**RESEARCH STRATEGY**

1. **SIGNIFICANCE**

Preterm labor (PTL), resulting in preterm birth (PTB), is the leading cause of child mortality under the age of five worldwide1. In the United States, one in ten pregnancies ends in PTL1, amounting to an annual healthcare cost burden of $26.2 billion2. While certain causative factors for PTL (e.g., multiple pregnancies, uterine or cervical abnormalities, infections) are known, two third of PTL cases occur spontaneously (sPTL) without clear medical risk factors3. Predicting and preventing sPTL pose significant clinical challenges. Current predictors for sPTL, including a history of previous PTL, ultrasonographic estimate of gestational age (GA), cervical length screening, and cervicovaginal-fluid- or blood-based biomarkers, have shown limited performance4,5. Additionally, preventive strategies, such as vaginal progesterone, cervical cerclage, and pessary have demonstrated limited to no efficacy in most patients6–9. Furthermore, PTB affects certain racial and ethnic groups disproportionately, with the highest prevalence (14.4%) found among African-American (AA) individuals3,10. Consequently, there is a critical need for identification of predictive biomarkers in diverse patient populations to improve the assessment of sPTL risk, enable patient stratification, and accelerate the development of new therapies.

While multiple factors contribute to sPTL, dysregulation of immunological mechanisms normally engaged in the progression from conception to labor is increasingly implicated in the pathogenesis of sPTL3,11–23. A thorough understanding of the immunological mechanisms involved in the initiation of labor is thus an essential first step towards identifying biologically plausible and clinically relevant predictive biomarkers of sPTL.

Maintenance of pregnancy relies on a finely tuned immune balance that engages a multicellular network of innate and adaptive mechanisms. In early pregnancy, tolerogenic mechanisms are largely mediated by the expansion and function of regulatory T cells (Tregs) and the exclusion of effector T cells from the decidua, the maternal-fetal interface where the maternal tissue is in direct contact with the placenta24–28. In late pregnancy, activation of innate immune mechanisms, including chemotactic recruitment of pro-inflammatory cells, e.g., neutrophils, monocytes (MCs)/macrophages (Mɸ), and Natural Killer (NK) cell subsets29–31, increased transcription of pro-inflammatory cytokines in the decidua32, and enhanced circulating NK and activated T cell signatures33 culminate in the initiation of the cascade of parturition34–40. In addition, emerging evidence from mouse models suggests the involvement of IL-33 and the IL-33 receptor (ST2)-expressing innate and adaptive immune cells in uterus-intrinsic inflammatory labor mechanisms41,42. However, these maternal-fetal immune mechanisms have been predominantly elucidated in animal models and remain understudied in the context of human pregnancy.

Compelling evidence from our group, utilizing high-dimensional monitoring of peripheral immune responses with mass cytometry, has shown that maternal immune adaptation during pregnancy follows precise temporal dynamics11,12,43,44. **Our goal is to determine the role of the local and peripheral innate immune systems in mechanisms initiating the onset of labor in human pregnancies and to identify clinically relevant biomarkers for predicting and preventing sPTL.** Our recent multi-omic modeling of peripheral immune cell, proteomic, and metabolic events during the last 14 weeks (wks) before spontaneous labor in term pregnancies identified a multivariable model that predicted the time to labor with high accuracy (Root Mean Square Error, RMSE = 2.3 wks)21. Remarkably, the most informative features of the predictive model were innate immune cell signaling responsiveness to inflammatory ligands (including MyD88 and JAK/STAT signaling in MCs and NK cells) and the synchronized surge of soluble (s)ST2 plasma concentrations. These findings resonate with recent murine studies41,42 and provide strong evidence implicating innate immune responses and the IL-33/ST2 system in inflammatory mechanisms of human labor. However, whether observed peripheral innate immune cell responsiveness indirectly reflects the progression of uterine-mediated inflammatory mechanisms of labor or whether circulating sST2, the IL-33 decoy receptor, functions as part of a regulatory network to control systemic inflammation with approaching labor are important knowledge gaps that we will address in this proposal.

Herein we propose the cross-tissue profiling of the immune cell and protein repertoire in the circulation and at the maternal-fetal interface to determine the contribution of peripheral and local innate immune responses to the inflammatory mechanisms initiating human labor (**Aim 1**). We will leverage a novel machine learning-based statistical framework to identify and prospectively validate clinically relevant predictive biomarkers of sPTL (**Aim 2**). **Aim 3** will employ computational drug repurposing45 and high-plex immune monitoring to identify candidate compounds that can modulate labor-associated innate immune responses for the prevention of sPTL**.** Thus, our proposal is highly significant, both for unraveling fundamental innate immune mechanisms underlying human labor and for elucidating the relationship between local and peripheral immune responses in labor pathobiology. It is also clinically significant as the data-driven discovery of blood-based immune biomarkers of sPTL, their validation in diverse patient cohorts, and the proposed drug discovery pipeline have important implications for 1) developing new diagnostic tools that can be used in the clinical management of individuals at risk for sPTL, and 2) accelerating clinical testing of new therapeutic strategies for sPTL prevention.

**Suitability, skills, and relevant experience of the investigative team.** Our research team has over ten years of expertise and a solid track record integrating single-cell immune profiling and other high-dimensional omic technologies with advanced machine learning methods to study human immune dynamics in pregnancies with term and preterm delivery11,12,14,15,44,46–49. Together, our team combines the required expertise in single-cell and plasma proteomics (**B. Gaudilliere**), feto-maternal immunology (**I. Stelzer, N. Gomez-Lopez**), obstetrics and PTB research (**J. Prins, D. Stevenson**), and machine learning and statistics **(N. Aghaeepour, M. Sirota, A. Tarca**) to accomplish the proposed studies.

1. **INNOVATION**

**B.1 Multiplex assessment of peripheral and local immune responses during pregnancy with single-cell suspension and imaging mass cytometry.** Our group has developed high-dimensional suspension mass cytometry (a.k.a. cytometry by time of flight mass spectrometry, CyTOF) immunoassays to interrogate maternal immune system dynamics in peripheral blood collected during healthy and pathological pregnancies. To assess the local maternal-fetal immune responses in the context of the intact cellular architecture of the placenta, we have also developed imaging mass cytometry (IMC) immunoassays. CyTOF merges traditional flow cytometry with inductively coupled plasma mass spectrometry to assess over 50 simultaneously measured proteomic parameters on a cell-by-cell basis50–52, offering unmatched proteomic information content from individual cells51,53. IMC (Hyperion, SBT) is a highly multiplex imaging platform that combines mass cytometry with microscopy imaging to enable the characterization and the spatial arrangement of cells within a solid tissue sample. In practice, combining CyTOF and IMC allows for precise phenotyping of all major cell subsets present in a blood or tissue sample via cell surface antibodies (abs) and the simultaneous assessment of multiple intracellular signaling proteins or cytokine responses (via phospho-specific abs or intracellular cytokine abs respectively) and mRNA transcripts (RNAscope)54–56. Development and implementation of CyTOF and IMC immunoassays to interrogate peripheral and local immune cell responses, particularly IL-33/ST2-dependent innate immune responses, will be the focus of **Aim 1**.

** Figure 1.** **Development and benchmarking of the Stabl statistical framework.** **A.** Stabl combines multivariable modeling with model-X knockoff noise injection and minimization of the false discovery proportion (FDP+) for the data-driven selection of reliable predictive biomarkers. **B.** Stabl demonstrates superior sparsity (left) and reliability at a similar predictive performance (right) in comparison to other algorithms (Lasso, Elastic Net, Sparse Group Lasso, and adaptive Lasso). An example is shown for the prediction of the time to labor comparing Stabl vs. Lasso.

**B.2 Data-driven selection of reliable and clinically relevant predictive biomarkers of sPTL using Stabl.** Omic technologies, such as mass cytometry, generate large datasets characterized by a high number of molecular features relative to the number of samples. These datasets often contain multiple data categories, including phenotypic, signaling, and spatial attributes of individual cells. From a diagnostics perspective, an integrated, multivariable analysis with sparse machine learning methods is a powerful approach as itcan reveal candidate biomarkers of pregnancy outcomes from several biological categories that provide higher predictive power when combined57. However, the objective selection of sparse (i.e., a reasonably small number of biomarkers allowing for feasibility of clinical development) and reliable (i.e., truly related to the clinical outcome) biomarker candidates remain important challenges that have hampered the clinical translation of multi-omic studies58,59. Our grouphas developed **a novel machine learning method, Stabl**, that combines injection of knockoff-modeled60 noise into the original data, a data-driven selection of reliable biomarkers, and integration of selected biomarkers into a cross-validated predictive model (*manuscript accepted in Nature Biotechnology*61).Extensive benchmarking of Stabl on synthetic data and on five real world omic datasets, including prediction of the time to labor (**Fig. 1**), demonstrated enhanced sparsity and reliability performances at similar predictive performance when compared to existing sparse algorithms, such as Lasso62, Elastic Net (EN)63, Sparse Group Lasso64, and adaptive Lasso65. As such, Stabl accounts for the trade-off between predictivity and sparsity, zeroing in on the most informative set (i.e., the “true set”) of predictive biomarkers. In this proposal (**Aim 2**), application of Stabl for the multivariable modeling of single-cell and plasma proteomic data will provide a rigorous statistical framework and data-driven approach for the selection and validation of predictive immune biomarkers of sPTL.

**B.3 Single-cell assessment of candidate drug immune modulatory properties using a high-throughput mass-tagged cell barcoding (MCB) assay.** sPTL is a complex disorder with largely ineffective therapies, emphasizing the urgency of exploring unconventional approaches to drug development. The highly multiplex capacity of mass cytometry offers a powerful approach for conducting high-throughput pharmacological assays. We propose a mass-tagged cell barcoding (MCB) assay to screen through an existing list of promising, FDA-approved compounds of interest (COIs) for the treatment of sPTL, previously selected using an established computational drug repurposing pipeline45 (**M. Sirota**, Co-I). Application of the MCB assay to healthy pregnant volunteer blood samples will allow modeling of the dose-dependent effect of individual COIs on hundreds of cell-type-specific immune signaling events (**Aim 3**). COIs with desired immune modulatory properties (i.e., that modulate labor-related innate immune responses) will be retained for further testing in an ex vivo placental tissue slice assay and, ultimately, in clinical studies.

**C. APPROACH**

**Figure 2.** **Research approach overview**

**Overview.** The innovative technologies described above will enable us to pursue three Specific Aims to address the **overall hypothesis** that *innate immune response trajectories preceding labor are accelerated in preterm compared to term pregnancies* (**Fig. 2**)*.* The study leverages existing peripheral blood, plasma, and placental tissue samples along with detailed clinical data collected from consenting pregnant individuals enrolled in three cohorts (see human subject section for details). In **Aim 1**, we will employ a combined IMC, CyTOF, and plasma proteomic strategy to determine the relationship between local and peripheral innate immune responses in the pathobiology of spontaneous *term* labor (sTL). Experiments in **Aim 1** will be performed on placental (decidua basalis) tissue samples as well as serial blood samples collected monthly during pregnancy from individuals who delivered via sTL (n=17), induced labor (n=17), or cesarean (C-)section (absent labor, n=17) in the Wayne State University (**WSU**)**-1** cohort. In **Aim 2**, we will perform an integrative single-cell (CyTOF) and plasma proteomic analysis of peripheral blood samples collected from pregnant individuals with sTL or sPTL from two independent cohorts: an AA cohort (**WSU-2**, n=100) and a European cohort (**NEXT**, n=200). Application of the Stabl machine learning framework will allow for construction and validation of a multivariable predictive model of sPTL and the data-driven selection of robust immune biomarkers. In **Aim 3**,we will employ an innovative high-plex MCB immunoassay to determine the effect of promising candidate drugs, previously selected from a drug repurposing pipeline45, on labor-associated innate immune responses. Validation of observed immune properties using a placental tissue slice assay will yield a shortlist of promising drug candidates that can be leveraged for the personalized preventative treatment of sPTL. Importantly, the three Aims are complementary, but not overlapping, as new mechanisms characterized in the context of labor in **Aims 1&2** will inform, but are not required for, the design of the MCB immunoassay in **Aim 3**.

**Scientific rigor and reproducibility.** We are using well-established and rigorous pipelines for single-cell and plasma proteomic data analyses in R and Python, many of which were developed by our groups61,66–70. All code will be made publicly available on Github, and all data will be uploaded to a data sharing platform (Dryad, **Aims 1-3**). Multivariable modeling of the high-dimensional data (**Aims 1&2**) will be performed using state-of-the-art sparsity promoting regularization machine learning methods (EN, Stabl)61–63. Predictive performances of multivariable models will be established using a Monte-Carlo cross-validation scheme stratified by patients, accounting for repeated sampling61,71. Model performances will be quantified using the Area Under the Receiver Operating characteristic Curve (AUROC) for binary (e.g., sTL vs. sPTL) or the RMSE for continuous outcomes (e.g., time to delivery, TTD). Analyses of placental tissue and peripheral blood samples are based on power calculations and expected results from previous studies as described below. We are considering **fetal sex as a biological variable** and will include other demographic and clinical variables (maternal age, fetal sex, race/ethnicity, parity, and gravidity) as additional features in our multivariable modeling.

**C1. Aim 1:** **Determine** **the contribution of local and peripheral innate immune cells to mechanisms of term labor.**

**Figure 3.** **A surge in sST2 plasma levels wks before labor coincides with decelerated innate immune signaling responses. A-B**. Split line regression modeling identifies a non-linear breakpoint (dotted line) in immune feature trajectories 2-4 wks before labor.Plasma sST2 levels (**C**), MyD88 (**D**), and JAK/STAT (**E-F**) signaling responses in peripheral NK cells, cMC, and mDC.

**C1.1 Scientific premise.** Our recent study of human immune dynamics in term pregnancies identified a surge in the plasma level of sST2, the soluble decoy receptor for IL-33, occurring 2–4 wks before labor44. This surge coincided with the dampening, or deceleration, of innate immune cell signaling responses to inflammatory ligands, includingthe JAK/STAT and MyD88 responses in CD14+ classical (c)MCs, CD56dimCD16+NK cells, and myeloid dendritic cells (mDC) following their steady increase during pregnancy (**Fig. 3**).Complementing these findings, recent studies in mice suggest that IL-33 is secreted locally by the uterine endo/myometrium41,72, the choriodecidua42, and the fetal membranes73 prior to the onset of labor and initiates uterus-intrinsic inflammatory mechanisms of labor via activation of ST2-expressing innate immune cells41.

IL-33 is a pleiotropic alarmin known to regulate a wide signaling network and functional phenotypes in human immune cells expressing ST2, including activation of canonical (MyD88) and non-canonical (e.g., JAK/STAT) signaling pathways72,74–78. Ourfindings thus prompt us to consider whether the decelerating (i.e., increased then dampened) responses of peripheral innate immune cells with approaching labor is mediated, in part, by expression of ST2, and whether these ST2+ cells could serve as early peripheral sensors of labor, reflecting the activity of tissue-resident ST2+ cells at the maternal-fetal interface. Alternatively, the surge of plasma sST2 could function as part of a broader negative feedback mechanism to minimize the systemic effect of uterine IL-33-mediated inflammation. *We hypothesize that, as labor approaches, there is an increased responsiveness of peripheral ST2+ innate immune cells, which occurs prior to the rise in sST2 plasma levels and correlates with the inflammatory profile of decidual ST2+ innate immune cells.*

In **Aim 1**, we will address this hypothesis using a cross-tissue, combined IMC/CyTOF analysis of placental tissue and matched longitudinal blood samples (**WSU-1** cohort, **N. Gomez-Lopez**, Co-I) to investigate: 1) the distribution, spatial organization, and signaling activity of all innate immune cells, including ST2+ immune cells, in the human decidua basalis, comparing individuals who did or did not experience spontaneous term labor (**Aim 1a**); and 2) the relationships between local (decidual) innate immune cell profiles and dynamic changes in peripheral innate immune cells and circulating proteins (including sST2) that precede the spontaneous onset of term labor (**Aim 1b**).

**C1.2 Preliminary data**

*WSU-1 clinical cohort and biorepository.* The **WSU-1** cohort will include 51 participants selected from a larger biorepository (median age 27, IQR [22–31], pre-pregnancy median BMI 30.2 [25.7–34.7], 8.6% nulliparous, 94% AA) who delivered full term (median GA at delivery 39w0d [38w3d–39w4d]) spontaneously (n=17), after induction of labor (n=17), or via primary C-section (n=17), recruited at GA 10–12 wks and followed through delivery. This collaboration was conducted in partnership with **Dr. Gomez-Lopez** during her tenure at the Perinatology Research Branch (NICHD, WSU) until 01/31/2023. Samples include formalin-fixed paraffin embedded (FFPE) placental tissue blocks collected at time of delivery. For 35 participants, over 230 whole blood (collected, stimulated with IFN-⍺, IL-2/4/6, and LPS and fixed for CyTOF analyses) and plasma samples were collected at 3–4 wk intervals during pregnancy (~6–8 samples per participant).

*Development of an IMC assay for high-dimensional assessment of placental tissue immune architecture.* Our lab has extensive experience in high-dimensional, multi-omic monitoring of maternal immune responses during healthy and pathological pregnancies11,12,44. In a preliminary study, we developed and validated a high-dimensional IMC assay that enables simultaneous imaging of over 35 phenotypic and functionalimmunologic markers in FFPE placenta (decidua basalis) tissue. Our pilot antibody (ab) panel allowed for high-resolution immunophenotyping of innate immune cells (including neutrophils, Mɸ subtypes, mDC, and NK subsets), single-cell level expression of ST2, and intracellular expression of signaling protein phosphorylation, such as phospho (p)STAT1, pCREB, pP38, prpS6 (**Fig. 4**), as well as their spatial organization within the placental cellular architecture. The panel also allowed detailed phenotyping of non-immune cells, including endothelial, trophoblast, and stromal cells.

**Figure 4. Development of a multiplex imaging mass cytometry (IMC) assay for single-cell assessment of innate immune system architecture in placental tissue. A.** IMC workflow. Placenta anatomy highlighting fetal villi and decidua basalis shown on a raw IMC image acquired using a 35-plex metal-conjugated antibody (ab) panel. **B.** *Bottom.*A segmented IMC image colored by cell types (white squares indicate ROI shown on top). *Top.* ST2 expression in CD68+Mɸ and CD66b+neutrophils (arrows). **C.** ST2 expression in peripheral whole blood samples showing negative control (metal minus one, MMO, black line) and anti-ST2 ab signal in circulating cMCs, neutrophils, and NK cells (red line).

*Development of a new IMC analytical framework integrating spatial subsetting with sparse predictive modeling.* Analysis of multiplexed imaging data presents considerable challenges, including feature reproducibility and dataset dimensionality. In particular, the selection of multiple regions of interest (ROIs) can introduce significant bias as intra-patient variability between ROIs can be attributed to either the sampling process (technical variability) or underlying tissue heterogeneity (biological variability). To address these issues, our group has developed a novel spatial subsetting approach to standardize cell population metrics to tissue zones, extract reproducible feature classes (e.g., zonal cell density, cell-cell interactions, functional cell attributes) and integrate curated single-cell and spatial information into a sparse multivariable modeling pipeline. We demonstrate that spatial subsetting improves both intra-patient and inter-cohort feature reproducibility (**Fig. 5**).

** Figure 5. Spatial subsetting and multivariable modeling of IMC data. A.** Feature classes extracted from IMC datasets. **B.** Integrative modeling approach combining each feature class into a unique multivariable model. **C.** Correlation coefficient for pairwise comparisons of absolute cell count of all cell populations between ROIs from the same patient before spatial subsetting (*top*) and ofcell population densities per spatial zone between ROIs from the same patient after subsetting (*bottom*). The example shown is comparing oral cancer tissue biopsies.

**C1.3 Experimental approach**

***Aim 1a: Comparative IMC analysis of placental tissue from individuals with or without spontaneous labor.*** We will analyze FFPE placental tissue samples (decidua basalis) collected at the time of delivery from the 51 participants, including 17 with spontaneous labor, 17 with induction of labor (i.e*.,* when the decidual immune landscape can be assumed to be a consequence, not an initiator of labor), and 17 who did not experience labor (primary C-section).

*Placental sample processing and IMC* *analysis.* Decidua basalis tissue sections 4μm thick will be analyzed using a 48-parameter metal-tagged ab panel (**Fig. 6**) allowing for the single-cell analysis of 1) major innate (neutrophils, Mɸ, DC subsets, and innate lymphoid cell subsets including NK cells) and adaptive (CD4+T, CD8+T, Tregs, B cells, and their subsets) immune cell populations, 2) detailed phenotyping of structural and non-immune cells (including endothelial, trophoblast, and stromal cells in the decidua), 3) ST2 and intracellular IL-33 expression, and 4) signaling activities of key elements of the MyD88 (pP38, pERK1/2, prpS6, pCREB, pNFκB) and JAK/STAT (pSTAT1/3/5) pathways. To complement ST2 and IL-33 protein expression analyses, RNAscope-based probes, compatible with ab-based markers, will be utilized in conjunction with anti-ST2 and anti-IL-33 abs to quantify ST2 and IL-33 mRNA expression55. Three ROIs per sample will be selected by a trained placental pathologist to ensure representation of major maternal and fetal compartments, such as remodeled spiral artery, endometrial gland, and extravillous trophoblast (EVT). Tissue sections will be stained using established protocols79 and acquired on a Hyperion IMC system. Single-cell segmentation will be achieved using Mesmer within the Steinbock pipeline80. Single cells will be classified into cell types using an unsupervised method (FlowSOM)81.

** Figure 6. Proposed IMC and CyTOF ab panels.** IMC (blue), CyTOF (red) or shared between IMC and CyTOF (black) ab targets. RNAscope probes are italicized.

*Standardized IMC feature extraction and assembly of a multi-class dataset.* We will generate structural tissue masks to define decidual areas for classification of cells into distinct biological spatial zones (e.g., glandular area, EVT, vascular area, non-glandular stromal area, border to intervillous space)82. This will allow generation of a spatially standardized multi-class dataset (**Fig. 2**) composed of single-cell features, such as zonal cell abundances (cell density, mm‑1), localization information (e.g., distance from gland, EVT, intervillous space, spiral artery), intracellular signaling states (e.g., Arcsinh transformed pP38 expression) and cell-cell interaction patterns (neighborhood coefficients via the Louvain algorithm83). Features will be reported as medians on a per cell population per spatial zone level for each patient.

*Multivariable modeling of IMC features classifying samples from spontaneous vs. non-spontaneous labor.* A multivariable modeling method using the Elastic Net (EN) algorithm will be utilized to classify samples from spontaneous labor, induction of labor, or C-section deliveries. Each IMC feature class (function, abundance, distance to vasculature/gland/trophoblast, neighborhood) will be treated as an individual omic data layer in a stacked generalization84. Model performance will be evaluated using a cross-validation method and multiclass AUROC61. Selected IMC features (i.e., with non-zero EN coefficients) will be validated using traditional immunofluorescence techniques. This model will identify a set of spatial features that differentiate the various labor conditions, a subset of which will be included in the cross-tissue analysis in **Aim 1b** (e.g., surface marker expression or intracellular signaling activities, as spatial features will not be shared between IMC and CyTOF datasets). *Power analysis:* For analysis of the primary endpoint (spontaneous, induced, or absent labor), we estimated the sample size to identify a multivariable model with predictive performance of 0.8 (AUROC) and an alpha of 0.05, at 80% power, as 13 patients per group (85% power, 15 patients; 90% power, 17 patients)85.

***Aim 1b: Cross-tissue analysis of peripheral and local immune cell responses associated with spontaneous labor.*** Serial whole blood samples collected from pregnant individuals (n=35, 239 samples) with spontaneous, induced, or absent labor will be analyzed using established CyTOF protocols44. A 48-plex CyTOF ab panel overlapping with our IMC panel (**Fig. 6,** **Aim 1a**) will be used for 1) the phenotypic characterization of peripheral innate and adaptive immune cell subsets, including ST2 expression and 2) the comprehensive assessment of intracellular signaling activities at baseline and in response to ex vivo stimulations (LPS, IFN-α, IL2/4/6), including MyD88 and JAK/STAT signaling. In parallel, the plasma concentration of sST2, along with >1,500 proteins will be analyzed using an aptamer-based platform (Somalogic)44,86. A cross-tissue analysis will be performed to determine whether local immune features associated with spontaneous labor are present in the blood before the onset of labor. The analysis will focus on immune cell features (cell frequencies and signaling responses) shared between IMC and CyTOF datasets derived from matched blood and placental samples. We will apply a two-stage modeling approach47. In the first stage, multivariable modeling of placental tissue will provide spatial features differentiating spontaneous vs. non-spontaneous labor (**Aim 1a**). In the second stage, each of these features will be supplied as response variables to a second multivariable EN model that uses peripheral blood immune features as predictors. To account for repeated sampling at multiple time points, within each cross-validation fold, univariable mixed-effects regression models will be used to select blood immune features for input into the EN model. Secondary correlation network analyses will quantify interactions between peripheral blood and placental immune features.

**C1.4 Expected results.** IMC analyses in **Aim 1a** will yield a comprehensive, single-cell atlas of the maternal-fetal interface in term pregnancies with spontaneous labor vs. induced or absent labor. We anticipate identifying a robust (AUROC>0.8) multivariable model differentiating labor conditions and pointing at an immune signature unique to spontaneous labor. We expect that in spontaneous labor, ST2+ innate immune cells will be more abundant compared to non-spontaneous labor, and that ST2+ cells will have increased inflammatory signaling activity compared to ST2- immune cells. Furthermore, the cross-tissue analysis in **Aim 1b** will generate a high-resolution profile of peripheral immune cell dynamics in healthy term pregnancies in relation to decidual immune cell profiles at the time of delivery. We expect that MyD88 and JAK/STAT intracellular signaling responses will 1) increase in ST2+ innate immune cells with progressing pregnancy then decelerate as sST2 plasma levels surge and 2) correlate with the signaling profiles of decidual, labor-specific ST2+ innate immune cells. Alternatively, peripheral ST2+ immune cell dynamics may not correlate with decidual immune ST2+ cell profiles, which will suggest that plasma sST2 may be part of a negative feedback preventing undesired systemic immune activation upon IL-33 leakage from local tissue sources.

**C1.5 Potential Pitfalls and Alternative Strategies.** We note that, by design, the proposed IMC assay focuses on functional properties of immune cells in the decidua basalis, such that other important tissues (e.g., fetal membranes/choriodecidua, endometrial/myometrial junction, cervix), and information levels (e.g., metabolomics, microbiota, transcriptomics) remain under-characterized. However, the decidua basalis is a primary site for maternal-fetal immune interaction which, during labor, resemble those occurring in the decidua parietalis87–93. Alternative strategies include investigating fetal membrane sections and punch biopsies of maternal endo/myometrium, and expanding our assay to include additional tissue cell and functional markers. While the analysis focuses on innate immune cells, the IMC analysis will include comprehensive analysis of adaptive (including T and B cell subsets) as well as non-immune cell types (e.g., decidual stromal cells, trophoblast cells) thus generating the first high-dimensional spatial proteomic atlas of the laboring vs. non-laboring decidua. The resulting dataset will build on and be integrated with other high-plex imaging efforts focused on 1st and 2nd trimester decidual tissue94. While our preliminary data shows ST2 protein expression in several decidual innate cell subsets, the anti-ST2 ab signal may be below the instrument detection level for certain cell subsets. However, inclusion of ST2 RNA-probes compatible with our IMC55 and CyTOF95 ab panels will provide a complementary transcriptomic assessment of ST2 expression. Our whole blood assay indirectly quantifies the effect of IL-33 on signaling responses previously linked to the time to labor. However, we will be poised to directly test immune cell IL-33 responsiveness using peripheral blood mononuclear cell (PBMC) samples available from patients in the same cohort. Finally, we recognize the multivariable modeling of labor-associated immune responses in human tissue does not imply causation. However, strong correlations between the single-cell data, labor type, and peripheral immune signatures will guide future experiments in mouse models aiming to establish causative mechanisms (**I. Stelzer**, Co-I).

**C2. Aim 2: Integrative modeling of single-cell immune responses and plasma proteomics using sparse machine learning to predict sPTL.**

**C2.1 Scientific premise.** Accurate predictive biomarkers of sPTL are needed to guide preventive treatment and the development of novel therapeutic strategies. While the etiology of sPTL is multifactorial, intra-amniotic inflammation and local innate immune activation are increasingly recognized as key factors in the pathobiology of sPTL96. However, detecting intra-amniotic immune dysfunctions clinically is impractical. *We hypothesize that multifactorial mechanisms of sPTL converge on innate inflammatory pathways that drive labor, occur early in sPTL pregnancies, and are detectable in the peripheral blood.*

Several multi-omic studies, including from our group, have successfully integrated genomic, transcriptomic, proteomic, and metabolomic profiling of peripheral blood samples to identify predictive models of sPTL46,97–99. However, the predictive performance of prior models (RMSE = 4–6 wks) is only moderately improved compared to the clinical gold standard (i.e.,delivery at 40 wks GA). While these studies show promise in uncovering blood-based biomarkers for sPTL, none of them assessed immune responses at the single-cell level. Hence, strong signals may have gone undetected as specific immune cell subsets would have been functionally and phenotypically under-characterized. In addition, machine learning methods used in prior studies did not utilize data-driven feature selection metrics during the multivariable modeling process, which may have hindered the identification of reliable and clinically applicable candidate biomarkers.

In **Aim 2**, we propose a single-cell proteomic approach (CyTOF) to comprehensively characterize peripheral innate immune cell phenotype and signaling responses in samples from individuals with sTL or sPTL enrolled in the **WSU-2** (training, n=100) and the **NEXT** (validation, n=200) cohorts. The CyTOF data will be integrated with the high-content assessment of the plasma proteome, as plasma proteomic platforms have compared positively compared to other technologies in our prior multi-omic analyses of sPTL44,47. Multivariable modeling will be performed using the Stabl framework, allowing for the data-driven selection of the most reliable biomarker candidates. Importantly, available cohorts will offer a unique opportunity to examine pregnancy immune responses in individuals from diverse racial/ethnic groups, socio-economic backgrounds, and geographic locations.

**C2.2 Preliminary data.** In a preliminary study aiming at identifying a sparse and reliable predictive model of the TTD in sPTL, we performed a new analysis of our previously published dataset using the Stabl framework (**Fig. 7**)44. The longitudinal dataset collected in sTL (n=128 samples, 48 patients) and sPTL (n=14 samples, 5 patients) pregnancies comprised 1,317 plasma proteomic analytes (Somalogic) and 1,502 single-cell CyTOF features, including the frequency and intracellular signaling activities of major innate and adaptive immune cell subsets, measured at baseline and in response to inflammatory stimulations. The CyTOF and plasma proteomic data were integrated and combined with clinical variables into a single multivariable model that predicted the TTD. When trained on 128 samples from sTL pregnancies only and applied to sPTL pregnancies, the model predicted the TTD with higher accuracy than prior predictive models of sPTL (RMSE = 2.7 wks). The Stabl model contained ten single-cell immune features, including the responsiveness of innate immune cell subsets to inflammatory stimulations (e.g., JAK/STAT signaling response to IFNα in cMCs and NK cells), and seven plasma proteomic features, of which sST2 was the most informative.

**Figure 7. Stabl predictive modeling of the time to delivery (TTD) in spontaneous term (sTL) and preterm labor (sPTL) - pilot study. A**. A multivariable model combining CyTOF and plasma proteomic features trained on 128 samples from individuals with sTL using the Stabl framework (*middle panel*) accurately predicted the TTD in an independent cohort of sTL (*left panel*) and sPTL (*right panel*). **B**. Data-driven selection of reliable features (stability path graphs) identify sST2 and the pSTAT1 response to IFNα in NK cells among the most informative model features.

The results suggest that analysis of innate immune cell signaling responses can provide biologically plausible and clinically relevant immune biomarkers. However, while these preliminary results from a single-center study provide a promising multivariable framework to identify candidate biomarkers of sPTL, the analyses proposed in **Aim 2** are critically needed to test the generalizability of the findings in larger and more diverse cohorts.

**Table 1: WSU-2 cohort demographics**

**C2.3 Analysis plan**

*Clinical study design, training and validation cohorts, and biorepositories.* The **WSU-2** cohort (**A. Tarca**, Co-I) includes 200 participants enrolled at GA 10-12 wks who delivered spontaneously at term (sTL, n=100) or preterm (sPTL, n=50, **Table 1**). For each participant, PBMC and plasma samples were collected at 2-3 time points between 24 and 37 wks of gestation. The **primary clinical outcome is the** **TTD**, i.e., the difference in wks between sampling time and time of delivery.Two samples per participant (i.e., 200 samples) will be selected from 100 participants, matched on major demographic and clinical variables to ensure a similar distribution of the TTD variable between the sTL (n=50) and sPTL (n=50) groups. Secondary analyses will be performed on a subset of GA-matched samples to identify GA-specific features that differ between term and preterm pregnancies. The **NEXT** prospective cohort(**J. Prins**, Co-I) includes 200 participants enrolled at 10 wks GA at the UMCG in the Netherlands (median age 32 [30–35], BMI 23 [20-25], parity 1 [0-2], GA at delivery 39, range [26–42], 100% European). The estimated incidence of sPTL in the **NEXT** cohort is 15-20% (high-risk pregnancies). Peripheral blood collected for CyTOF analysis and plasma samples obtained at two timepoints per patient (~GA 12 and 28 wks) will be used for independent validation of identified candidate biomarkers of sPTL in a cohort with different racial/ethnic and socioeconomic distribution.

*Single-cell analysis of peripheral immune cell signaling responses.* PBMC samples will be analyzed at baseline and in response to a series of stimulations, including IL-1β, IL-6, IL-33, IFN⍺, and TNF⍺, chosen to evoke canonical signaling responses implicated in the peripheral immune response to pregnancy. We will employ a 48-plex CyTOF assay as described in **Aim 1b** allowing for: 1) the extensive phenotypic characterization of innate and adaptive immune cell subsets, including all innate cell subsets, and 2) the comprehensive assessment of intracellular signaling activities at baseline and in response to ex vivo stimulations, including elements of the MyD88 and JAK/STAT pathways (**Fig. 6**).

*Derivation of single-cell CyTOF and plasma proteomic immune features.* We will use dimension reduction (UMAP)100 and cell-subset identification (FlowSOM)81 algorithms to agnostically define and quantify the distribution of all innate immune cell subsets in individual patient samples, based on cell lineage and phenotypic marker expression. While the panel will be tailored towards innate immune cell phenotyping, ~10 markers will be included, allowing for identification of major adaptive immune cells. The intracellular signaling activities of each immune cell cluster will be quantified as the relative change (arcsinh ratio) in intracellular ab signal from the unstimulated condition. For *plasma proteomic analysis,* plasma samples collected in parallel will be analyzed using the Somalogic platform for multiplex analysis of circulating proteins.

*Multivariable predictive modeling of the time to delivery (TTD) and identification of biomarker candidates.* The Stabl algorithm will be applied to the combined CyTOF and plasma proteomic data generated from the **WSU-2** cohort (training set, n = 100) to identify a predictive model of the TTD (primary outcome). Each immune feature class (cell frequencies, signaling responses, plasma proteomics) will be treated as an omic data layer84. Additionally, clinical and demographic data (GA at time of sampling, obstetric history, fetal sex, BMI, co-morbidities) will be integrated as a distinct model data layer. Importantly, during the multivariable modeling, Stabl uses knockoff-modeled noise injection to determine the most frequently selected features by minimization of the false discovery proportion (FDP), allowing for data-driven identification of the most informative predictive biomarkers. Features selected via minimization of the FDP while building sparse multivariable models on individual omic data layers will then be integrated into a final model using a stacked generalization method. Model performance will be evaluated using an established Monte-Carlo cross-validation procedure stratified by patients, accounting for repeated sampling61.

*Independent validation and secondary analyses.* The multivariable predictive model, as well as individual features selected by Stabl, will be independently validated using samples from the **NEXT** cohort (n = 200). To minimize site-specific batch effects, the CyTOF datasets from both cohorts will be normalized using an established normalization method (CytoNorm)69. Secondary analyses will be performed to test model performance in early (GA<34 wks) vs. late (GA<37 wks) sPTL, and preterm premature rupture of membranes (PPROM) vs. sPTL with intact membranes. Additional post hocanalyses will quantify the effect of race/ethnicity as well as fetal sex and determine the effect of potential clinical or demographic confounders. Correlation network analyses will also be performed to interpret the relationships between single-cell and plasma proteomic model features within the broader context of the entire dataset.

*Power analysis.* A power analysis was conducted using Monte Carlo simulations71,101,102 based on our preliminary data and under a worst-case scenario assumption that a multivariable Stabl model enhances prediction accuracy exclusively in cases of sPTL. Ten thousand simulations were executed for varying sample sizes of the training and validation cohorts. The assumption of normal distribution of the errors for each model following the preliminary data experimental values was used to simulate the data points. Each simulation employed the Mann-Whitney U test to consider rejection of the null hypothesis, which posits no significant difference in error between the GA-based and Stabl model predictions. For the training cohort, the power analysis forecasted a statistical power greater than 90% with a sample size of 60, adopting an alpha level of 0.05. In the validation cohort, assuming a 20% incidence rate of preterm birth and consistent with the worst-case scenario assumption, a statistical power of 84% was achieved at an alpha level of 0.05, using a sample size of 200.

**C2.4 Expected outcomes.** Analyses in **Aim 2** will identify and validate a multivariable model that accurately predicts the TTD in sTL and sPTL pregnancies (expected RMSE < 2.5 wks). We expect that employing the Stabl method will yield a sparse model containing a number (~10–20) of reliable single-cell and plasma proteomic features that can be translated into a scalable clinical test. Model features will point at cell-type specific and plasma proteomic events that are shared between sTL and sPTL and occur earlier in sPTL. We anticipate the most informative model features will include: 1) intracellular signaling activities of cMCs and NK cell subsets in response to inflammatory ligands (MyD88 response to IL-1β or IL-33, JAK/STAT response to IFNα or IL-6) and 2) the plasma concentrations of proteins previously implicated in inflammatory changes preceding labor, including sST2, Siglec-6, or Angiopoietin. Identified single-cell and plasma proteomic biomarkers will be confirmed using traditional fluorescence flow cytometry and ab-based protein detection assays to facilitate translation into routine clinical workflows.

**C2.5 Potential Pitfalls and Alternative Strategies.** Our analysis is designed to identify predictive mechanisms common to sTL and sPTL that occur earlier in preterm pregnancies. However, we recognize that immune responses that differ between sTL and sPTL pregnancies may also provide a source of robust biomarkers. Identification of GA-dependent mechanisms that differentiate sTL from sPTL pregnancies will therefore be the subject of secondary analyses using available GA-matched samples between the term and preterm groups. In these analyses, the model predictive performances will be assessed using the AUROC. The lack of availability of samples collected before 20 wks is another limitation of the study. However, the range of GA for samples included in the analysis will allow construction of clinically meaningful predictive models applicable for the earliest cases of sPTL. We also recognize the significant demographic differences between the training and validation cohorts, as our goal is to identify predictive biomarkers that are generalizable across diverse cohorts. If the multivariable model fails to validate, a less stringent approach will be employed using univariate statistics (FDR approach) to determine whether individual model features validate in the test sample set. Finally, we note that the proposed assays focus on functional properties of innate immune cells and plasma proteomic markers, such that other important biological features, including additional adaptive immune responses, metabolomic, microbial, and transcriptomic events are under-characterized. However, at the completion of the project, we will be poised to expand the assay and bioinformatic pipeline to include additional omic data layers to further improve the predictive performance of the models.

**C3. Aim 3: High-plex MCB assay to identify candidate compounds for modulation of labor-associated innate immune responses and prevention of sPTL.**

**C3.1 Scientific premise.** Developing new drugs for the prevention of a complex disease like sPTL is time-consuming and costly. Therefore, there is an urgent need to consider unconventional strategies, such as repurposing drugs currently utilized for other conditions103,104. Our computational drug repurposing pipeline yielded a series of 83 promising COIs for the treatment of sPTL, including 13 FDA category A or B compounds45. However, the mechanisms by which identified COIs may prevent sPTL is inferred from analyses of transcriptomic data derived from cancer cell line experiments. Before considering their use in patients at risk for sPTL, it is essential to determine the effect of promising COIs on cellular mechanisms in the context of primary human tissue. *We hypothesize that the most promising COIs for the treatment of sPTL will prolong gestation, in part, by exerting cell-specific inhibition of innate inflammatory signaling responses*.

**Figure 8.** **Overview of** **the high-throughput assessment of the COIs’ immune-modulatory properties using a MCB assay.**

In support of this hypothesis, several COIs have known immune-modulatory properties, including the steroid hormone progesterone105,106, a previously approved albeit poorly effective treatment for PTL. Other COIs alter immune cell responses through plausible mechanisms, although they are prescribed for non-inflammatory conditions. For example, the proton pump inhibitor lansoprazole has been shown to inhibit TLR4 signaling responses in human neutrophils107. The experiments in peripheral blood immune cells proposed in **Aim 3** will evaluate the effect of COIs on key inflammatory signaling responses implicated in immunological regulation of labor, including IL-33-mediated signaling responses, while monitoring off-target and potentially undesirable effects on other innate or adaptive immune cells. COIs with cell-type and pathway-specific effects targeting labor-regulating immune responses will be retained for validation in human placental tissue and, ultimately, for further preclinical and clinical testing.

We will first develop a multiplex MCB assay for the high-throughput pharmacological assessment of 13 COIs or COI combinations in peripheral blood immune cells collected in healthy pregnant individuals. Using this assay, we will create a comprehensive dataset of the dose-dependent effect of the COIs on pro-inflammatory signaling responses (including the MyD88 and JAK/STAT signaling responses to IL-1β, IL-6, IL-33, IFNα, and TNFα) across all major immune cell subsets. Multivariable simulations based on previously published44,61, or newly identified (**Aim 2**), predictive models of labor onset will evaluate the capacity of each COI to alter individual participants’ predicted TTD (**Fig. 8**). COIs with promising immune modulatory properties,i.e.,those delaying the predicted TTD, will then be evaluated in the context of whole placental tissue using an ex vivo placental tissue slice assay.

**C3.2 Preliminary data.** The MCB of peripheral blood samples with palladium isotopes is a powerful tool for the multiplexed and high-throughput characterization of the immune-modulating properties of pharmacological agents108. Using binary combinations of six palladium isotopes, up to 64 (26) cell samples can be barcoded and simultaneously analyzed with CyTOF. **Fig. 8** outlines the experimental paradigm of an MCB assay that is currently utilized in the Gaudilliere lab and requires minimal (100μl) blood volume per tested condition. This MCB protocol will be adapted to evaluate the capacity of COIs to modulate key inflammatory signaling responses implicated in the pathobiology of labor.

In a proof-of-concept experiment, we applied the MCB assay to examine the immune modulatory effect of the COI lansoprazole on innate and adaptive immune responses in samples from four GA-matched pregnant volunteers (**Fig. 9**). The MCB assay allowed simultaneous measurement of >4000 intracellular signaling responses (including key elements of the MyD88 and JAK/STAT signaling pathways) across 20 innate and adaptive immune cell subsets in whole blood samples incubated with increasing doses of lansoprazole (10-7-10-4M) and stimulated with four different inflammatory ligands. The results showed cell-type and pathway-specific effects of lansoprazole that are consistent with inhibition of MyD88 pathways in myeloid cells and with recent in vivodata showing that lansoprazole prolongs gestation in a mouse model of inflammatory PTL45. This preliminary data provides the experimental framework to evaluate the immune modulatory properties of promising COIs in human immune cells.

**Figure 9.** **Proof-of-concept MCB assay using lansoprazole.** The analysis reveals cell-type and pathway-specific inhibition of MyD88 signaling responses (**A**), resulting in a delay in the simulated time to delivery (TTD n=4) (**B**).

**C3.3 Experimental approach.** Experiments in **Aim 3** will utilize whole blood samples from ten GA-matched pregnant volunteers collected between 20–24 wks GA as part of an ongoing biobanking effort at Stanford University (human subject section, **B. Gaudilliere**, PI). We will develop an MCB assay to comprehensively study the immunological properties of 13 COIs and COI combinations (**Fig. 8**). Placental samples collected at delivery from ten participants (see **V. Winn** letter of support) will also be prepared for confirmatory experiments using an ex vivo placental tissue slice assay evaluating the effect of select COIs on immune and decidual cells in the context of the intact placental tissue.

*MCB of human primary immune cells.* Whole blood aliquots (100μl, ~2x105 cells per condition) will be incubated with increasing concentrations of each COI, then left unstimulated (baseline condition) or stimulated with a cytokine panel, including IL-1β, IL-6, IL-33, IFNα, or TNFα, at 0.2μg/mL for two hours. Since the COIs are repurposed drugs, we will use publicly available pharmacological data to determine the range of concentrations that will be used for each COI. After stimulation, immune cells will be fixed, permeabilized, and barcoded using our established protocol109 and processed for CyTOF analysis.

*Derivation of cell-type and pathway-specific IC50 matrix and simulation of the COI’s effect on the TTD*. The CyTOF ab panel described in **Aim 2** will be employed for the single-cell assessment of intracellular signaling responses. For each COI concentration, cell population, and stimulation condition tested, signaling responses will be calculated as the difference (arcsinh ratio) in median signal intensity between the stimulated and unstimulated conditions. We will fit sigmoid functions to determine the concentration at which a given COI inhibits each cell-specific signaling response by 50% (IC50). The goodness-of-fit will be reported along with the inferred IC50 confidence interval. Statistical significance will be assessed using a Kolmogorov-Smirnov test adjusted using the Benjamini-Hochberg procedure for multiple testing across all conditions to assess the difference with a null effect distribution (COI=0M). For each COI, the analysis will provide an IC50 matrix of significant drug effects on cell type-specific signaling responses. In a simulation experiment, the IC50 matrix will be applied to immune profiles obtained from prior CyTOF analyses of patient samples collected during pregnancy44,61 (including newly generated data from **Aim 2**), resulting in a new dataset simulating COI-exposed samples. Multivariable predictive modeling of the TTD using the simulated dataset will estimate the effect of each COI on the predicted TTD. COIs associated with delayed simulated TTD prediction and an IC50 within an acceptable range (i.e., within one log of publicly available IC50 data) will be retained for further evaluation in placental tissue. *Power analysis*. Samples from ten volunteers will provide 80% power to detect an inhibitory effect >30% controlled for an alpha of 0.05.

*Validation of the immune modulatory effect of select COIs in an ex vivo placental tissue slice assay.* Precision-cut tissue slices (PCTS) will be prepared from fresh placental samples collected within 1-2 hours after delivery. PCTS is an ex vivomodel currently in use at UMCG (**J. Prins,** CO-I) that closely resembles the in vivo tissue state, as the tissue-specific cell types and tissue matrix-cell-cell interactions are maintained110*.* Slices of 250-300µm thickness will be cut from the basal plate and villous parenchyma separately. PCTS will be incubated for six and 24 hours with increasing concentrations of each COI and in the presence or absence of the cytokine panel. After FFPE processing of PCTS, the effect of selected COIs on placental immune cell subsets will be evaluated with our 48-plex IMC immunoassay (**Aim 1a**).

**C3.4 Expected Outcomes.** Results from the MCB assay will provide a single-cell readout of immune mechanisms by which COIs may dampen inflammatory innate immune responses associated with approaching labor and sPTL. Multivariate modeling analyses will integrate all single-cell responses into a simulated readout identifying whether a COI shortens or prolongs individual volunteers’ predicted TTD. We anticipate observing three categories of COI effects on human immune cells: 1) no inhibition of inflammatory signaling responses (IC50 >2 logs of publicly available IC50), indicating that modulation of sPTL-related immune responses is an implausible mechanism; 2) inhibition of multiple signaling responses across many immune cell-types, indicating a non-specific effect; or 3) cell-type- and/or signaling-specific inhibition of innate immune cell signaling (e.g., IL-1β, IL-6, IL-33, or IFNα-mediated) within an acceptable pharmacological range (IC50 within 1 log of publicly available IC50). These COIs will be retained for further preclinical and clinical evaluation. With respect to experiments in placental tissue slices, we anticipate that a subset of COIs will inhibit local inflammatory signaling responses in both immune and non-immune cells of the placenta. Overall, analyses in **Aim 3** will provide important mechanistic results in primary human tissue to facilitate future clinical studies in patients at risk for sPTL.

**C3.5 Potential Pitfalls and Alternative Strategies.** The MCB assay is currently in use in the Gaudilliere lab, and we do not expect major technological hurdles. We recognize that the list of COIs is derived from a pilot computational drug repurposing study. However, further studies in the Sirota laboratory will include additional publicly available transcriptomic datasets (including single-cell RNAseq datasets), expanding the list of COIs to be included in the MCB assay. In addition, the MCB is limited to the functional analyses of proximal signaling responses. To address this limitation, we will preserve PBMCs from individual patients to perform additional functional analyses, such as intracellular cytokine expression (e.g., IL-33). Similarly, our assay is designed to identify inhibitory properties of COIs on pro-inflammatory responses. However, COIs may prevent sPTL by activating regulatory signaling responses. To ensure that signaling activation is not overlooked, we will include Effective Concentration 50 (EC50) analyses of the baseline condition in the overall analytical plan. Finally, we recognize that the MCB assay interrogates peripheral immune responses, which may be only indirectly implicated in the pathogenesis of sPTL. However, the MCB ex vivo assay offers a powerful system to evaluate the immunological properties of multiple COIs, across a large array of human cell-types. While experiments in third trimester placental tissue aim to address this limitation, further testing of promising COIs could be performed using placenta-on-a-chip systems that mirror the placental tissue architecture at an earlier GA110,111.

**C4. Summary and timeline.** The overarching goal of our research is to determine the role of local and peripheral innate immune responses in inflammatory processes driving human labor and to identify mechanistic and targetable biomarkers for the prediction and prevention of sPTL. At the completion of the project, we will have generated a comprehensive single-cell atlas of the human maternal-fetal interface in laboring vs. non-laboring pregnancies (**Aim 1a**). Results from the proposed cross-tissue analyses will significantly improve our understanding of how local and peripheral innate immune responses contribute to the onset of spontaneous labor in humans (**Aim 1b**). We will have identified and validated in diverse patient cohorts a set of reliable predictive immune biomarkers of sPTL (**Aim 2**) and identified a shortlist of promising drug candidates targeting biologically plausible mechanisms for the prevention of sPTL (**Aim 3**). We will also have assembled a diverse and multi-disciplinary team of investigators with a track record in feto-maternal immunology research that will be primed to 1) test the generalizability of identified sPTL biomarkers in large, prospective studies across various demographic and socio-economic groups, 2) test the causality of innate immune mechanisms observed in the context of human pregnancy in preclinical models already established by the investigators, and 3) conduct clinical studies testing the safety and efficacy of promising drug candidates and their use as part of personalized regimens in at-risk individuals for the prevention of sPTL.

A screenshot of a computer

Description automatically generated**Table 2: Timeline**