxidative-stress-	UPLC?QTOF?MS-	66-
Salicylic-acid,-palmitoyl-lysolecithin,-citrulline-	Equine-gut?Induced-sepsis-	Fatty-acid-synthesis-	UPLC?TOF?MS-GC?MS-	69-
S?(3?methylbutanoyl)?dihydrolipoamide?E,-lactitol-dehydrate,-N? nonanoyl?glycine,-and-S?)henyl?D?ysteine-	Serum-from-septic,-SIRS,-and-healthy-patients-	Coagulation-	HPLC?MS/MS-	71-
Multiple-carnitine-esters,-fatty-acids,-fatty-acid-transporters,-GPC-and-GPE-esters-	Sepsis-patient-serum-at-multiple-time-points-	Fatty-acid-oxidation,-gluconeogenesis,-TCA-cycle-	UPLC?MS/MS-GC?MS-	72-
C10:1-and-PCaaC32:0-	Blood-samples-from-septic-patients-	Lipid-Production-	Targeted-LC?MS/MS-	74-

Proteomics:-				
Biomarker-	**Model/Sample-Type-**	**Altered-Pathways-**	**Method-**	**Reference-**
α?2-HS-glycoprotein-	CLP-mouse-model-	Calcium-homeostasis-	2D-gel?based-LC? MS/MS-	92-
Pentraxin-3-	LPS-mouse-model-	PTX3-oligomerization-	LC?MS/MS-	95-
C4,-CRP,-plasminogen-precursor,-apolipoprotein-A?II,-plasma-protease-C1-inhibitor-precursor,-transthyretin-precursor,-and-serum-amyloid-P? component-precursor-	Serum-from-septic,-SIRS-patients-	Acute?phase-response,-Complement-activation,-and-Blood-coagulation-	Immunodepletion-LC? MS/MS-	104-
Kininogen,-cathepsin? L1,-periostin,-and-neutrophil-gelatinase? associated-lipocalin-	Septic-patient-plasma-	Complement-and-coagulation-cascade-	N?linked-glycoproteome-enriched-LC?MS/MS-	105-
EFCAB7,-actin,-IL?1 β,-GPIX,-and-GPIIB-	Platelets-from-septic-patients-	Calcium-binding,-Acute-phase-response,-Cell-structure-and-integrity-	2D3?MALDI?TOF?MS-	115-