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

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CHAPTER VI

Conclusion and Perspectives

Conclusion

Preterm birth constitutes a disruption of the natural trajectory of fetal development and represents a critical inflection point in perinatal healthcare.[1] Beyond its immediate threat to neonatal survival, preterm birth significantly increases the risk of long-term adverse outcomes, with ramifications that extend to families, healthcare systems, and society. While advances in perinatal care have enabled the survival of preterm neonates even at the limits of viability, ongoing efforts must extend beyond initial stabilization to prevent long-term sequelae and ensure lifelong well-being and high quality of life. The clinical management of preterm birth and its associated complications are particularly challenging due to the considerable heterogeneity in etiology and clinical presentations, distinct physiological characteristics of preterm neonates, and their increased vulnerability to rapid clinical deterioration. This clinical instability demands prompt and appropriate interventions, but the lack of sensitive and specific diagnostic tools often leads to the administration of empirical treatments based solely on suspicion, increasing the risk of overtreatment and iatrogenic harm.

Clinical mismanagements that carry downstream consequences for the neonate may occur both before and after birth, often stemming from diagnostic uncertainty, as observed in the management of spontaneous preterm birth and late-onset sepsis. In the absence of definitive diagnostics, clinicians often err on the side of caution, resulting in the empirical administration of interventions such as antenatal corticosteroids and postnatal broad-spectrum antibiotics.[2,3] While indispensable for survival, these treatments become unwarranted in cases of false positives, exposing fetuses and neonates to pharmacological agents without clinical necessity, leading to adverse metabolic, growth, and neurodevelopmental outcomes. Unlike term neonates or adults, preterm neonates possess not only developmentally immature but also functionally distinct physiological systems, which profoundly affect disease presentation, progression, and response to interventions. These limitations underscore the need for a deeper mechanistic understanding of pathophysiological processes to enable precise and personalized diagnostic and therapeutic strategies. Therefore, dedicated research targeting this population is of paramount importance. However, progress is inherently limited by their low circulating blood volume and ethical constraints surrounding invasive, painful procedures, which pose significant barriers to sample collection for research purposes. Conventional plasma-based workflows require relatively large volumes and complex handling, while the more feasible dried blood spot (DBS) sampling suffers from analytical limitations, including hematocrit-dependent variability, non-uniform sample distribution, and reduced quantitative reliability. These limitations

underscore the need for improved sampling techniques that are not only physiologically compatible with preterm neonates and resource-efficient but also analytically robust, ensuring that each of the extremely precious samples yields maximal information without any loss or compromise.

Therefore, the overarching aim of the thesis was to advance the understanding of pathophysiological mechanisms underlying spontaneous preterm birth and late-onset sepsis, to support the development of more timely and accurate diagnostics and targeted therapeutic strategies, thereby reducing unnecessary interventions and improving preterm outcomes. To this end, the thesis employed targeted metabolomics to investigate signaling lipids and amines, molecules implicated in inflammatory pathways that precipitate parturition and drive the host response to an infection, to uncover biologically meaningful molecular signatures reflective of the disease states. In addition, the thesis addressed the major methodological barrier inherent to research in preterm population through the evaluation of state-of-the-art microsampling techniques, with the goal of establishing a volume-efficient, analytically robust sampling strategy compatible with preterm neonates and translational implementation. This final chapter consolidates the key findings of the preceding core research chapters, reflecting on their contribution in addressing critical challenges in preterm care, while acknowledging methodological and interpretative limitations, and outlining future directions. It concludes with a broader outlook on the implications of this work, positioning it within the wider context of preterm neonatal research, diagnostic innovation, and clinical translation.

Mechanistic decoding of preterm conditions through targeted metabolomics for enhanced neonatal care

Chapter II investigated the maternal urinary metabolome at the critical clinical juncture of suspected imminent preterm birth, when therapeutic decisions must be made, to identify molecular signatures that may improve the accuracy of spontaneous preterm birth prediction. By retrospectively classifying cases from an exploratory subset of a prospective cohort into non-infectious preterm, infectious preterm, and term groups, the study not only captured the limitations of current predictive tools but also reflected the real-world diagnostic ambiguity and the etiological heterogeneity that challenge obstetric decision-making. By including preterm cases with histological chorioamnionitis, the most prevalent infectious etiology, alongside non-infectious preterm and term cases, the study enabled an initial investigation into mechanisms underlying preterm birth and whether distinct pathways are implicated across etiological subtypes. This comparative framework revealed metabolic profiles suggestive of two divergent mechanistic pathways driving spontaneous preterm births: one marked by oxidative stress and impaired inflammation resolution, predominant in the non-

infectious preterm group, and the other characterized by heightened immune activation, driven by microbial exposure, observed in the infectious preterm group. Notably, the metabolic profiles of non-infectious preterm showed marked divergence from term births, suggesting a distinct pathophysiological state that may be readily identifiable using signaling lipid profiling. In contrast, infectious preterm cases exhibited minimal differences with term profiles at the point of clinical suspicion, suggesting an activation of shared inflammatory pathways associated with the onset of labor. While such signaling may represent a physiological, transient priming in term pregnancies, such as cervical remodeling, it may become exaggerated and sustained in the presence of an infection, prematurely driving the cascade that leads to birth. This overlap in pathways may limit the diagnostic utility of signaling lipids in identifying infection-associated preterm births and reinforces the need for complementary approaches, such as volatile organic compound analysis, to detect infection-specific signatures with greater sensitivity. Beyond endotype-specific differences, the identification of shared, etiology-independent metabolites, such as 9-HODE, offer insights into the core downstream pathways involved in the premature initiation of labor, irrespective of the causative trigger. When combined with clinical symptoms, such markers hold potential to improve the prediction of spontaneous preterm birth when presented with clinical signs of imminent birth.

Chapter III focused on characterizing the pathophysiological mechanisms underlying late-onset sepsis in preterm neonates, with the aim of identifying biomarkers capable of distinguishing sepsis from non-infectious systemic inflammation (SINS). The use of 227 biobanked plasma samples collected during routine clinical care exemplifies an ethically sound, resource-efficient approach to generate new insights without imposing additional burden on the high-risk population. As one of the largest metabolomic studies in this context, the dataset captured a diverse clinical landscape, including healthy controls, SINS cases, sex differences, culture-positive and culture-negative sepsis, and gram-positive and gram-negative infections, offering a unique opportunity to explore the heterogeneity of sepsis presentation and host response. This heterogeneity, which is often overlooked in existing studies, is critical in understanding the biological basis for divergent clinical trajectories among neonates who present with comparable signs of illness but experience markedly different outcomes. The study revealed a spectrum of metabolic alterations across the inflammatory continuum, identifying both the similarities and distinctions between SINS and sepsis. Several metabolites were similarly dysregulated in both SINS and sepsis, illustrating the inherent complexity in distinguishing between these conditions. These shared signatures likely represent common downstream responses to systemic inflammation irrespective of the causative trigger, offering viable targets for therapeutic modulation aimed at restoring the

immunometabolic equilibrium in critically ill neonates. A subset of metabolites exhibited progressive trends from control to SINS to sepsis, revealing the greater severity and metabolic dysregulation in sepsis compared to SINS and the distinct pathophysiological impact of infection-driven systemic inflammation. These trends serve as quantitative indicators of escalating inflammatory burden and may offer a framework to establish threshold-based markers to support clinical differentiation. Distinct metabolic alterations unique to sepsis provided insights into sepsis pathophysiology and promising biomarker candidates. Further, sex-specific metabolic profiles were observed, with males exhibiting a pro-inflammatory phenotype and females an anti-inflammatory phenotype, suggesting a biological underpinning that may warrant sex-informed clinical management. Similarly, divergent metabolic responses to gram-positive and gram-negative bacterial sepsis, highlights the potential of host metabolomic profiles for early pathogen identification and targeted therapies. Finally, the integration of metabolic and inflammatory markers into a single diagnostic panel demonstrated the potential of metabolomics to enhance timely and accurate diagnosis of sepsis.

Together, **Chapter II** and **Chapter III** demonstrate the potential of targeted metabolomics in advancing the mechanistic understanding of underlying disease processes and identifying distinct disease-specific metabolic signatures. These findings reveal how metabolic profiling can differentiate true pathological cases from clinically similar presentations across critical windows of perinatal care. By enabling an early and accurate diagnosis, this approach reduces the risk of unnecessary treatments occurring due to misdiagnosis, thereby minimizing iatrogenic harm and improving preterm outcomes. Beyond diagnostic utility, these insights underscore the emerging role of metabolomics in guiding individualized therapeutic strategies. The identification of the underlying etiology, such as distinguishing between an infectious and non-infectious origin of preterm birth, or between gram-positive and gram-negative bacterial infection in sepsis, opens the door to interventions tailored to the specific pathophysiological context. Such etiological clarity can inform critical clinical decisions, including whether to initiate antimicrobial therapy upon suspicion of preterm birth to protect both mother and fetus, and guiding the selection of pathogen-specific antibiotics for neonates with sepsis, thereby avoiding unnecessary broad-spectrum antibiotic use. Further, the mapping of altered pathways such as PPARγ-mediated signaling in preterm birth or BCAA metabolism in sepsis, reveal potential novel targets for therapeutic innovation. For instance, pharmacological activation of the nuclear receptor PPARγ or targeted nutritional supplementation to correct amino acid imbalances may hold potential to modulate inflammatory processes and re-establish metabolic homeostasis, improving health outcomes.

These precision-guided strategies hold significant promise in transforming perinatal care toward a more stratified, biologically informed, evidence-based clinical framework.

Although this work demonstrates the potential of targeted metabolomics to enhance diagnostic precision and support personalized interventions, translating these insights into clinical practice requires further research. As novel investigations addressing previously unexplored clinical contexts, these studies were conducted on a small scale within a single, well-characterized cohort to enable a focused, controlled, and in-depth exploration of candidate biomarkers and underlying mechanisms. Therefore, external validation in larger, multi-center cohorts with diverse populations is essential to ensure the robustness, generalizability, and clinical applicability of these findings. Further, while distinct metabolic associations were observed across etiologies and clinically relevant subgroups within individual strata, the limited sample size precluded a formal evaluation of whether variables such as sex or pathogen type function as modifiers of the metabolic response. Future studies with sufficient power should incorporate interaction terms into the regression models to determine whether subgroup characteristics significantly influence the relationship between metabolites and disease state. Similarly, due to limitations in sample size, the diagnostic panels presented in this work were developed for overall classification of spontaneous preterm birth and late-onset sepsis, without accounting for subgroup-specific differences. Subsequent efforts should therefore prioritize stratified diagnostic evaluations to uncover more precise or discriminative biomarkers that may have been obscured in aggregate models. Future research should also focus on functional studies to elucidate the plausible mechanistic links identified in this work and determine whether the dysregulated metabolites actively contribute to disease pathogenesis or merely reflect downstream consequences. *In vitro* and *in vivo* models could be used to study their mechanistic roles or potential as therapeutic targets, ultimately supporting their translation into clinical applications. Integration with other omics layers, such as genomics, transcriptomics, and proteomics may enable a more comprehensive systems-level understanding of disease processes. Finally, development and clinical validation of rapid, cost-effective, high-throughput metabolomics-based diagnostic tools, such as point-of-care assays, will be essential for real-world implementation to support clinical decision-making in perinatal care to improve preterm outcomes.

Innovations in microsampling technologies for improved blood sample collection to advance preterm neonatal research

Chapter IV presented a comprehensive review on the recent advancements in blood microsampling technologies and their diverse applications. Microsampling technologies represent a significant shift in the paradigm of blood collection, offering promising

alternatives to conventional phlebotomy. By enabling minimally invasive sampling with substantially reduced volume requirements, they lower procedural burden and are particularly suited for vulnerable populations. These approaches improve patient comfort and compliance, minimize complication risks, and streamline logistics through compatibility with home-based or remote sampling. Collectively, these advantages support the advancement of decentralized diagnostics and enhance the feasibility of large-scale population studies, especially in settings where traditional blood collection is impractical or resource-intensive. The annual-style review captured the growing portfolio of both dried and liquid matrix microsampling approaches, highlighting innovations in device design and analytical workflows, aimed at improving volumetric accuracy, analytical and clinical compatibility, user-friendliness, and overall sample integrity. The technologies evaluated ranged from traditional DBS, the oldest microsampling technology, to more refined approaches designed to overcome longstanding limitations such as the hematocrit effect. Volumetric DBS devices, which use integrated capillaries to deliver precise volumes onto filter paper, exemplify this shift toward improved accuracy and reproducibility. Alternative sampling formats, including dried plasma spots, dried blood spheroids, volumetric tip microsampling, and microtube liquid microsampling, illustrate the increasing diversification of microsampling technologies. This diversity allows for the strategic selection of sampling strategy optimized for the target analytes, biological context, or user requirements, while lowering barriers to implementation in both research and clinical environments. Despite the growing use of these technologies across several domains, including pharmacokinetics, therapeutic drug monitoring, omics-based biomarker research, and toxicology, their widespread adoption into clinical practice is an ongoing process. This transition is often constrained by the lack of regulatory consensus, need for matrix-bridging studies, and absence of standardized protocols for sample handling and downstream analysis.

While the scope of this review extended beyond neonatal applications, the insights gained provided a strong foundation for identifying innovations with translational potential in preterm neonatal blood sampling. The review revealed that the majority of commercially-available devices continue to rely on lancet-based collection, inherently limiting their potential to reduce procedural pain in preterm neonates, where heel pricks with a lancet remain the standard approach. Lancet device designs may be optimized through automation and control of puncture parameters such as depth and velocity, in efforts to minimize tissue trauma and improve reproducibility. Notably, the review also highlighted microneedle-based technologies as a promising alternative for blood sampling. Due to their substantially smaller dimensions compared to lancets, microneedles may not penetrate the skin deep enough to activate pain receptors located deeper in the dermis, thereby offering a less painful sampling

alternative. However, commercial implementation remains limited, with only the TAP II device currently available that uses solid microneedles coupled with vacuum-assisted extraction, primarily designed for adult use. Although the device offers a minimally-invasive approach, the suction force required for sample collection poses a significant risk of damage in preterm neonates due to the exceptional fragility of their skin. Based on these insights, I propose that hollow microneedles may offer a more viable option for preterm neonatal blood collection. Unlike solid microneedle systems, hollow microneedles would enable vacuum-assisted blood extraction through their internal channels, eliminating direct suction on the skin surface and reducing the risk of mechanical trauma. However, the translation of novel conceptual designs into clinically approved devices is often protracted, hindered by extended phases of prototype development and iterative optimization, complex regulatory pathways, and the requirement for extensive clinical validation.

The review also highlighted several potential near-term opportunities to improve sample quality and logistical feasibility by leveraging existing microsampling technologies. In particular, volumetric absorptive microsampling (VAMS) emerged as a promising alternative to traditional DBS sampling, offering a fixed-volume, hematocrit-independent collection while retaining advantages of DBS, such as minimal invasiveness, simplified storage, and ease of transport. VAMS facilitates more efficient and controlled sampling by directly absorbing the entire available blood drop onto a volumetric polymer tip, minimizing sample loss during collection. In contrast, DBS involves manual spotting onto filter paper, which can result in uneven spreading, partial absorption, and inconsistent sample volumes. Moreover, while DBS typically analyzes only a portion of sample through spot punching, contributing to sample wastage, VAMS ensures full utilization of the collected volume. Therefore, VAMS supports both analytical rigor and ethical responsibility required for preterm neonatal care. VAMS has been gaining popularity as a versatile platform for diverse applications, including neonatal screening, therapeutic drug monitoring, pharmacokinetic studies, and biomarker research. Its analytical performance, however, is intrinsically linked to the physicochemical properties of the target analytes, making rigorous method validation indispensable for each specific application. To date, metabolomic studies with VAMS have largely focused on highly abundant and relatively stable metabolites, such as amines and organic acids. As demonstrated in **Chapter II** and **Chapter III**, signaling lipids hold significant potential in advancing our understanding of inflammation-associated preterm conditions and in serving as biomarker candidates. Given the biological relevance of these lipids, which are typically low in abundance and chemically labile, Chapter V assessed the feasibility of using VAMS as a reliable strategy for their targeted profiling.

In **Chapter V**, a modified extraction protocol, adapted from a validated plasma method incorporating additional steps, including rehydration, vortexing, incubation, and sonication, was applied to systematically evaluate the performance of VAMS in comparison to DBS and liquid whole blood (WB). The method assessment focused on key analytical parameters, such as liquid-liquid extraction recovery, precision, and matrix effects, for major classes of signaling lipids, including oxylipins, lysophospholipids, free fatty acids, bile acids, and endocannabinoids. The findings revealed that VAMS demonstrated higher analytical precision than both DBS and WB, indicating its potential as a more consistent sampling matrix and supporting its suitability for generating reproducible data in low-volume metabolomics workflows. The adapted protocol proved effective in achieving moderate extraction recoveries (50-80%) that were largely comparable between VAMS and DBS for most lipid classes and consistently superior to WB, suggesting that dried matrices may reduce matrix-associated interference during extraction. Future research should focus on enhancing extraction recoveries by optimizing sample preparation protocols, with strategies to reduce analyte interactions with the substrate and matrix interferences. Repeated extraction cycles and increased time for extraction may further facilitate a more complete recovery of the analytes. Matrix effects were consistent across VAMS, DBS, and WB, indicating that ion suppression or enhancement during mass spectrometry was not substantially influenced by the choice of sampling substrate. However, to mitigate the substantial matrix effects observed for endocannabinoids, free fatty acids, and lysophospholipids, future research should implement additional cleanup techniques such as Hybrid-SPE precipitation to selectively remove matrix components like phospholipids that are known to contribute to ion suppression and enhancement.

Notably, VAMS demonstrated superior alignment of endogenous metabolite levels with WB compared to DBS, suggesting that its 3D porous polymeric structure provides a more protective environment during drying and short-term storage of 24h. This structural advantage likely reduces platelet activation and analyte degradation through pathways such as oxidation and hydrolysis, which are more likely to occur in the exposed 2D cellulose format of DBS. However, most metabolite levels were relatively lower in VAMS compared to WB, likely reflecting an incomplete analyte release from the polymeric tip during extraction, potentially due to stronger interactions between lipids and the sampling substrate. Given the 3D nature of the polymeric matrix, diffusion of analytes may be slow and uneven, particularly for larger or more hydrophobic molecules such as free fatty acids. Future research should focus on evaluating and improving the extraction efficiency of analytes from the VAMS tip. Strategies such as increasing rehydration volume and incubation duration prior to solvent extraction may facilitate a more complete desorption of analytes from the tip,

thereby enhancing overall analyte recovery. Further, the short-term stability of VAMS was tested at 24h, 48h, and 1 week under room temperature to capture real-world challenges, such as limited cold chain infrastructure in low-resource settings and logistical delays, and to serve as a stress test simulating worst-case scenarios involving prolonged ambient exposure. While only subtle changes were observed in metabolite levels from 24h to 48h storage, extended storage of 1 week resulted in pronounced alterations across most lipid species, underscoring the critical importance of defining acceptable storage windows for preserving metabolite integrity. Stabilization measures such as desiccant-assisted storage or pre-treatment with antioxidants may offer avenues to extend analyte stability under ambient conditions. Further work should explore the efficacy of these approaches as well as investigate the early kinetics of degradation by investigating shorter storage intervals (*e.g.* 4h, 8h, 12h) and varying temperature conditions. Such studies will be essential for establishing handling guidelines that preserve the integrity of endogenous metabolite profiles, ensuring accurate reflection of *in vivo* concentrations at the time of sampling.

Overall, this study establishes VAMS as a highly promising microsampling technology for signaling lipid profiling, highlighting clear practical and analytical advantages over traditional DBS. Alongside the methodological refinements identified to improve analytical performance, future studies should employ higher-sensitivity instrumentation to expand analyte coverage, particularly for low-abundance oxylipins that were underrepresented in this exploratory study due to instrument limitations. Together, **Chapter IV** and **Chapter V** address critical challenges in blood collection, including procedural pain, sample loss, and analytical variability, supporting the advancement of a more ethical, reliable, and data-rich framework for research in preterm neonates. This framework would enable a deeper biological understanding of preterm conditions and support robust biomarker discovery for early and accurate diagnosis and personalized treatment, with the potential to revolutionize the clinical management of preterm neonates.

Outlook and Future Perspectives

The primary focus of perinatal care for preterm neonates has long centered on ensuring survival, often prompting aggressive interventions, such as the administration of empirical or unnecessary treatments as precautionary measures amidst diagnostic uncertainty to prevent rapid health deterioration. However, accumulating evidence of iatrogenic harm associated with such practices has contributed to the growing awareness that perinatal care must evolve beyond immediate life-saving measures toward approaches that ensure long-term health, developmental outcomes, and overall quality of life. This thesis contributes to that evolving paradigm by advancing both the biological understanding of preterm conditions

and the methodological feasibility of studying them in vulnerable preterm neonatal populations. By focusing on two high-risk inflammatory clinical contexts, spontaneous preterm birth and late-onset sepsis, it demonstrates the potential of targeted metabolomic profiling of signaling lipids and amines to differentiate true pathology from clinically ambiguous presentations, enhance etiological stratification, and identify factors that contribute to heterogenous outcomes. In parallel, the thesis provides a transformative framework using advanced microsampling techniques to ethically and effectively advance such scientific research in preterm neonates, where frequency, quality, and safety of sample collection remain a major barrier. Positioned at the intersection of urgency, complexity, and vulnerability, this thesis lays the foundation for a more proactive, biologically-informed evidence-based model of care, supporting precision diagnostics and personalized treatment strategies to improve preterm outcomes.

Through its integration of both antenatal and postnatal phases, the thesis illustrates that the trajectory of preterm outcomes can be profoundly shaped by clinical decisions even before birth, highlighting a critical window of opportunity to improve preterm outcomes. Research aimed at improving obstetric care often focuses on understanding maternal pathophysiology, which is relatively more feasible than preterm neonatal research due to the accessibility of biological specimens. This thesis demonstrates the potential of urine as a non-invasive, readily accessible, and low-burden matrix for biomarker discovery, establishing its utility in the accurate prediction of spontaneous preterm birth. The advancement of obstetric diagnostics not only improves neonatal health, but also reduces maternal stress, prevents unnecessary treatments, and alleviates strain on hospital resources. In contrast, postnatal research in preterm neonates is constrained by their limited volumes, clinical fragility, and ethical considerations around invasive procedures. This thesis navigates these challenges by capitalizing on residual plasma samples from routine clinical testing, underscoring the value of repurposing surplus clinical material to advance neonatal research without imposing additional burden on the infant. When applied to late-onset sepsis, one of the leading causes of neonatal mortality, the findings of the thesis carry far-reaching implications. By demonstrating the potential of metabolic biomarkers to improve their diagnostic accuracy, they pave the way for a more judicious use of antibiotics, that would help mitigate the risk of long-term complications, including obesity, asthma, and adverse neurodevelopmental outcomes. This would ultimately contribute to improved health trajectories and quality of life for affected individuals, while also reducing prolonged hospitalizations, alleviating emotional and financial strain on families, and lowering broader societal costs associated with healthcare burden and productivity loss. While the use of residual samples offers ethical advantages, it is not always ideal, particularly for longitudinal studies, due to inconsistent

sample availability, variable volumes, and pre-analytical variability that may compromise data quality and comparability. Dedicated collection of precise sample volumes would offer the most robust approach; however it would necessitate frequent sampling, which is not ethically suitable with the current lancet-based methods due to the associated pain and tissue trauma.

Microneedle-based sampling techniques represent a promising avenue for minimally-invasive, pain-free blood sampling in preterm neonates. This thesis also explored the potential of hollow microneedle-based sampling as an alternative to the conventional lancet-based collection. Although a fully functional prototype was not completed within the scope of this work, significant progress was achieved in the foundational development phase. Hollow microneedles were successfully fabricated using 3D printing and underwent preliminary evaluation, including fluid uptake experiments with water and blood-mimicking fluid, as well as skin insertion testing to assess mechanical integrity and penetration performance. While these findings remain unpublished, the work is documented in the **Supplementary Material** and establishes a foundational technical basis for the future development of pain-free microsampling technologies suitable for fragile neonatal populations. Blood sampled through these hollow microneedles may be directly absorbed onto volumetric polymeric tips, such as those used in VAMS technology, allowing for precise volumetric control, improved analyte stability, and simplified transport under ambient conditions. The findings of this thesis, which demonstrate the analytical viability of VAMS for signaling lipid profiling, reinforce its potential as a downstream platform for microsampled blood. Together, these innovations lay the groundwork for an ethically responsible and high-quality sampling workflow that could facilitate longitudinal monitoring and expand access to preterm neonatal research. While such a device may be well-suited for research applications, supporting mechanistic studies and the identification and validation of diagnostic and prognostic biomarkers, its translation into routine clinical diagnostics presents challenges. Mass-spectrometric analysis of metabolic biomarkers, while renowned for their high sensitivity and specificity, remains dependent on centralized laboratory infrastructure, skilled personnel, and time-intensive workflows. While recent advances in portable and miniaturized mass spectrometry systems are making rapid point-of-care metabolite-based diagnostics increasingly feasible, these platforms are still often limited by high costs, operational complexity, and ongoing maintenance needs, restricting their widespread adoption, especially in low-resource settings.[4] The emergence of on-chip mass spectrometry presents a compelling advancement in overcoming these limitations. By integrating mass spectrometric components onto microfabricated chips, these systems offer significant reductions in size, power requirements, and operational complexity.[5] Moreover, their potential for scalable manufacturing may substantially lower production costs, enhancing the

accessibility of high-performance metabolic analysis. As the technology matures, on-chip MS holds promise for delivering rapid, cost-effective, and user-friendly diagnostics at the point of care.

Alternatively, biosensors may also offer a promising solution for translating validated biomarkers into point-of-care diagnostics with minimal sample requirements and user intervention. Biosensors are bioanalytical systems typically consisting of two basic functional units: a biorecognition element, such as antibodies, aptamers, or enzymes, that selectively interacts with the target, and a transducer that converts this interaction into a measurable signal, via optical, electrochemical, colorimetric, or other physiochemical modalities.[6] When coupled with microneedle-based sampling systems, these platforms enable minimally invasive, on-site quantification of metabolites from low-volume blood samples. Alternatively, the biosensing elements may be incorporated into the microneedle structures themselves, enabling a real-time continuous monitoring of analytes, typically from interstitial fluid, which is in close dynamic equilibrium with blood.[7] These microneedle-based wearable biosensors would eliminate the need for fluid extraction and generate dynamic molecular readouts that reflect an individual's evolving physiological state. When coupled with digital infrastructure, such continuous biochemical monitoring could feed into centralized health dashboards, allowing for biomarker trends to be visualized alongside clinical parameters. The integration of machine learning algorithms and artificial intelligence may further support the interpretation of these complex temporal data, supporting early diagnosis and guiding individualized management strategies, ultimately enabling timely, data-driven decision-making in preterm neonatal care.

Taken together, the work presented in this thesis redefines the boundaries of possibilities in both the clinical care and scientific investigation of preterm neonates. Its dual focus on unraveling complex pathophysiological mechanisms to inform precision diagnostics and treatments, and on reimagining how such insights may be ethically and practically obtained, reflects a deliberate shift from working within existing clinical constraints to actively reshaping them. This simultaneous advancement of scientific depth and feasibility positions the thesis not just as a body of knowledge, but as a transformative framework, demonstrating how future research in these vulnerable populations can be both scientifically rigorous and ethically responsible. Ultimately, this thesis advances a vision for a future where the survival of the littlest is not merely about being alive but ensuring healthy developmental trajectories and long-term quality of life.

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Supplementary Material

The human skin is a structurally complex organ composed of three primary layers: the epidermis, dermis, and hypodermis, each contributing to its barrier, regulatory, and sensory functions.[1] The stratum corneum, the outermost layer of the epidermis, serves as the robust physical barrier against physical, chemical, and pathogenic insults. Beneath the epidermal layer, the dermis houses an intricate network of blood vessels, lymphatic vessels, and nerve endings that play essential roles in immune surveillance, thermoregulation, and tissue homeostasis. The hypodermis, composed primarily of adipose tissue, provides insulation and mechanical cushioning. Although vital for physiological defense, this multilayered architecture of the skin presents a major barrier to transdermal access for blood sampling. Conventional hypodermic needles, typically 8-40 mm in length, penetrate deep into the dermis or subcutaneous tissue, triggering nociceptors causing pain, tissue trauma, and procedural anxiety.[2] Lancet-based methods offer a modest reduction in invasiveness, typically employing penetration depths of 1-3 mm. However, their relatively large incision length, typically 1.75 mm, produces a sizable wound which poses a significant risk of discomfort and bruising, particularly in preterm neonates whose skin is structurally immature, thinner, and more susceptible to trauma.[3]

Microneedles have emerged as a promising alternative due to their micron-scale structures, typically 150-2000 μm in length and 150-300 μm in width, designed to breach the stratum corneum and access the superficial dermal compartment without stimulating pain receptors or compromising deeper tissue integrity.[3] By enabling access to capillary blood or interstitial fluid through a minimally invasive interface, microneedles reduce procedural burden positioning them as an ideal technology for preterm neonatal applications. Microneedles are broadly classified based on their structural design into two principal types: solid and hollow. Solid microneedles function by creating micro-perforations in the skin, thereby facilitating fluid access through secondary mechanisms such as manual skin compression or vacuum-assisted extraction. In contrast, hollow microneedles are engineered with internal lumens that permit direct fluid extraction, either through passive capillary action or controlled negative pressure. This design eliminates the need for lateral or compressive forces, reducing mechanical stress on the skin, an essential consideration in vulnerable populations such as the preterm neonates. Therefore, in this thesis, a hollow microneedle-based prototype was conceptualized and developed as a proof-of-concept to demonstrate the feasibility of this approach for blood sampling.

Design and Fabrication

The design of hollow microneedles for blood sampling requires careful consideration of key parameters, such as needle length and lumen diameter, to strike an optimal balance between

minimizing pain perception and ensuring functional performance. A study by Gill *et al.* demonstrated that microneedles shorter than 700 µm were perceived as virtually painless by adults, with pain perception increasing with needle length.[4] However, effective access to the capillary beds for reliable blood extraction has been shown to require microneedle lengths of at least 1000 µm. While solid microneedles have demonstrated efficient sampling at this threshold, hollow microneedles typically require greater lengths, typically ranging from 1500 to 1800 µm, to ensure that the tip of the internal lumen is positioned deep enough within the dermal microvasculature to facilitate direct fluid uptake, thereby supporting consistent and efficient blood sampling.[5,6] Furthermore, a minimum lumen diameter of 50 µm has been reported as necessary to mitigate the risks of clogging and excessive flow resistance.[7]

Guided by these design considerations, the initial design of the prototype comprised an array of 5 microneedles, each featuring a conical geometry with a length of 1800 µm, a base diameter of 300 µm, and a 30° bevel tip, designed to facilitate efficient skin penetration while minimizing insertion force and tissue disruption. An internal lumen diameter of 60 µm was incorporated, informed by existing literature, to permit unobstructed flow of blood through the microneedle channel.[7] The five microneedles were integrated into a cylindrical housing measuring 3.5 mm in height. Internally, the individual lumens were designed to converge into a common outlet channel to streamline fluid collection. The base structure incorporated a wider flange for structural support and a threaded connection to allow secure attachment to standard microfluidic tubing via Captite fitting. This modular interface was designed to enable sample extraction through conventional syringe actuation for early-phase *in vitro* evaluations (**Figure 1**). The prototype was fabricated using projection micro-stereolithography (PµSL), a high-resolution 3D printing technique based on additive manufacturing, employing a biocompatible acrylate-based photopolymer (BIO RESIN, Boston Micro Fabrication, USA).[8] The system provided a lateral resolution of 20 µm, which was critical for accurately reproducing both the sharp external microneedle geometries and the narrow internal lumens required for fluid transport.

In-vitro Sampling Evaluation

Preliminary *in vitro* experiments were conducted to evaluate the functional performance of the microneedles, with a focus on assessing the integrity and patency of the microneedle channels for effective fluid extraction. Two test media were employed for this assessment: distilled water and a blood-mimicking fluid, composed of a glycerol-water (40:60 v/v) mixture, to approximate the viscosity and surface tension properties of whole blood and simulate physiologically relevant flow dynamics within the microchannels. These fluids were placed in open reservoirs, into which the microneedle array was manually immersed to establish fluid contact. The base of the microneedle prototype was connected to a 1 mL Luer-

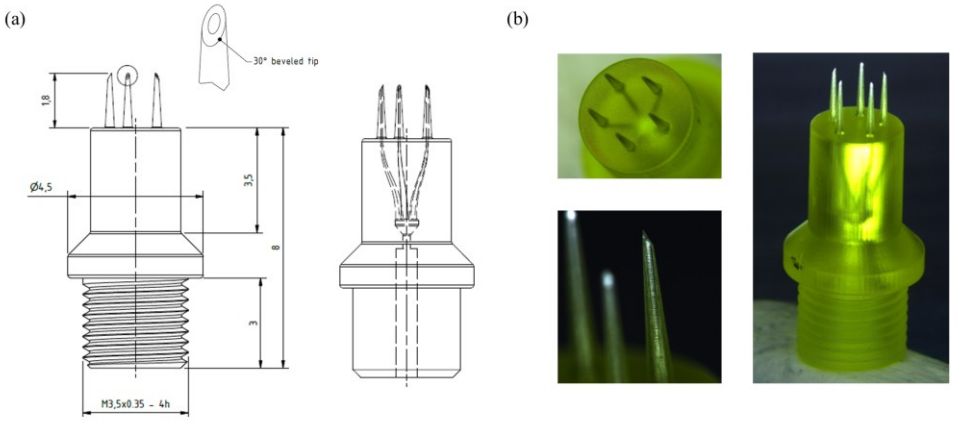

Figure 1. Design and fabrication of the initial hollow microneedle prototype. (a) Technical drawings of the microneedle array prototype featuring five conical microneedles (1800 µm in height, 300 µm base diameter, and 30° bevel tip), integrated into a cylindrical housing with a threaded base (M3.5×0.35 - 4h) for interfacing. Each microneedle included a 60 µm internal lumen, converging into a common outlet for sample extraction. (b) Microscopic images of the 3D-printed prototype fabricated using projection micro-stereolithography (PµSL) with 20 µm resolution using a biocompatible acrylate-based photopolymer.

lock syringe via a Captite fitting and intermediary tubing, establishing a secure interface for fluid withdrawal. Negative pressure was applied through gentle manual retraction of the syringe plunger, initiating fluid withdrawal through the hollow microneedles and into the collection chamber. Successful fluid aspiration was observed in both media, indicating unobstructed flow through the needle lumens. To further assess the patency of each individual channel, complementary delivery-mode testing was performed with distilled water, wherein the fluid was observed exiting uniformly from all five microneedle tips, indicating bidirectional flow capability. No leakage or structural deformation was detected during repeated trials, further validating the mechanical integrity of the design.

Skin Insertion Test

To evaluate the mechanical performance and skin penetration capability of the microneedles under physiologically relevant conditions, insertion testing was performed using *ex vivo* human abdominal skin. The skin was obtained from a local hospital within 24 hours following cosmetic surgery, in accordance with the Declaration of Helsinki. Excess fat was removed using a scalpel, and the skin was stored at -80°C until use. Prior to experimentation, the skin was thawed at 37°C for one hour in a humidified Petri dish and subsequently mounted on a parafilm covered Styrofoam using pins to ensure uniform tension across the surface and minimize lateral movement during insertion (**Figure 2a**). The epidermal surface was cleaned with Milli-Q water followed by 70% ethanol to remove residual contaminants. The microneedles were inserted using a digitally controlled applicator for impact-driven

insertion at a velocity of 3.2 m/s to eliminate variability associated with manual application, thereby ensuring reproducible and efficient skin penetration (**Figure 2b**).[9]

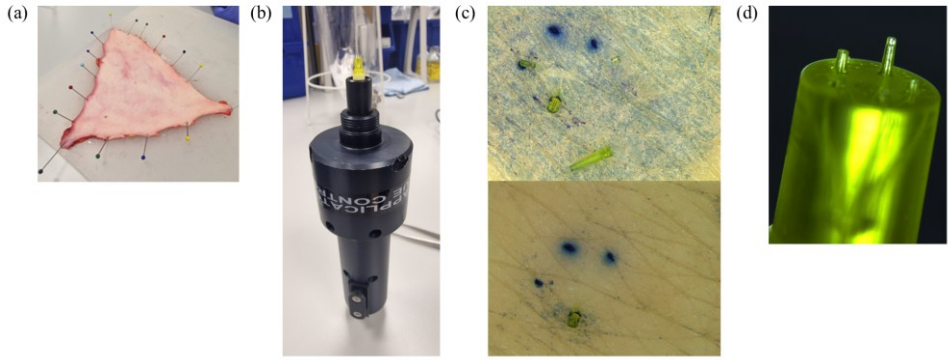

Figure 2. Evaluation of microneedle skin insertion using *ex vivo* human skin. (a) *Ex vivo* human abdominal skin mounted on parafilm covered Styrofoam for insertion testing. (b) Digitally controlled microneedle applicator for impact insertion. (c) Trypan blue staining of insertion entry points before and after tape stripping. (d) Post-insertion microscopic images of prototype showing structural failure of all five microneedles.

Microneedle penetration was assessed using trypan blue staining as a qualitative indicator of the stratum corneum disruption. Following microneedle application, 70 µL of an aqueous 0.4% trypan blue solution was applied to the site and left undisturbed for one hour to allow dye penetration into disrupted sites. The area was subsequently washed twice with distilled water and once with 70% ethanol to remove residual surface dye. To facilitate clear visualization of stained insertion points, the stratum corneum was gradually removed through sequential tape stripping until the skin displayed a smooth, glossy appearance, indicative of complete layer removal. Microscopic images were captured before and after insertion to document changes in microneedle structures, verify penetration, assess the number of successful insertions, and evaluate the uniformity of entry points across the array. Post-insertion assessment showed that 4 out of 5 microneedles penetrated the skin, yielding an 80% penetration efficiency. However, the trypan blue staining revealed variability in entry point morphology, suggesting inconsistent penetration depths across the microneedle array (**Figure 2c**). All 5 microneedles exhibited varying degrees of structural failure: three fractured at the base, one near the base, and one at the tip (**Figure 2d**). These findings revealed that while the applied velocity was sufficient to breach the skin barrier, the design lacked the structural integrity required to withstand high-velocity insertion, necessitating further refinement of the material properties or geometry to improve mechanical robustness.

Design Optimization

Following the structural failure observed during skin insertion testing, the microneedle design was geometrically refined to improve the mechanical strength and structural integrity. Specifically, the aspect ratio was reduced by shortening the needle length from 1800 µm to 1200 µm, thereby lowering the bending moment during impact. Additionally, the original conical geometry with a 300 µm base diameter was replaced with a tetrahedral design featuring a triangular base inscribed within a 533 µm circumscribed circle (**Figure 3a**). This configuration would increase the base contact area and redistribute stress more uniformly at the junction with the substrate, reducing the risk of fracture under high-velocity insertion. The modified array was fabricated using PµSL and evaluated under identical *ex vivo* skin conditions as previously described. Upon application with the velocity-controlled impact applicator, all microneedles successfully penetrated the *ex vivo* human skin in a uniform manner, with no evidence of bending, fracture, or deformation (**Figure 3b**). These results confirmed that the geometric optimization substantially improved both penetration reliability and mechanical resilience, validating the new design for further development.

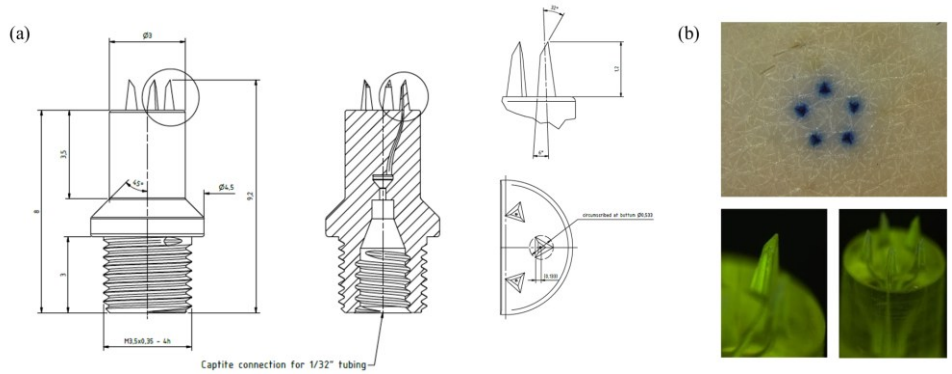

Figure 3. Design and testing of optimized hollow microneedle prototype. (a) Technical schematics of the optimized microneedle prototype featuring a triangular pyramidal geometry with reduced aspect ratio and increased base width for enhanced mechanical strength. (b) Post-insertion assessment showing trypan blue stained entry points on *ex vivo* human skin and corresponding microscopic images of the microneedle array, confirming successful penetration and preserved structural integrity.

Future Work

Future development of the prototype should focus on a comprehensive mechanical and functional characterization to further optimize performance and reliability for clinical translation. Detailed assessments such as axial fracture force testing, insertion force profiling, and mechanical durability under repeated use will be essential to establish structural robustness. Additionally, the evaluation of skin penetration efficiency and safety may be performed with optimal coherence tomography to quantify insertion depth and uniformity,

and histological analyses to assess tissue damage and inflammatory response. To advance towards an autonomous sampling platform, the development of a miniaturized actuator capable of controlled insertion and sampling is envisioned. Integration of the prototype with storage units such as VAMS tips would facilitate precise volumetric collection and simplify downstream processing. Finally, translational *in vivo* studies using appropriate animal models will be critical for validating biological compatibility, sampling performance, and analyte stability, thereby laying the foundation for eventual human application.

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