March of Dimes Prematurity Research Center Stanford University

The nation's first transdisciplinary research center dedicated to identifying the causes of preterm birth

Newsletter: Fall 2017

What's inside?

Greetings

This fall has been a generative time at our Prematurity Research Center. We have established a new theme: the immunome and preterm birth, with preliminary work by Brice Gaudilliere, MD, PhD, Martin Angst, MD, and Nima Aghaeepour, PhD. For more on their findings published in September in *Science Immunology*, see page 2 featuring a Q&A with Drs. Gaudilliere and Aghaeepour. Their particular description of the immune clock is what set the stage for the March of Dimes wanting to establish a new theme focused primarily on the immunology of parturition and some of the pathologies of parturition. With this addition, we maintain three themes associated with our Center: (1) microbiome and preterm birth, (2) transcriptome and preterm birth, and the newly-minted (3) immunome and preterm birth. A fourth area of responsibility is the maintenance of the repository housing data from multiple March of Dimes Centers, an effort that is being overseen by Marina Sirota, PhD.

Our immunome theme links with our other two themes directly because it draws on the same samples that are now being collected for the microbiome and transcriptome work. This longitudinal cohort allows us to thematically integrate different elements related to the timing of pregnancy. We will now be able to explore the genome, microbiome, transcriptome, proteome, and immunome—all of these aspects simultaneously—and get a more complete description of normal parturition and pathologic parturition. Of course, our focus remains on preterm birth; however, we are also now investigating preeclampsia, so expect to see more work on that in the next year. Since we're looking at genes and the expression of gene pathways, we now can identify new diagnostics and targetable pathways for interventions. We will employ unique, distinguishing tools for defining gestational age, timing of birth, and the predictions of the pathologies of pregnancy.

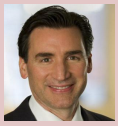
To this end, the Center has had several high-impact articles published this fall, including the *Science Immunology* paper and another out of Stephen Quake's lab that was featured on the cover of *Clinical Chemistry*. For more on the latter, see page 4. Finally, we replicated our findings showing differences in the vaginal microbiome of women who delivered at term versus those who delivered preterm in a paper in the *Proceedings of the National Academy of Sciences*.

From all of us at the Center, we hope you have a happy holiday season and look forward to working with you in 2018.

Leadership

Principal Investigator: David Stevenson, MD

Co-Principal Investigators:
Gary Darmstadt, MD
Maurice Druzin, MD
Gary Shaw, DrPH
Paul Wise, MD, MPH




Theme 1: The Microbiome and Preterm Birth
Lead Investigator: David Relman, MD

Theme 2: The Transcriptome and Preterm Birth
Lead Investigator: Stephen Quake, DPhil

Theme 3: The Immunome and Preterm Birth
Lead Investigator: Brice Gaudilliere, MD, PhD, Nima Aghaeepour, PhD, & Martin Angst, MD

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Q&A with Brice Gaudilliere and Nima Aghaeepour

Brice Gaudilliere, MD, PhD
Assistant Professor of Anesthesiology, Perioperative and Pain Medicine

Nima Aghaeepour, PhD
Baxter Laboratory in Stem Cell Biology

What happens to immune cells during pregnancy, a singular immunological event where tolerance for a fetus has to be maintained? Reported in a recent paper in *Science Immunology*, our Center's findings indicate the immune system follows a predictable pattern during a full-term pregnancy. Knowing the precise timing of immunological events during a normal pregnancy may help us spot when a woman's clock is not in sync and identify what cells or pathways may trigger preterm birth.

Combining single-cell mass cytometry (CyTOF) with a novel elastic net algorithm that is primed with existing knowledge about the immune system from the literature, Stanford researchers were able to show commonalities in the behavior of immune cells during pregnancy. Using peripheral blood samples from 18 women with full-term pregnancies, investigators assessed three factors: (1) abundances of immune cells present, (2) the different activities of these cells at resting state, (3) and the cells' receptor-specific responses. The analysis uncovered both innate and adaptive immunological changes during the course of human pregnancy, leading them to coin the expression the "immune clock of pregnancy."

This immune clock work was led by Brice Gaudilliere, MD, PhD (senior author) and Nima Aghaeepour, PhD (first author). Drs. Gaudilliere and Aghaeepour along with Martin Angst, MD, were officially given PI status and will now run an independent theme: *The Immunome and Preterm Birth*. This work will be done in conjunction with the microbiome and transcriptome efforts, led by David Relman, MD, and Stephen Quake, PhD, respectively. (See our other newsletters for Q&As with Drs. Relman [Fall 2015] and Quake [Spring 2016].)

Recently, we met with Drs. Gaudilliere and Aghaeepour to discuss their work.

Laura Hedli [LH]: Now that you know the immunological adaptations that take place during full-term pregnancy, do you anticipate this chronology will be condensed in preterm birth, or will steps be skipped?

Brice Gaudilliere [BG]: The short answer is: We don't know, but we now have the methodology and computational framework to determine whether the immune clock of pregnancy is accelerated or disrupted in a preterm pregnancy.

Nima Aghaeepour [NA]: It's not just that we'll be able to detect abnormalities, but we will be able to act upon them. In the same way that we have used our understanding of how the immune system responds to cancer to develop treatments, if we understand how the immune system responds to pregnancy, we could utilize it to affect outcomes.

LH: Analysis at the cellular level produces so much data. How did you prime the algorithm to manage that output with knowledge from the literature?

NA: There are in fact so many measurements that we needed to reduce some of them systematically. One thing that we did that I don't think I've seen anywhere before is that we went through the existing immunology literature and decided that this specific stimulation is likely to hit these four signaling pathways. Then, we found a way to incorporate that knowledge into the algorithm, and that significantly increased our predictive power. As a computer scientist, that was surprising to me. I usually think we can infer everything from the data, assuming that it will be more accurate than anything we can mine from the literature.

"We were not interested in looking at one thing at a time in isolation. We were interested in the modularity of the immune system and how that changes over time." – Dr. Nima Aghaeepour

BG: It's important to note that we were not biased on what we know about pregnancy. We're not telling the algorithm: *We think that pregnancy is going to do this to the immune system*. What we're telling the algorithm: *We know that if we take this ligand and we stimulate this one cell type, usually this is what happens*. For instance, you know that if you put your key in the car and turn right, it's going to turn on. This is very established previous knowledge that has nothing to do with pregnancy. This is a fundamental piece of the paper—that our approach is agnostic. We're throwing the data into this algorithm and letting the algorithm point us at the major immunologic events that track with pregnancy.

NA: In a perfect world we wouldn't need to do this. But in an imperfect world with limited sample sizes and a lot of noise, you can get rid of a lot of that noise by using preexisting knowledge of the immune system.

LH: Your study size was 18 women with full-term pregnancies, with three peripheral blood samples from each. At what points in time were the samples taken?

BG: Every woman was sampled three times throughout, but there is variability in when these three samples happen between patients. The analysis is based on using the gestational age (weeks of pregnancy) as a continuous variable. The analysis is agnostic to the notion of trimester. We didn't want the analysis to be biased by the fact that there are these arbitrary limits.

NA: For our algorithms to work effectively, you need more than just discrete trimesters and comparisons between one time point and another. The immune system itself is modular, the cell types working together through various signaling pathways. Usually when one thing goes up, it takes a whole bunch of other measurements up with it. We had to use an algorithm that could account for that interconnectivity between different cell types and signaling pathways. We were not interested in looking at one thing at a time in isolation. We were interested in the modularity of the immune system and how that changes over time.

LH: Do you think your research would eventually support us doing away with the arbitrary concept of trimesters in favor of a more nuanced chronology of pregnancy?

BG: The idea is that by just looking at how immune cells are behaving, we can precisely track the gestational age of women who deliver at term. Eventually, this approach will allow us to describe pregnancy in terms of immunological time rather than trimesters.

NA: You could start thinking about this concept of pseudotime or biological time.

BG: I like the concept of redefining the gestational age based on biological markers. You can only get at this by looking at the entire system, by taking a systems-immunology approach. That's where you get the precision, where you get the global view of how these adaptations are happening across time.

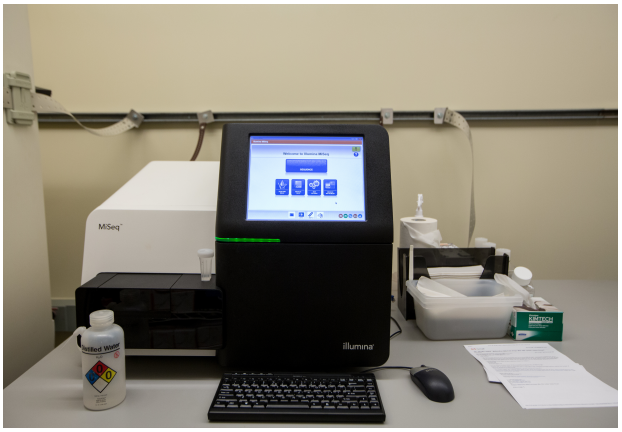
LH: The second part of your paper explores the specific signaling pathways that are important for producing these immunological adaptations. What did you find?

BG: The most informative feature of the immune clock model points to the Interleukin-2/STAT5 signaling pathway in CD4+ T cells. This signaling pathway is very important in the differentiation and stability of peripheral regulatory T-cells (T-regs), which are a subset of CD4+ T cells with immunosuppressive function that play a key role in feto-maternal tolerance. The role of peripheral T-regs in pregnancy has been extensively studied in mouse models by other groups. What we're seeing in humans is that the IL-2/STAT5 signaling activity progressively increases during the second half of pregnancy. A possible interpretation for these findings is that as the fetal antigens are being shed in the mother's blood stream, peripheral T-regs are mobilized to suppress the maternal immune response to these foreign antigens.

Investigating the transcriptome and the microbiome, concurrently

By Laura Hedli

Stephen Quake, DPhil, is an ace innovator when it comes to noninvasive prenatal diagnostics, and cell-free RNA sequencing (cfRNA-seq) is his latest tool of choice. "It's like a molecular stethoscope," says Dr. Quake, senior author of a recent MOD PRC paper exploring the capabilities of cfRNA-seq.



This cell-free RNA sequencing machine processes a single sample of peripheral maternal blood and allows us to simultaneously monitor immune response and microbial infections.

Indeed, cfRNA-seq is a tool that shows great promise in helping us sense what's happening to cell-free RNA during pregnancy. It's allowing us to learn not only about human RNA (maternal and fetal), but also non-human RNA (bacterial and viral). From samples of peripheral maternal blood, researchers were able to simultaneously detect both the immune response and also the presence of microbial infection. This is the hallmark of the study published in September in *Clinical Chemistry*—that both can be observed at the same time in a single sample. There is an up-regulation of anti-inflammatory genes and an increased abundance of anti-microbial genes, which makes sense given the nature of parturition. A mother's body has to tolerate a fetus for up to nine months and also protect it from infection. Taken together with the findings from Drs. Aghaeepour and Gaudilliere and colleagues (page 2), what we're beginning to uncover is a story of pregnancy that is more nuanced than we had previously imagined.

Lead investigator of our transcriptome theme, Dr. Quake, explains how scientists historically have conceptualized how the immune system responds to pregnancy. "One school of thought says: The immune system is cranked up. It's the healthiest you'll be and you're resistant to infection. And others say, it's actually cranked down. The fetus is a foreign object in the body and you've got to turn down the immune system so you don't attack it. Our take on this, based on the data, is that it's too simplistic to say that the immune system is monolithic, that it goes one direction or another.

If you actually dig into it, some parts of it are cranked up, some parts of it are turned down, and they compensate for each other."

In our Spring 2016 newsletter, we reported on the first paper out of the Quake lab to explore cfRNA-seq for analyzing peripheral blood samples from 11 women enrolled in our March of Dimes cohort. Through that study published in 2014 in the *Proceedings of the National Academy of Sciences*, Dr. Quake and his team learned about the normal path of development. They detected RNA transcripts from both the mother and fetus from samples of maternal blood and found that their expression levels changed as pregnancy progressed. They also identified several novel, non-coding RNAs and circular RNAs that had not before been known to be associated with pregnancy. This paper confirms those findings.

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"We had spent years [in my lab] working it out from cell-free DNA, and this is really the first attempt at trying to understand what's going on with RNA. Immediately we ran into challenges," says Dr. Quake. "We spent a lot of time trying to understand that. An important part of this paper's contribution is showing how to separate the signals, what you can trust and what you can't trust."

Because the input is fragmented RNA molecules, the products of cfRNA-seq are highly susceptible to contamination and degradation. The first part of this paper involved testing four different commercial cfRNA-seq library preparation methods for efficiency, and proposing certain recommendations for sample processing that may produce better results.

The second part of the paper characterized the changes of the human transcriptome and the plasma microbiome during pregnancy. First author Wenying Pan, PhD, says there were several biological questions they had when they began the study. "During pregnancy big changes occur in the women's body. An immune modulation happens because the fetus carries the paternal antigen. There's an immune suppression that happens," says Dr. Pan. "At the same time during pregnancy, the fetus and the mom are also very susceptible to infection, so how is that being balanced with the immune modulation? What is the interaction between the maternal immune system and the environment of the microbiome?" Investigators found over the course of pregnancy, the plasma microbiome remained relatively stable. However, the bacteria *Ureaplasma* was more prevalent and in greater concentrations after delivery, indicating a possible association with postpartum infection.

One woman in the study was found to have had human parvovirus B19 that went undiagnosed by her doctors. Our March of Dimes physicians were excited about the clinical applications of this methodology—it told them something they wouldn't have otherwise known but in the future would have the potential to treat. "Because we happened to capture it by chance in our sample collection, we could study not only the viral load and the presence of this viral infection from the RNA, but we could see also the correlative effects, the consequent effects on the woman and her immune system," says Dr. Quake. Human parvovirus B19 usually results in flushing, rash, swelling of joints. It can be passed from mother to fetus, putting the baby at risk and, in certain instances, causing fetal death.

Unlike cfDNA, cfRNA sequencing can detect certain viral pathogens like human parvovirus B19, and also Zika and norovirus. In the next 5-10 years provided the cost for this type of testing becomes more affordable and there are successful clinical trials, Dr. Pan hopes that we will adopt more comprehensive methods for screening during pregnancy to detect asymptomatic and rare viruses. Currently, clinics only screen for rubella, hepatitis B &C, HIV, tuberculosis, syphilis, chlamydia and Group B streptococcus.

Dr. Pan thinks it may be interesting to recruit women with known infectious diseases for clinical trials in order to validate cfRNA-seq as a diagnostic method. "What's the sensitivity or specificity compared to the other target diagnostics? When they have this infection, how do their immune cells respond?" she says. She also expects cfRNA-seq may be able to help us understand how the fetus reacts. "In the case of Zika virus, we should also see some difference in the fetal specific gene. If their brain becomes smaller, can we see that in the blood?"

To investigate these questions, we will need larger samples and cheaper sequencing methods. The *Clinical Chemistry* study's sample size of 60 is modest. Only 22 of the samples were from women who had a premature birth. Researchers caution that the results cannot yet be extrapolated to make any predictions about how the immune system behaves in mothers who deliver preterm.

Using peripheral blood samples from our March of Dimes cohort, Dr. Quake and his team will continue to explore the breadth of application for cfRNA-seq. Future research will involve using this method to look at different causes of preterm birth (like inflammation, infection, or genetics). The cfRNA data may allow us to separate out those different causes or see what's correlated.

"We're continuing to use cell-free RNA sequencing as a probe for mom and baby's health, and we'll see where it all takes us," says Dr. Quake. "We're interested in tracking the general path of development of the fetus and learning when things are getting off track and when the doctors should intervene to help."

Faculty Highlights

Gary Darmstadt, MD, MS, has been awarded a $2 million grant from the Bill & Melinda Gates Foundation to fund his work on preterm birth in collaboration with the Ottawa Hospital Research Initiative and the Ontario Newborn Screening Initiative. The grant will fund research to explore the feasibility and validity of using newborn metabolic profiles from cord blood and newborn heel prick blood spots (along with select clinical data) for gestational age dating of newborn infants born in primary health facilities. David Stevenson, MD, and Karl Sylvester, MD, are co-PIs.

David Stevenson, MD, was elected a fellow of the American Association for the Advancement of Science. He is among nearly 400 new AAAS fellows this year, chosen for his distinguished contributions to advance science and its applications.

Recent Publications

1. Tsur A, Mayo JA, Wong RJ, Shaw GM, Stevenson DK, Gould JB. 'The obesity paradox': a reconsideration of obesity and the risk of preterm birth. *J Perinatol*. 2017 Jul 27. doi: 10.1038/jp.2017.104.
2. Mayo JA, Shachar BZ, Stevenson DK, Shaw GM. Letter to the Editor: Response to: Interpregnancy Interval and Adverse Pregnancy Outcomes: An Analysis of Successive Pregnancies. *Obstetrics & Gynecology*. 2017 Aug; 130(2):463.
3. Carmichael SL, Kan P, Padula A, Rehkopf D, Oehlert J, Mayo J, Weber A, Wise P, Shaw GM, Stevenson DK. Social disadvantage and the black-white disparity in spontaneous preterm delivery among California births. *PLoS One. 2017* Aug 11; 12(8):e0182862.
4. Weber KA, Mayo JA, Carmichael SL, Stevenson DK, Winn VD, Shaw GM. Occurrence of selected structural birth defects among women with preeclampsia and other hypertensive disorders. *Am J Epidemiol*. 2017 Aug 23. doi: 10.1093/aje/kwx269.
5. Callahan BJ, DiGiulio DB, Goltsman DA, Sun C, Costello EK, Jeganathan P, Biggio JR, Wong RJ, Druzin ML, Shaw GM, Stevenson DK, Holmes SP, Relman DA. Replication and refinement of a vaginal microbial signature of preterm birth. *PNAS*. 2017 Sept 12; 114(37): 9966-9971.
6. Wise PH, Shaw GM, Druzin ML, Darmstadt GL, Quaintance C, Relman DA, Quake SR, Butte AJ, Angst MS, Muglia LJ, Macones G, Driscoll D, Ober C, Simpson JL, Katz M, Howse J, Stevenson DK. Risky business: Meeting the structural needs of convergent science. *J Pediatr.* 2017 Dec; 191:255-258.
7. Shaw GM, Yang W, Roberts E, Kegley SE, Stevenson DK, Carmichael SL, English PB. Residential agricultural pesticide exposures and risks of spontaneous preterm birth. *Epidemiology*. 2018 Jan; 29(1):8-21.