AARHUS UNIVERSITY

Amniochorionic membrane cells in the maternal blood as a biomarker for preterm birth

December 1st 2020

Principal Investigator:
Niels Uldbjerg, DMSc, Professor
Department of Obstetrics and Gynecology
Aarhus University Hospital
Palle Juul-Jensens Boulevard 99
8200 Aarhus N
Phone: +45 20679420
Email: uldbjerg@clin.au.dk

Coordinating Investigator:
Emmeli Mikkelsen, MD, PhD-student
Department of Obstetrics and Gynecology
Aarhus University Hospital
Palle Juul-Jensens Boulevard 99
8200 Aarhus N
Phone: +45 29652328
Email: emmeli.mikkelsen@clin.au.dk

AARHUS UNIVERSITY

Project description

Academic title and name: Emmeli Fredsgaard Ravnkilde Mikkelsen, Medical Doctor

Project title: Amniochorionic membrane cells in the maternal blood as a biomarker for preterm birth

Supervisors

- Niels Uldbjerg (main supervisor), Professor, DMSc, Department of Gynecology and Obstetrics, Aarhus University Hospital.
- Ramkumar Menon, Associate Professor, PhD, Department of Gynecology and Obstetrics, University of Texas Medical Branch at Galveston.
- Torben Steiniche, Professor, DMSc, Department of Histopathology, Aarhus University Hospital

Collaborators

- Palle Schelde, MSc, ARCEDI Biotech Aps.
- Ripudaman Singh, PhD, MBA, ARCEDI Biotech Aps.
- Berthold Huppertz, Professor, PhD, Medical University of Graz, Austria.

Aim

The aim of this PhD protocol is 1) to characterize circulating fetal amniochorionic membrane cells (ACM cells) in pregnant women and 2) to investigate if they can function as biomarkers of amniochorionic membrane dysfunction, including risk of preterm birth.

Hypotheses

1) It is possible to identify specific cells of the fetal membranes by their protein expression (ACM cells), and confirm the expression and localization of these cells by immunohistochemistry.
2) ACM cells can be identified and isolated in maternal blood using the same antibodies that identify the cells in the fetal membranes.
3) ACM cells circulating in maternal blood can serve as a biomarker for fetal membrane physiologic or pathologic status.
4) The concentration of circulating ACM cells reflects the risk of certain adverse pregnancy outcomes, including Preterm Prelabor Rupture of the Fetal Membranes (PPROM).

Abbreviations

- ACM cells: Amniochorionic Membrane Cells
- PPROM: Preterm Prelabor Rupture of the Fetal Membranes
- PLC: Preterm Labor Contractions

Background

Preterm birth: Globally, preterm birth (15 million per year) is the leading cause of mortality (1 million per year) and morbidity among children under 5 years of age [1]. Important pathways include preterm labor contractions (PLC), Preterm Prelabor Rupture of the Fetal Membranes (PPROM), and iatrogenic

delivery due to preeclampsia and fetal growth restriction [2]. In 2015, the Sustainable Development Goal of the United Nations defined that by 2030 the world should reduce neonatal mortality to at least as low as 12 per 1,000 live births. In order to do so, we must increase our understanding of fetal signaling during parturition in order to tailor better treatments to avoid preterm birth.

ACM cells: The fetal membrane, i.e. the amniochorionic membrane, consists of a single layer of cuboidal amnion epithelial cells, amnion mesenchymal cells (scattered fibroblasts), chorionic trophoblasts, and a collagen-rich extracellular matrix. During pregnancy, the fetal membrane cells multiply, grow and age, which in the end leads to telomere-dependent cellular senescence, labor and human parturition [3, 4]. These changes are expected in membranes at term, but similar features are expected to be seen in cases with PPROM [5].
Recently, in collaboration with ARCEDI Biotech Aps and The University of Texas Medical Branch at Galveston, Aarhus University has identified specific fetal membrane cell markers, i.e. specific proteins highly expressed by the ACM cells. These proteins were identified as followed: mRNA was extracted from fetal membrane cells, and subjected to RNAseq analysis to identify the genes that were differentially expressed as compared to the maternal blood cells. The highest expressed mRNAs and their corresponding proteins were identified. Commercially available antibodies specific for those identified proteins were obtained. These specific antibodies were used for isolating circulating ACM cells from maternal blood from pregnancies carrying male fetuses by Magnetic Activated Cell Sorting (MACS). After isolation, the cells were stained using a fluorescent-labeled pool of cytokeratin antibodies and visualized by scanning microscopy. The individual fetal cells were verified by XY-FISH analysis. Our preliminary studies indicate that circulating ACM cells are present in the second half of pregnancy but not in the first half of the pregnancy (results not yet published).
Nevertheless, we still do not know how the ACM cells are dislocated to the maternal blood. By the use of an advanced histological technique, the laboratory of one of the supervisors, Ramkumar Menon, has demonstrated “microfractures” in fetal membranes [6], which may constitute an important source of ACM cells.

We want to confirm by immunohistochemistry that specific antibodies can identify ACM cells in the fetal membranes, and that they can be a platform for isolating ACM cells from maternal circulation by MACS. Identification, quantification, and analysis of ACM cells in the maternal circulation can give us a better understanding of pregnancy physiology and pathophysiology. Furthermore, ACM cells might constitute a new clinical tool for the identification and correct treatment of pregnant women at risk of adverse outcome.

Materials and methods

Study 1: To confirm the protein expression profile of ACM cells by immunohistochemistry
Aim: To confirm that the selected proteins by RNAseq are highly expressed in the fetal membranes, and investigate how specific the expression or expression pattern are for ACM cells.
Hypotheses:

1. The expression or expression pattern of the selected proteins are specific for ACM cells in the fetal membranes and can be used for identification of ACM cells in the maternal circulation.

Materials: 1) Normal fetal amniochorionic membranes. 2) Placental tissue. 3) Multi tissue block containing several types of maternal tissue. 4) Tissue of the placental bed in the uterus collected from placenta previa cesarean sections.

Methods: Immunohistochemistry will be conducted using the specific antibodies previously used for isolation of ACM cells in the maternal circulation. For visualization of antibody binding, standard immunohistochemical protocols will be used. The analysis will be performed on automated staining platforms (Ventana). Interpretation of the staining will be done by bright field microscopy under the supervision of Associate Professor Ramkumar Menon, Professor Berthold Huppertz, and professor Torben Steiniche, in Texas, Graz, and Aarhus, respectively.

Study 2: Optimizing the method for the isolation of ACM cells from the maternal circulation.
Background: Presently, we use a pool of specific antibodies for markers expressed on the cell surface in both the amnion and chorion to enrich ACM cells from maternal blood.

Aim: We wish to:

1. Evaluate the performance of each antibody to see if it is possible to reduce the number of antibodies used for isolation, in order to make the test more simple and robust.
2. Evaluate if the expression pattern of the selected proteins changes during different gestational ages.

Hypotheses:

1. The antibodies perform differently concerning the number of ACM cells they identify.
2. The expression pattern of the selected proteins do not change during gestation.

Materials:

1. Cross sectional cohort of second and third trimester pregnant women. One blood sample from each woman. The number of participants depends on the results; might be between 10 and 100.
2. Longitudinal cohort of 15 women with normal pregnancies. Blood samples will be collected in gestation weeks 12, 20, 28 and 34, as well as at labor and post partum.

Methods: We will isolate the ACM cells from maternal blood by Magnetic Activating Cell Sorting (MACS) using different specific antibodies for ACM cells. The enriched ACM cells will be stained using fluorescent-labeled cytokeratin and vimentin antibodies, and sorted individually by Fluorescence Activated Cell Sorting (FACS). The true identification of the fetal derived ACM cells will be done by Short Tandem Repeat (SRT) analysis. If appropriate, we will optimize the protocol for the antibodies that perform best and are stabile expressed regardless of gestational age.

Study 3: The number of ACM cells in the maternal circulation in normal and pathological pregnancies.
Aim: To evaluate the number of ACM cells in the maternal circulation during pregnancy and at term in normal and pathological pregnancies.

Hypotheses:

1. Normal pregnancies: The number of ACM cells depends on gestational age and increases during labor.
2. Pathological pregnancies: The number of ACM cells is increased above the normal level for the gestational age.

Materials:

1. Longitudinal cohort of normal pregnant women: We will recruit 25 participants at their nuchal translucency scan at 12 weeks gestation. Blood samples will be collected at week 12, 20, 28, 34 and 36. Furthermore, we will collect blood at term, 2 days, and 4, 8 and 12 weeks after birth.
2. Term pregnant women with and without labor contractions: We will recruit 25 women in active labor at the delivery ward and 25 women with planned cesarean sections within a few days.
3. Preterm labor contractions (PLC): We will recruit 25 participants treated at the ward with tocolytics because of preterm labor contractions before 34 weeks gestation.
4. Preterm Prelabor Rupture of the Fetal Membranes (PPROM): We will recruit 25 participants treated at the ward because of PPROM before 34 weeks gestation.
5. Control group: We will recruit 25 women at gestation week 25+0 to 37.

Methods: The ACM cell analyses will be assessed according to the protocol developed in study 2. From these blood samples, we will establish a biobank in order to evaluate RNA expression in a later project.

Perspectives

Perspective 1: This study is based on the expression of selected proteins in normal pregnancies at term. A more broader gene expression profile at different times of gestational ages in relation to different pathological conditions could be interesting and done by RNAseq.
Perspective 2: We intend to conduct a large clinical trial on the use of ACM cells for identification of pregnancies at risk of adverse outcome, and investigate if the ACM cells can be used as biomarkers for early detection of threatening preterm birth.
Perspective 3: Interventions to reduce preterm labor have primarily been designed based on the signaling effects emitted by maternal compartments. By investigating the pathophysiological mechanisms of the fetal amniochorionic membrane, we will be able to tailor treatments based on signals from the fetal compartments as well. This may enable us to develop more advanced treatments in the future.

Internalization

- ARCEDI Biotech Aps: This private firm has established their primary technology for rare circulating fetal cell isolation and analysis at the laboratory at the Department of Gynecology and Obstetrics, Aarhus University Hospital, and has presently also established collaborations and laboratories in the United States.
- Ramkumar Menon from the Department of Gynecology and Obstetrics at University of Texas Medical Branch at Galveston is an honorary professor at Aarhus University Hospital, Department

of Gynecology and Obstetrics. Through many years of research, he has achieved profound knowledge of the fetal membranes and its pathophysiology during preterm birth.
- Professor Berthold Huppertz from Medical University of Graz, Austria, has a profound knowledge of the antibody staining of placental biopsies and is internationally leading the use of this method.

Ethical aspects
All three studies will be reported to and approved by The Danish Ethical Committee and the Danish Data Protection Agency. All results are stored in RedCap and analyzed in pseudo-anonymized form.

Statistical Analysis Plan
The mean number of isolated ACM cells in the different groups will be compared by using a chi-square test. A significant result is defined at a p-value < 0.05. The mean number of isolated ACM cells in each group will be presented with a 95% confidence intervals (Cl). The primary outcome will be compared with pregnancy and birth related secondary outcomes.

References
1 Liu, L., Oza, S., Hogan, D., Chu, Y., Perin, J., Zhu, J., Lawn, J. E., Cousens, S., Mathers, C., Black, R. E. 2016 Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet (London, England)*. **388**, 3027-3035. (10.1016/S0140-6736(16)31593-8)
2 Goldenberg, R. L., Culhane, J. F., Iams, J. D., Romero, R. 2008 Epidemiology and causes of preterm birth. *The Lancet*. **371**, 75-84. (https://doi.org/10.1016/S0140-6736(08)60074-4)
3 Behnia, F., Taylor, B. D., Woodson, M., Kacerovsky, M., Hawkins, H., Fortunato, S. J., Saade, G. R., Menon, R. 2015 Chorioamniotic membrane senescence: a signal for parturition? *American Journal of Obstetrics & Gynecology*. **213**, 359.e351-359.e316. (10.1016/j.ajog.2015.05.041)
4 Menon, R., Behnia, F., Polettini, J., Saade, G. R., Campisi, J., Velarde, M. 2016 Placental membrane aging and HMGB1 signaling associated with human parturition. *Aging*. **8**, 216-230. (10.18632/aging.100891)
5 Menon, R., Boldogh, I., Hawkins, H. K., Woodson, M., Polettini, J., Syed, T. A., Fortunato, S. J., Saade, G. R., Papaconstantinou, J., Taylor, R. N. 2014 Histological Evidence of Oxidative Stress and Premature Senescence in Preterm Premature Rupture of the Human Fetal Membranes Recapitulated in Vitro. *The American Journal of Pathology*. **184**, 1740-1751. (10.1016/j.ajpath.2014.02.011)
6 Richardson, L. S., Vargas, G., Brown, T., Ochoa, L., Sheller-Miller, S., Saade, G. R., Taylor, R. N., Menon, R. 2017 Discovery and Characterization of Human Amniochorionic Membrane Microfractures. *The American Journal of Pathology*. **187**, 2821-2830. (10.1016/j.ajpath.2017.08.019)