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Survival of the littlest: improving preterm outcomes through metabolomics and microsampling

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CHAPTER I

General Introduction and Scope

Introduction

Global burden of preterm birth

The World Health Organization defines preterm birth as a live birth occurring before the completion of 37 weeks of gestation or within 259 days from the onset of the mother's last menstrual cycle.[1] Every year, an estimated 13.4 million babies are born too soon, accounting for 1 in 10 live births worldwide.[2] The spectrum of preterm birth spans a critical window of fetal development, with approximately 85% occurring between 32 and <37 weeks of gestation (moderate-to-late preterm), 10% between 28 and <32 weeks of gestation (very preterm), and 5% before 28 weeks of gestation (extremely preterm).[3] Along this gradient of prematurity, lower gestational age at birth is associated with a decline in survival probability and a pronounced increase in the risk and severity of health complications.[4] Prematurity-associated complications are the leading cause of mortality in children under the age of five, with approximately half of these deaths occurring within the first 28 days of life (neonatal period).[2,5,6] Beyond the risk of mortality, preterm birth survivors are at an increased risk of developing a myriad of short- and long-term morbidities, profoundly impacting their quality of life.[7] These adverse health outcomes stem from the premature termination of intrauterine development, arising from heterogenous etiologies and resulting in the incomplete maturation of vital organs (**Figure 1**). This physiological immaturity manifests as functional deficits that preclude effective extrauterine adaptation, predisposing preterm infants to multisystem morbidities. These include neurological conditions, such as intraventricular hemorrhage and cerebral palsy; respiratory disorders, like respiratory distress syndrome and chronic obstructive pulmonary disease; cardiovascular dysfunctions, such as patent ductus arteriosus and hypertension; metabolic disorders, like hypoglycemia and dyslipidemia; gastrointestinal complications, such as necrotizing enterocolitis and irritable bowel syndrome; and immune dysregulations, like sepsis and allergy.[7,8]

The consequences of preterm birth extend far beyond clinical implications for individuals born preterm, imposing substantial socioeconomic burdens on families, healthcare systems, and societies worldwide (**Figure 1**).[9,10] Parents of preterm infants are at a heightened risk of mental health challenges, with studies reporting increased prevalence of anxiety, depression, and parenting stress, which can persist for years after birth.[10,11] The high out-of-pocket expenditures for specialized medical care and frequent hospitalizations, compounded by lost income from extended parental leave and career disruptions, can lead to economic instability and place significant strain on family dynamics and relationships.[12] The financial burden on the healthcare system is equally profound, as demonstrated by the 2016 United States birth cohort analysis, which estimated the total cost of preterm births at $25.2 billion, disproportionately driven by extremely preterm infants whose mean incremental lifetime

costs were 12 times higher than those born moderately preterm.[13] The escalating costs are primarily driven by the need for prolonged hospitalization in neonatal intensive care units, advanced medical interventions, and a highly specialized workforce. These factors necessitate a significant reallocation of hospital resources, potentially impacting other areas of hospital operations. Furthermore, preterm-born individuals often require long-term medical, psychological, educational, or vocational support due to neurodevelopmental impairments, learning disabilities, and chronic health conditions. Longitudinal studies have shown that preterm-born individuals exhibit lower academic achievement with reduced university enrollment and graduation rates, lower earning potential, and greater employment instability, compared to their term-born peers.[9] These challenges translate into significant productivity loss due to diminished workforce participation and human capital. This societal impact is more severe in low-income countries due to higher preterm birth rates, limited access to quality neonatal care, and inadequate long-term support systems.[2]

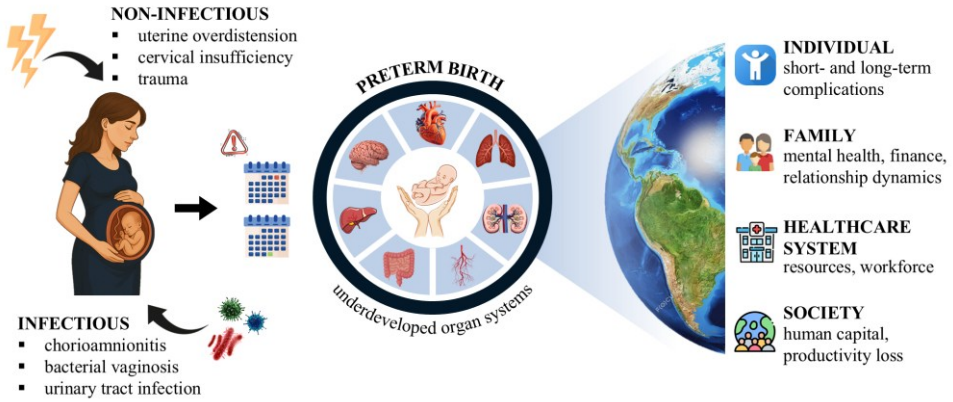

Figure 1. Illustration of the global burden of preterm birth. The figure depicts the heterogenous etiologies of preterm birth, the resultant immaturity across multiple organ systems, and the broad-reaching impact on the individual, families, healthcare systems, and society at large.

Therefore, preterm birth represents a major multifaceted global healthcare challenge with far-reaching clinical, economic, societal, and intergenerational repercussions. To minimize these consequences and improve preterm outcomes, advancements in perinatal care, such as early interventions, accurate diagnostics, and precision medicine, are imperative.[14,15] However, effective management of these critical needs, which offers a promising avenue to enhance survival rates, reduce health complications, and promote healthier developmental trajectories, is complicated by the unique vulnerabilities of preterm infants.

Challenges in preterm perinatal healthcare

Preterm perinatal care presents a complex set of challenges due to the distinct and rapidly evolving physiology of fetuses and preterm neonates, which differs markedly from term-born

neonates and adults.[16–18] The physiological immaturity resulting from premature birth places preterm neonates at a functional disadvantage, with deficits in surfactant production, cerebrovascular regulation, renal clearance, and gut and skin barrier integrity, compared to the term-born neonates.[19,20] Beyond immaturity, neonatal physiological systems are functionally distinct from those of adults, as they are uniquely configured to support the transitional demands of early extrauterine life. Key processes, such as hepatic metabolism, immune surveillance, and cardiovascular regulation, operate through different mechanisms, prioritizing developmental needs over efficiency or resilience; metabolic pathways in the liver are oriented towards anabolic processes rather than detoxification and maintenance of homeostasis, immune system is primarily biased towards tolerance, and cardiovascular system is characterized by transitional circulation rather than optimized hemodynamics.[21–23] This immaturity coupled with functional distinctiveness across multiple organ systems collectively engenders heterogenous, atypical, subtle, or non-specific presentations of medical conditions and responses to treatments, complicating diagnostic precision and therapeutic decision-making.[24,25]

Such challenges may arise even before birth, at the point of antenatal risk assessment and management of preterm labor. While some cases of preterm birth are the result of planned interventions due to maternal or fetal complications, a substantial proportion occurs spontaneously. Spontaneous preterm births often arise from multifactorial and poorly understood mechanisms, including both infectious and non-infectious origins, that are challenging to detect at the time of presentation.[26] This diagnostic uncertainty frequently leads to precautionary interventions which may prove unnecessary if preterm birth does not follow. After preterm birth, physiological vulnerabilities complicate neonatal care and increase the propensity for rapid clinical deterioration. A major contributing factor is compromised host defense, which renders preterm neonates highly susceptible to infections, where even minor insults can escalate into life-threatening conditions such as sepsis.[27] Vigilant monitoring and prompt interventions are therefore essential to prevent fatal outcomes. However, this urgency increases the risk of misdiagnosis, leading to overtreatment or delayed care, potentially resulting in unintended adverse preterm outcomes. Furthermore, iatrogenic complications may inadvertently also occur from application of broad, generalized, treatment protocols, underscoring the need for precision medicine approaches tailored to their unique profiles and requirements.[28] Therefore, a critical challenge in preterm perinatal care involves striking a precarious balance between diagnostic accuracy and timely, tailored interventions. To achieve this, a deeper understanding of preterm neonatal pathophysiology and behavior is crucial but remains largely limited by three key barriers. Foremost, ethical considerations inherent to research in vulnerable populations, which necessitate stringent protective measures, have led to strict federal regulations, imposing

significant constraints in conducting neonatal research.[29] Secondly, the physiological instability of preterm neonates imposes methodological limitations. For instance, the limited blood volume in preterm neonates renders frequent sampling for research purposes clinically inadvisable and ethically contentious as it poses significant risk of iatrogenic anemia or hemodynamic instability.[30] Lastly, parental apprehensions surrounding informed consent further restricts participant recruitment, as caregivers understandably hesitate to subject their fragile infants to experimental protocols.[31] These factors impede the advancement of preterm neonatal research, thereby rendering accurate diagnosis and evidence-based treatment strategies an ongoing healthcare challenge.

Spontaneous preterm birth prediction

Preterm birth (PTB) may either occur spontaneously (sPTB), with intact membranes or following preterm premature rupture of membranes, or be medically indicated (iatrogenic PTB) in response to maternal or fetal complications.[26] Iatrogenic PTBs are typically associated with well-defined, identifiable, clinical indications such as maternal hypertension, fetal growth restriction, and placental abnormalities, allowing for informed clinical decision-making and better management of maternal and neonatal outcomes. In contrast, the multifactorial and heterogenous etiology of sPTB, arising from factors such as inflammation, maternal infections, cervical insufficiency, uterine distension, and genetic predisposition, remain poorly understood complicating accurate prediction and risk stratification. Current approaches for sPTB prediction focus on structural, biochemical, or clinical markers that primarily reflect risk associations or downstream effects rather than mechanistic determinants.[32] Cervical length assessment via transvaginal ultrasound represents a key screening modality, with cervical shortening below 25 mm serving as a robust indicator of increased risk for sPTB. However, its predictive performance, particularly when employing a universal cut off, may be suboptimal due to variability influenced by gestational age and population-specific characteristics.[33–35] Moreover, the requirement of ultrasonographic equipment and operator expertise constrains its widespread implementation across diverse healthcare ecosystems.[32] Similarly, fetal fibronectin testing detects an extracellular matrix glycoprotein released from fetal membranes in cervicovaginal secretions, indicating a disruption of the maternal-fetal interface. Although the test demonstrates a high negative predictive value, its poor positive predictive value limits its ability to reliably identify true cases of imminent PTB.[36] Further, PTB risk stratification often involves assessing maternal risk factors such as demographic characteristics (*e.g.* age, ethnicity, socioeconomic status), obstetric history (*e.g.* prior preterm births, multiple gestations), behavioral factors (*e.g.* smoking, alcohol consumption), and comorbid medical conditions (*e.g.* depression, diabetes).[37] While such assessments offer a broad framework for identifying at-risk individuals, their performance remains limited due to significant heterogeneity in risk

associations across populations and PTB subtypes.[37,38] Despite employing diverse techniques, these current predictive tools misclassify a substantial proportion of pregnancies, with studies showing that nearly 40% of those suspected of imminent PTB ultimately result in term deliveries.[39]

The high rate of false-positives in sPTB prediction has critical implications for preterm neonatal health. The suspicion of imminent PTB prompts immediate initiation of preventive and protective interventions, such as tocolytics to delay labor to allow time for fetal therapy, antenatal corticosteroids to accelerate fetal lung maturation, and antibiotics to manage and treat potential maternal infections. While these interventions are life-saving for fetuses truly at risk of PTB, their administration to those that reach full term can lead to unintended adverse consequences. Unnecessary fetal exposure to corticosteroids has been associated with short- and long-term adverse outcomes, including reduced fetal growth velocity, increased risk of admission to neonatal intensive care units, neonatal hypoglycemia, enhanced susceptibility to infections, and a higher likelihood of neurodevelopmental and behavioral disorders.[39–41] In addition, the efficacy of corticosteroids is maximized with minimal side effects when administered 2-7 days prior delivery, and multiple courses are not recommended due to increased risk of adverse outcomes.[42,43] This critical challenge underscores the urgent need for highly precise tools for accurately predicting sPTB to ensure an effective management of at-risk pregnancies, ultimately safeguarding the health and development of preterm infants while minimizing iatrogenic harm to those born at term. Development of such predictive tools requires a shift from reliance on non-specific clinical indicators toward the identification of underlying mechanistic pathways, thereby facilitating targeted and timely interventions tailored to individual risk profiles.

Diagnosis of late-onset sepsis

Neonatal sepsis refers to a systemic infection of bacterial, viral, or fungal origin that occurs within the first month of life. While the third international consensus definition for sepsis, defined as a life-threatening organ dysfunction caused by a dysregulated host response to an infection, provides a valuable diagnostic framework for adult sepsis, its translation into the neonatal context is limited.[44] This limitation stems from its failure to accommodate the unique physiological characteristics of a neonate and the gestational- and postnatal-age dependent variability in clinical presentation.[45–47] The neonatal immune system is functionally distinct compared to adults, primarily due to its reliance on innate immunity and perinatal adaptations driven by epigenetic reprogramming. Such adaptations develop to preserve feto-maternal tolerance, navigate the transition from a sterile intra-uterine milieu to an environment rich in foreign antigens, ensure protection against infections, and promote tolerance of microbial colonization at birth.[48] These unique physiological demands of a neonate result in a bias against T_H1-cell-polarizing cytokines, rendering them susceptible to

microbial infections.[49] The defenses against infections are further compromised in preterm neonates as a result of diminished and immature immune cell populations, and deficiency of crucial immune regulatory molecules, heightening the risk of sepsis.[16,50,51] Notably, the neonatal immune response, particularly in the context of bacterial sepsis, manifests as a disease tolerance phenotype rather than the disease resistance phenotype observed in adults.[52] This tolerogenic response aims to minimize harm from immunopathology at the cost of uncontrolled pathogen proliferation due to the high energy demands of a neonate, which necessitates prioritization of vital functions over combating pathogens.[52]

Neonatal sepsis can arise either through vertical transmission from the mother during birth or environmental sources postnatally. Based on the time of onset, neonatal sepsis can be categorized into early-onset sepsis (EOS), occurring within the first 72 hours of life, and late-onset sepsis (LOS), which manifests after 72 hours. While the incidence rate of EOS has gradually declined over the years due to advancements in screening and intrapartum antibiotic prophylaxis, LOS rates have remained unchanged posing a persistent burden to neonatal healthcare. The diagnosis of LOS is challenging due to the presence of subtle and non-specific symptoms, such as fever, abnormal heart and respiratory rates, feeding intolerance, and lethargy, which often resemble symptoms of other preterm conditions. Evaluation of these atypical manifestations are inadequate for sepsis diagnosis, necessitating a comprehensive approach incorporating various clinical criteria, laboratory thresholds for inflammatory markers and hematological indices, and microbiological data.[53] Biomarkers such as c-reactive protein (CRP), interleukin-6 (IL-6), and procalcitonin (PCT), have been shown to demonstrate a robust negative predictive value to rule out the presence of an infection and serve as prognostic markers for sepsis severity and treatment response. However, they lack the sensitivity and specificity to accurately diagnose sepsis from other non-infectious inflammatory conditions.[54] Blood cultures are the golden standard for sepsis confirmation, but their reliability is compromised by the high rate of false negatives due to limited blood sample volumes, non-culturable pathogens, and exposure to antibiotics prior sampling. Additionally, the prolonged turnaround time for microbiological results impedes their utility in clinical decision-making to initiate antibiotic therapy. Given the high-risk nature of neonatal sepsis, characterized by the rapid deterioration of health within a few hours in the absence of prompt intervention, clinicians administer broad-spectrum antibiotics to all neonates at clinical suspicion. This empirical administration results in unnecessary antibiotic use in non-septic cases leading to antibiotic resistance, development of multidrug-resistant pathogens, and gut microbiome disruption, which could worsen premature outcomes and result in long-term complications such as obesity and asthma.[55,56]

The current challenges in the diagnosis and management of LOS in preterm neonates highlight the critical need for a deeper understanding of sepsis pathophysiology in this

vulnerable population, as well as the development of more accurate and timely diagnostic tools capable of distinguishing LOS from other non-infectious inflammatory conditions. Additionally, early identification of causative pathogens is essential to guide personalized therapeutic strategies to improve preterm outcomes.

Blood sampling in preterm neonates

Blood sampling in preterm neonates presents significant challenges due to their physiological fragility, low circulating blood volume (90-105 mL/kg), and limitations of current sampling techniques. Traditionally, blood is obtained via a heel prick, arterial catheterization, or venipuncture. While arterial or venous access allow for the collection of larger sample volumes, these procedures are invasive and painful, carrying inherent risks such as infections, thrombosis, and vascular injury.[57] Heel pricks, although less invasive and widely used for capillary microsampling in neonates, are not without complications; repeated lancet-based punctures of the calcaneal region are painful and can lead to bruising, hematoma formation, local tissue damage, scarring, and an increased risk of infection.[58] Frequent blood sampling in preterm neonates can contribute to iatrogenic blood loss, necessitating blood transfusions which have been associated with increased risk of complications of prematurity.[59,60] In an observational cohort study conducted by Councilman *et al*., extremely preterm infants underwent a median of 47 blood sampling procedures within the first month of life, resulting in a cumulative blood loss equivalent to nearly one-third of their total blood volume.[59] Exposure to pain during the neonatal period has been linked to long-term neurosensory and cognitive impairments, negative impacts on psychosocial behaviors, and altered pain sensitivity.[61] These challenges necessitate careful consideration of sampling frequency and volume to minimize procedural risks while balancing the need for accurate clinical monitoring and research advancements.

Beyond the procedural risks of blood sampling, challenges arise in preserving the quality of blood samples. Given the limited volume that can be ethically and safely drawn, each sample represents an extremely valuable clinical and research resource, underscoring the imperative to ensure its optimal handling, processing, and storage. However, the integrity of these precious specimens may be compromised by technical and logistical limitations. In routine preterm clinical care, blood is often collected via heel lancing into microtubes, as liquid whole blood, or more commonly as dried blood spots (DBS). Liquid blood samples require immediate post-collection processing to isolate plasma, the preferred matrix for most biochemical analyses. This requirement imposes strict time constraints and demands access to specialized equipment and personnel, which can be particularly challenging in low-resource settings. Delays in processing can lead to degradation of analytes, compromising data quality and downstream interpretation. Furthermore, plasma samples require cold-chain storage and transportation to ensure stability, adding logistical complexity and increasing

overall costs.[62] DBS sampling circumvents these limitations by enabling the collection of small volumes of blood without the need for immediate processing or cold-chain requirements. DBS samples are collected by spotting blood droplets onto specialized filter paper, which are air-dried at room temperature to produce a stable dried matrix that allows short-term storage and transportation under ambient conditions.[63] The drying process inhibits enzymatic activity, thereby enhancing the stability of several analytes and preserving overall sample integrity.[64] However, DBS is prone to hematocrit bias, a well-documented source of variability in DBS analysis. Hematocrit refers to the percentage volume of red blood cells to the total volume of blood. Variations in hematocrit levels influence the viscosity and spread of blood on the filter paper; higher levels result in smaller, denser spots due to reduced spread, while lower levels lead to larger, more diffused spots. This inconsistency affects the homogeneity of analyte distribution across the dried spot, complicating accurate quantification of analytes when fixed-sized punched discs are utilized for analysis. Consequently, the hematocrit effect introduces significant challenges in ensuring reliable measurements in DBS-based analyses.[65]

Therefore, in this context of preterm neonatal care where sample volumes are inherently limited, these analytical inconsistencies pose a significant risk to both clinical interpretation and scientific advancement. The implementation of improved sampling strategies that combine practical feasibility with the generation of high-quality, analytically robust specimens is essential to maximize the informational value of each sample. Such advancements are pivotal for driving progress in preterm neonatal research and ultimately improving clinical outcomes in this highly vulnerable population.

Metabolomics

Metabolomics is the study of small, low-molecular weight (<1.5 kDa), biochemical molecules within biological specimens such as cells, tissues, or biofluids.[66,67] These molecules, known as metabolites, are the intermediate or end products of metabolic reactions that provide a dynamic readout of an organism's physiological or pathological state. Metabolites exhibit a wide range of physicochemical properties based on polarity, molecular weight, structural complexity, and functional groups, reflecting their diverse roles in metabolic pathways.[68] The comprehensive set of these metabolites within a biological system is known as the metabolome. The metabolome is highly sensitive to both internal factors, such as enzymatic activity, hormonal signaling, and immune responses, and external factors such as diet, lifestyle, and environmental exposures. It reflects the cumulative outcome of the complex interactions between upstream genomic, transcriptomic, and proteomic regulation, and the exogenous influences, offering real-time functional insights into biochemical processes.[66] Moreover, monitoring of metabolites within specific pathways facilitate the inference of activation states or metabolic switching, revealing which routes are upregulated,

suppressed, or reprogrammed in response to physiological or pathological stimuli. Thus, metabolomics offers a valuable framework for uncovering disease mechanisms, identifying diagnostic and prognostic biomarkers, and guiding the development of therapeutic strategies. It is a powerful tool in the advancement of precision medicine, offering the potential to tailor interventions based on individual metabolic profiles.[69]

The two most commonly employed analytical technologies for metabolomic data acquisition are the nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).[70] NMR spectroscopy is a non-destructive technique that uses radiofrequency radiation to provide detailed structural information with high reproducibility and minimal sample preparation. It enables absolute quantification of metabolites without the need for external reference standards. However, its application is limited by low sensitivity, which restricts the detection of low abundance metabolites and reduces overall metabolite coverage.[71] In contrast, MS offers superior sensitivity and dynamic range, enabling the detection of metabolites at concentrations as low as nanograms per milliliter.[72] It operates by ionizing chemical species and analyzing their mass-to-charge ratios, thereby facilitating both the identification and quantification of metabolites across a broad chemical space. The Human Metabolome Database 5.0 reports more than 220,000 metabolite entries, highlighting the compositional complexity of biological specimens.[73] To reduce matrix interference and improve analytical resolution, MS is often coupled with separation techniques such as capillary electrophoresis (CE), gas chromatography (GC), and liquid chromatography (LC).[74] While CE and GC are particularly suited for specific applications, such as the analysis of highly polar and volatile compounds respectively, their widespread use is constrained by limited metabolite coverage, need for derivatization, and technical challenges in coupling with MS.[75,76] In contrast, LC offers a broad metabolite compatibility, including polar, non-polar, non-volatile and thermally labile metabolites. Separation in LC is governed by the differential interaction of metabolites with a solid stationary phase and a liquid mobile phase, primarily influenced by their polarity, hydrophobicity, and ionic properties. LC-MS has emerged as the most versatile and widely adopted hyphenated technique in metabolomics due to high sample throughput, sensitivity, and metabolite coverage, and suitability for both untargeted and targeted workflows.[77]

Untargeted metabolomics is a comprehensive, discovery-driven approach that aims to profile all detectable metabolites within a biological sample, including both known and unknown compounds.[78] This approach is typically used in exploratory investigations for hypothesis-generation, biomarker discovery, and exploration of novel metabolic pathways. However, untargeted analysis suffers from challenges such as complex data processing, difficulties in identifying unknown metabolites, reliance on relative quantification, and a bias toward detecting higher abundance compounds.[79] Conversely, targeted metabolomics is a

hypothesis-driven approach that focuses on the precise quantification of a predefined set of known, biochemically annotated metabolites, often selected based on prior established knowledge of metabolic pathways.[80] This approach leverages optimized extraction protocols, isotopically labeled internal standards, and highly selective analytical methods such as liquid-chromatography tandem mass-spectrometry (LC-MS/MS), to achieve high sensitivity, specificity, and reproducibility. LC-MS/MS allows for multiple reaction monitoring of predefined precursor to product ion transitions for each metabolite of interest, enabling accurate metabolite quantification even in complex matrices.[81] Targeted analysis is ideal for clinical studies with translational relevance, including mechanistic understanding of diseases, biomarker discovery and validation, therapeutic monitoring, pathway-specific investigations, and longitudinal analyses.

Signaling lipids, amino acids, and amines as modulators of immunity and inflammation

Signaling lipids are a diverse class of bioactive lipid molecules, predominantly derived from polyunsaturated fatty acids (PUFAs) and membrane phospholipids.[82] Unlike structural lipids that serve as energy reservoirs or cellular membrane components, signaling lipids function as dynamic mediators of cellular communication, particularly in the regulation of inflammation, immune responses, vascular tone, and homeostasis. These lipids exert their effects via membrane-bound or nuclear receptors and are typically synthesized and degraded rapidly in response to stimuli such as inflammation, infection, or injury.[83] Major classes of signaling lipids include oxylipins, lysophospholipids, free fatty acids, endocannabinoids and bile acids (**Figure 2**). Oxylipins are produced from the oxidation of PUFAs, mediated either by enzymatic pathways, including the cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 (CYP450), or by non-enzymatic free radical-driven lipid peroxidation. They encompass both pro-inflammatory mediators such as prostaglandins and leukotrienes, which drive immune cell recruitment and activation, as well as pro-resolving mediators like resolvins and lipoxins that promote inflammation resolution and restoration of homeostasis.[84] Oxylipins also include various hydroxylated and dihydroxylated fatty acid derivatives, such as HETEs, HODEs, DiHETEs, DiHODEs, DiHETrEs, and HDoHEs, many of which arise from the enzymatic transformation of epoxy fatty acid intermediates, and contribute to diverse inflammatory signaling processes.[85] Lysophospholipids, such as lysophosphatidic acid and sphingosine-1-phosphate, are largely produced via the enzymatic hydrolysis of membrane phosphospholipids and mediate physiological processes such as immune cell trafficking, cellular proliferation, vascular permeability, and immunomodulation.[86] Free fatty acids serve not only as metabolic substrates but also as signaling entities, with their immunomodulatory effect determined by their chemical structure; saturated fatty acids have been shown to induce inflammation while omega-3 PUFAs exert anti-inflammatory effects.[87]

Endocannabinoids, such as anandamide and 2-arachidonoylglycerol, mediate immunomodulatory effects via cannabinoid receptors CB1 and CB2, by modulating cytokine signaling, promoting immune cell apoptosis, and suppressing innate and adaptive immune responses.[88] Bile acids, synthesized from cholesterol and modified by the gut microbiota, function beyond lipid metabolism as signaling molecules, regulating cytokine production and immune cell differentiation.[89,90] Dysregulation of lipid signaling pathways have been implicated in a wide range of diseases, including cancer, metabolic disorders, and inflammatory conditions, highlighting the central role of lipid mediators in pathophysiology.[82]

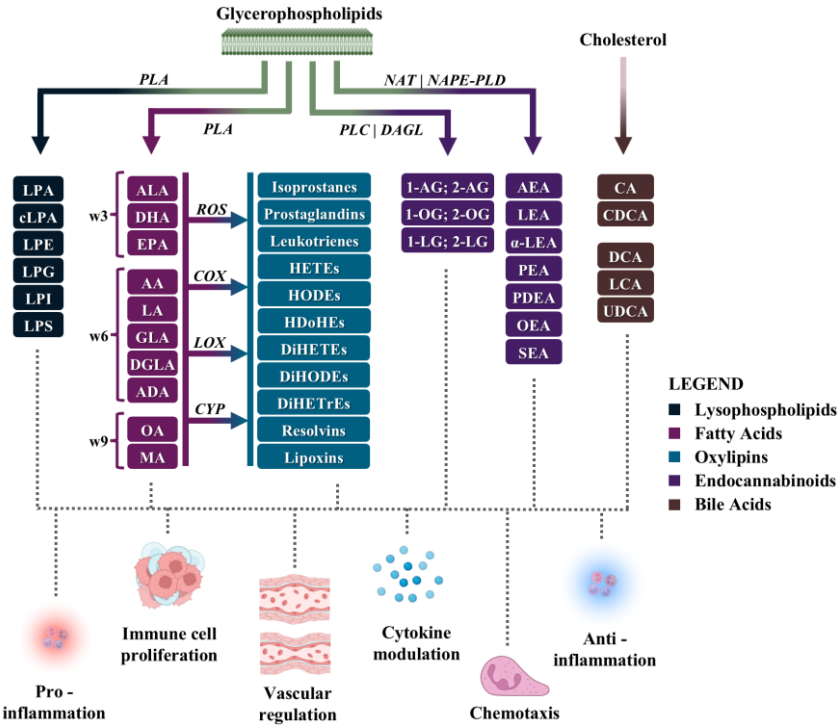

Figure 2. Overview of major signaling lipid classes and their functions in inflammation and immune response. The figure illustrates the major classes of signaling lipids, including lysophospholipids, fatty acids, oxylipins, endocannabinoids and bile acids, and their biosynthetic origins from membrane phospholipids, polyunsaturated fatty acids, and cholesterol. Functional roles in inflammation and immune modulation are broadly represented across lipid classes. Arrows depict various production pathways, including both enzymatic conversions (*e.g.*, COX, LOX, CYP450) and non-enzymatic mechanisms such as oxidation by reactive oxygen species.

Amino acids are organic compounds composed of an amino group, a carboxyl group, a hydrogen atom, and a distinctive side chain attached to a central carbon atom. Beyond their fundamental role as building blocks of proteins, amino acids play critical roles in modulating immune cell proliferation, cytokine production, antioxidant defense, and the balance between

pro- and anti-inflammatory responses.[91–93] For example, glutamine acts as a major fuel for immune cells and supports T cell proliferation. Arginine is a precursor for nitric oxide, a key signaling molecule in pathogen defense and inflammation.[93] Tryptophan metabolism can influence macrophage polarization and the production of anti-inflammatory metabolites such as kynurenine.[92,94] Branched-chain amino acids, including leucine, isoleucine, and valine, contribute to immunometabolic control by activating the mTORC1 pathway, thereby stimulating protein synthesis, promoting immune cell growth, and modulating cytokine production.[95] In addition, amino acids serve as precursors for a wide array of bioactive amines, predominantly generated via enzymatic decarboxylation reactions. Amines are a broad class of nitrogen-containing organic compounds derived from ammonia by replacing one or more hydrogen atoms with alkyl or aryl groups, resulting in primary, secondary, or tertiary amines depending on the degree of substitution. Several of these amino acid-derived amines play essential regulatory roles in immune function, inflammation, vascular tone, and cellular communication. For instance, histamine increases blood vessel permeability, promotes vasodilation, and facilitates the migration of immune cells to site of inflammation.[96] Serotonin, synthesized from tryptophan, modulates cytokine secretion and promotes platelet aggregation.[97] Polyamines, such as putrescine, spermidine, and spermine, are critical for cellular growth, proliferation, functional differentiation, and tissue repair.[98] Collectively, the capacity of amino acids and their amine derivatives to influence inflammatory and immune responses highlights their potential not only as biomarkers, but also as modulators of disease processes, opening avenues for novel therapeutic strategies in inflammation-driven pathologies.

Biological matrices for metabolomic analysis

The selection of an appropriate biological matrix is a critical consideration in metabolomic studies, as it fundamentally influences the range of detectable metabolites, the complexity of sample preparation, and the physiological relevance and biological interpretability of metabolic signatures.[99] Among the various biofluids available, such as whole blood, plasma, urine, saliva, and sweat, plasma is the most widely used matrix. This preference is due to its rich biochemical content, relatively standardized collection protocols, dynamic and holistic reflection of systemic physiological and pathophysiological changes, and availability of well-established reference ranges that support clinical translation. Despite its advantages, plasma has its disadvantages where collection remains invasive and technically demanding, typically requiring venipuncture or arterial access, immediate centrifugation, and strict cold-chain logistics to maintain analyte integrity.[62] These procedural complexities become particularly challenging in low-resource settings and vulnerable populations such as preterm neonates, where limited blood volume and heightened procedural risks severely constrain its practicality and ethical feasibility. Additionally, plasma preparation typically involves

protein precipitation to minimize matrix effects, which may lead to the loss of protein-bound metabolites and consequently limit metabolome coverage.[100]

Urine is an attractive alternative for metabolomic analysis due to its non-invasive, painless, and low-risk collection, capacity for yielding relatively large sample volumes, and feasibility for repeated sampling, making it particularly suitable for clinical monitoring or longitudinal studies. While urine also contains a wealth of endogenous metabolites, it represents a cumulative excretory output over time, influenced by renal filtration and excretion dynamics, which may obscure temporal resolution of biological events and complicate biological interpretation. Urinary composition is influenced by factors such as diet, hydration status, and time of collection, necessitating careful normalization during data analysis due to inherent variability in concentration.[101] Moreover, several metabolites, including signaling lipids, are excreted in conjugated forms to enhance their water solubility for renal elimination. Accurate quantification of the parent compounds therefore necessitates deconjugation steps, adding to the methodological complexity of the analytical workflow.[102]

Dried whole blood specimens, such as the DBS, are increasingly used in metabolomics due to their minimally invasive collection, low sample volume requirements, and simplified handling, storage, and transport, eliminating the need for immediate processing or cold-chain infrastructure. Dried blood sampling facilitates decentralized and remote sampling, making it highly suitable for large-scale, longitudinal, and geographically diverse metabolomic studies.[103] However, the presence of cellular components in whole blood introduces increased matrix complexity, potentially elevating background noise and necessitating more rigorous extraction protocols. While dried blood sampling ultimately improves metabolite stability by inhibiting enzymatic and hydrolytic activity post-drying, certain labile metabolites may still be vulnerable to degradation during the drying process itself.[104] Analytical variability may also arise from inconsistencies in sample volume, hematocrit effect, and heterogeneity in spot formation, further complicating reproducibility and standardization. Another limitation is the relative scarcity of established reference ranges and clinical interpretive frameworks for whole blood metabolomics, which are largely derived from plasma-based studies. This necessitates bridging studies to facilitate translation and contextualization of findings within existing biomarker landscapes.[103] These limitations are increasingly being addressed by recent advances in volumetric microsampling technologies and optimization and standardization of processing workflows, positioning dried whole blood metabolomics as a promising platform for translational metabolomic research.[65]

Volumetric absorptive microsampling

Volumetric Absorptive Microsampling (VAMS), developed by Neoteryx, is an innovative microsampling technology that addresses limitations of volumetric accuracy and hematocrit-

dependent variability associated with traditional DBS sampling.[105] The Mitra device, which employs the VAMS technology, consists of a plastic handler and a porous polymeric tip designed to absorb a fixed volume (10, 20, or 30 µL) of blood, independent of the hematocrit effect. Blood droplets, typically obtained via a lancet-induced finger prick, are passively drawn into the tip through capillary action, enabling precise volumetric sample collection. VAMS offers a promising alternative to DBS, retaining its advantages of minimal invasiveness, low sample volume, and simplified storage and transport, while providing improved precision and reproducibility for quantitative applications.[65] VAMS has been gaining traction across several research domains, including biomarker discovery, pharmacokinetics, therapeutic drug monitoring, and environmental exposure assessment.[65] Recent studies have demonstrated the successful application of VAMS in both targeted and untargeted metabolomic workflows for a wide array of endogenous metabolites.[106] For instance, a study by Kok *et al*. demonstrated that 36 major metabolites, including amino acids and organic acids, could be reliably profiled from just 10 µL of whole blood using VAMS. These metabolites remained stable for up to four days at room temperature, with the method showing good analytical repeatability and recovery.[107] However, despite its growing success, the performance of VAMS remains highly dependent on the physicochemical properties of specific metabolite classes. Comprehensive evaluation of pre-analytical variables, such as analyte recovery, matrix effects introduced by the polymeric tip, and extraction efficiency, remain essential to ensure method reliability and reproducibility. Therefore, rigorous validation tailored to the target analytes is critical before widespread adoption of VAMS in metabolomic workflows.

Metabolomic data analysis

Data analysis in metabolomics necessitates rigorous methodological considerations owing to the high dimensionality of the datasets, multifactorial sources of variability, and the risk of false discoveries or overfitting, particularly in studies with limited sample sizes.[108] Robust quality control (QC) protocols are essential to ensure reliability, reproducibility, and analytical integrity of metabolomics data. Tools such as mzQuality enable automated quality assessment by correcting for instrumental drift and technical variations through batch correction algorithms, performing signal normalizations using internal standards, identifying outliers via the Rosner test, and filtering features on stringent inclusion criteria based on background signal contribution and relative standard deviation across QC samples.[109] Metabolites with missingness are assessed for randomness using tests such as the fisher's exact test. Features with greater than 20% missingness across samples are typically excluded from analysis to reduce the influence of sparsely measured features and avoid inclusion of imputed noise.[110] For features with lower proportions of missing values, imputation is performed using appropriate methods, such as k-nearest neighbors or quantile regression

imputation of left-censored data (QRILC).[111] QRILC is employed when missingness is presumed to arise from values falling below the limit of detection, to preserve the distributional characteristics of low-abundance metabolites. Given the wide dynamic range and often skewed distribution of metabolite intensities, logarithmic transformation is commonly applied to stabilize variance and approximate normality.[112] Further, scaling techniques such as autoscaling are applied to harmonize feature magnitudes, ensuring comparability across metabolites within multivariate models.[112] For specific matrices like urine, additional normalization strategies are required to correct for sample dilution; these include normalization to osmolality, creatinine, or specific gravity, all aiming to reduce inter-individual variability arising from renal filtration and hydration status.[113]

Statistical analysis in metabolomics typically integrates both univariate and multivariate approaches to uncover significant metabolic alterations and interpret complex biological mechanisms.[108] Exploratory techniques such as principal component analysis are commonly employed for dimensionality reduction, enabling the identification of underlying patterns, detection of outliers, and visualization of sample clustering.[114] Univariate comparisons of metabolite levels between groups are performed using t-tests (for two groups) and ANOVA (for more than two groups), identifying differentially regulated metabolites.[115] To account for confounding factors, linear regression models are applied, enabling the estimation of the true association of metabolites with the outcome of interest. In studies involving repeated measures, hierarchical data, or random variation across subjects, linear mixed-effects models offer a flexible framework by incorporating both fixed and random effects.[116] When the outcome is binary or categorical, logistic regression is used to model the log-odds of the outcome as a function of metabolite levels and covariates, thereby facilitating the assessment of associations between the predictors and the probability of the outcome. In addition, correlation analyses are widely employed in metabolomics to assess associations between metabolite levels and continuous clinical variables. Pearson correlation is used to evaluate linear relationships under the assumption of normality, whereas Spearman's rank correlation, a non-parametric alternative, is applied when the data exhibit skewed distributions or contain outliers, capturing monotonic trends.[117] Given the large number of metabolites typically assessed in parallel, the risk of Type I errors, false positives resulting from the incorrect rejection of true null hypotheses, can be substantial. To address this, multiple testing correction procedures are applied, with the Benjamini-Hochberg false discovery rate correction being one of the most widely adopted in metabolomic studies.[118] This approach controls the expected proportion of false discoveries among significant findings, thereby enhancing the reliability of results.

Predictive modeling plays a central role in metabolomics-driven biomarker discovery, enabling the identification of molecular signatures that can accurately distinguish clinical

phenotypes, stratify risk, or predict disease outcomes. The high dimensionality and multicollinearity inherent to metabolomic data are effectively addressed using regularized techniques such as the Least Absolute Shrinkage and Selection Operator (LASSO), which simultaneously performs feature selection and model regularization by penalizing and shrinking coefficients of less informative variables to zero.[119] To ensure feature stability and model robustness, especially in studies with limited sample sizes, bootstrap aggregation is often employed, wherein models are iteratively trained on resampled subsets and evaluated on out-of-bag samples across multiple iterations.[119] Predictive performance is summarized using averaged metrics such as area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), and F1 score, and features consistently selected across iterations are prioritized as potential biomarkers.[120] For small, unbalanced datasets, Leave-One-Out Cross-Validation (LOOCV) offers an appropriate tool for model performance, by iteratively training the model on all but one sample and evaluating it on the excluded observation, thereby maximizing data usage while preserving independence between training and test sets.[120] To compare the classification performance of competing models, the McNemar test is used to assess differences in misclassification rates, providing statistical support for evaluating improvements in diagnostic accuracy.[121] Collectively, these strategies constitute a rigorous analytical framework for deriving reliable and clinically relevant metabolomic biomarkers.

Scope and outline of thesis

Preterm birth remains a leading cause of neonatal mortality and morbidity worldwide, posing a substantial global health burden. Preterm neonates traverse a high-risk developmental trajectory that begins in utero and extends into the neonatal period, which is characterized by dynamic physiological adaptations and heightened susceptibility to severe, potentially life-threatening complications. Despite substantial advances in perinatal care, the clinical management of preterm neonates continues to be hindered by diagnostic ambiguity, lack of precision therapeutics, and limited mechanistic understanding of the distinct pathophysiology of prematurity-associated conditions. Due to their potential for rapid clinical deterioration, preterm neonates receive empirical treatments at the earliest signs of suspicion. Although such precautionary measures may be life-saving in true pathological cases, they frequently result in overtreatment, inadvertently exposing vulnerable neonates to unnecessary therapeutic burdens and increasing the risk of both immediate iatrogenic complications and long-term adverse outcomes. Furthermore, progress in preterm neonatal research is constrained by the limitations of conventional blood sampling methods.

This thesis hypothesizes that the application of metabolomics and advanced microsampling techniques can provide a transformative framework to advance preterm neonatal research and outcomes. Specifically, it posits that characterization of metabolic dysregulations in signaling

lipids and amines can reveal key pathophysiological mechanisms underlying inflammation-associated preterm conditions, thereby enabling earlier and more accurate diagnosis as well as novel and personalized treatment strategies. Further, it hypothesizes that emerging state-of-the-art microsampling techniques can overcome practical and ethical limitations associated with conventional sampling methods that hinder the advancement of research in preterm neonates. **Figure 3** provides an illustration of the overview of the four core chapters in the thesis.

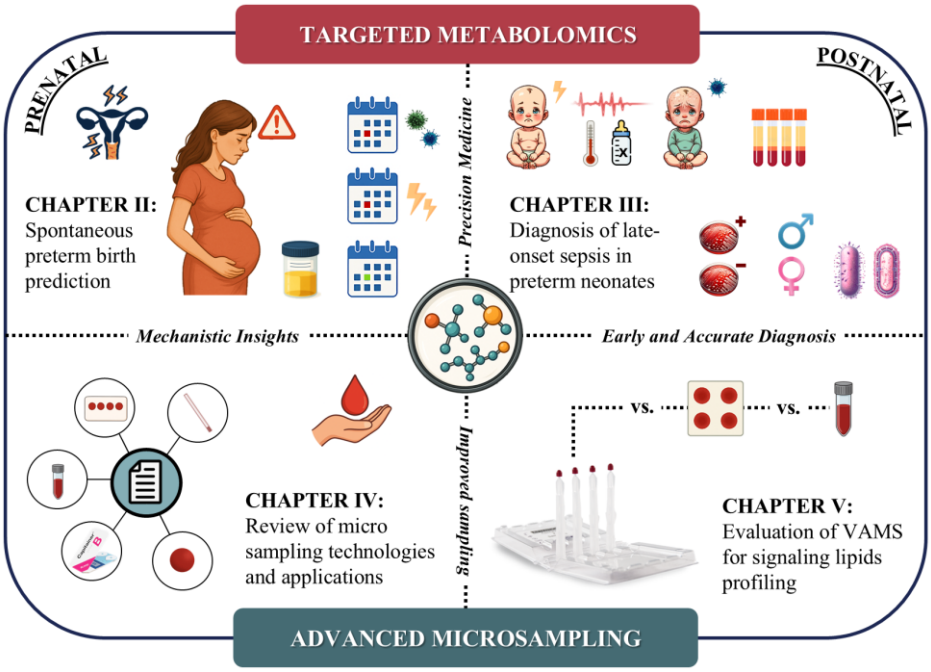

Figure 3. Overview of research chapters of the thesis. The figure provides an illustration of the four principal research chapters of the thesis, organized into two overarching domains: metabolomics and microsampling. Chapters II and III explore the application of targeted metabolomics to elucidate pathophysiological mechanisms underlying spontaneous preterm birth and late-onset sepsis, investigated through maternal urine and neonatal plasma samples, respectively, for biomarker discovery. Chapters IV and V focus on innovations in microsampling technologies, with a review of existing approaches to identify feasible alternatives for neonatal research, followed by the experimental evaluation of Volumetric Absorptive Microsampling for signaling lipid analysis. Together, these chapters integrate molecular insight with sampling innovation to advance preterm neonatal research and outcomes.

This thesis focuses on the investigation of spontaneous preterm birth and late-onset sepsis, two temporally distinct conditions mediated by inflammatory processes, to reveal how molecular insights across the perinatal continuum can inform clinical decision-making, minimize empirical interventions, and ultimately improve preterm outcomes. **Chapter II** aims to investigate the pathophysiological mechanisms underlying sPTB through targeted metabolomic profiling of maternal urine samples obtained at the time of clinical suspicion of

imminent PTB. This exploratory study focuses on the evaluation of signaling lipid mediators as potential biomarkers to differentiate between women who would deliver preterm from those who would ultimately deliver at term, despite presenting with similar clinical symptoms. To account for the etiological heterogeneity of sPTB, both infectious and non-infectious subtypes are examined, with the aim of identifying metabolic signatures that may be indicative of distinct underlying biological mechanisms, to support early and accurate risk stratification and improve clinical decision-making in preterm labor management.

Chapter III transitions to the postnatal period, focusing on LOS in preterm neonates with the aim of understanding its underlying pathophysiology and distinguishing it from non-infectious systemic inflammation (SINS) at the moment of clinical suspicion. Targeted profiling of signaling lipids and amines in plasma samples is employed to characterize metabolic perturbations associated with LOS and SINS. The study also examines the influence of clinical variables, such as sex and pathogen type, on metabolic responses, to provide deeper insights into the heterogeneity of LOS presentations. In addition, it assesses the diagnostic utility of these metabolites, both independently and in combination with conventional inflammatory markers, to support earlier and more accurate diagnosis and inform more personalized and judicious clinical decision-making in the management of suspected neonatal sepsis.

Chapter IV provides a comprehensive review on recent developments in microsampling technologies and their applications across clinical and research domains. The review surveys a broad range of dried and liquid matrix microsampling approaches to identify innovations with potential to improve sample collection and research outcomes in preterm neonates. It discusses the working principles, strengths, and limitations of commercially-available devices and highlights key advancements in bioanalytical assay development, matrix interchangeability studies, and device performance evaluations. It examines the extent of adoption of microsampling technologies in neonatal and pediatric applications to assess their translational potential for preterm care. This chapter aims to identify promising alternatives to conventional sampling methods that could alleviate the procedural burden, minimize sample wastage, and enhance analytical reliability, thereby addressing the critical limitations in advancing preterm neonatal research.

Chapter V builds on insights from the review which positions VAMS as a promising technology to enhance sample quality and alleviate logistical burdens within preterm neonatal applications. This chapter aims to assess the feasibility of VAMS for the targeted profiling of signaling lipids by adapting an existing plasma preparation protocol. The analytical performance of VAMS is evaluated in comparison to liquid whole blood and traditional DBS, based on liquid-liquid extraction recovery, matrix effects, and precision

parameters. The study also assesses the concordance of endogenous metabolite levels between sampling methodologies to determine their ability to preserve physiologically relevant metabolic profiles. Further, it examines the short-term stability of VAMS under ambient conditions to assess its logistical feasibility for use in low-resource settings.

Lastly, **Chapter VI** serves as the concluding chapter of this thesis, providing a synthesis of key findings and situating them within the broader context of neonatal research and clinical care. It summarizes the contributions of each chapter in addressing current preterm birth challenges through metabolomics and microsampling innovations. It further offers a critical reflection on the broader scientific impact and translational relevance of the work, and outlines future research directions alongside practical recommendations for clinical implementation. Collectively, this thesis establishes a transformative framework to overcome longstanding barriers in preterm neonatal research and management by integrating metabolomic profiling with state-of-the-art microsampling. This innovative approach provides deeper insights into the unique pathophysiology of this vulnerable population and opens new avenues for the development of more precise and individualized treatment strategies, with the potential to transform clinical outcomes and long-term health trajectories in preterm neonates.

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