Abstract

Background: The Vanderbilt Institute for Clinical and Translational Research piloted the development of Project PLACENTA (PathLink ACquired gEstatioNal Tissue bAnk). This project investigated the feasibility of a fresh gestational tissue biobank, which provides tissue linked to electronic medical records for investigators interested in maternal-fetal health.

Methods: We developed a pipeline for collection of placental tissue from Labor and Delivery within approximately 30 minutes of delivery. An email alert was developed, to signal delivery, with the ability to specifically flag patients with certain phenotypic traits. Once collected 4 to 8 mm punch biopsy cores were snap frozen and subsequently used for DNA, RNA and protein extraction. Tissue was also collected for Formalin Fixed Paraffin Embedded (FFPE) histology, flow cytometry, and quality control measures.

Results: Of 60 deliveries using the email notification system, 25 (42%) were sent to Pathology or assigned to other research protocols and were not available for collection, 10 (16%) were discarded prior to arrival at Labor and Delivery, and 25 (42%) were available for collection. Twenty placentas were collected and averaged 38 minutes per collection. DNA extraction yielded an average of 53 µg/µl per sample and RNA extraction yielded 679 ng/µl on average per sample. Proteomic studies showed no degradation of protein, abundant and similar quantities of protein across samples and differentiation between the amnion, decidua, and villi. Histological studies showed good quality for interpretation and occasional pathology including multifocal chronic villitis, meconium laden macrophages, and Stage 2 acute chorioamnionitis. Flow cytometry demonstrated good cell viability after isolation.

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Introduction

Despite advances in maternal fetal medicine, premature births continue to be a major problem in all countries (Salk and Salk, 1977; Driscoll and Gross, 2009). Every year there are an estimated 15 million preterm births worldwide, 1 million of which lead to death before five years of age (World Health Organization ‘Preterm Birth’, 2018). Many of the remaining 14 million survivors face lifetime disabilities and debilitating health ailments. Though medicine has made great steps toward improving maternal-fetal health, clearly much still needs to be accomplished. Today, scientists have broad access to large scale biobanks as the resources and technology have evolved to effectively capture, maintain and distribute a broadening array of organisms and tissues for study both locally and more globally (Eiseman and Haga, 1999; De Souza and Greenspan, 2013). With this, scientists have been better able to investigate specific research questions dealing with tissues, organs, and diseases which were previously either unavailable or difficult to obtain due to timing, population size, and maintenance constraints. Despite the fact that the field of maternal-fetal health and work on prevention of preterm birth has received much focus over the last several years, biobanks containing placental tissue are seriously underrepresented due to a high cost of maintenance and difficulties in retrieval and storage of the tissue (Rubens et al., 2014; Hod and Lieberman, 2015; Tan, Guaran, and Challis, 2012; Tenenbaum-Gavish and Hod, 2013; Monangi et al., 2015).

Indeed, two of the World Health Organization’s Millennium Development Goals included improvement in maternal health and reduction in child mortality, which have been important areas of research for well over a decade (World Health Organization ‘Millennium Development Goals’ 2018). Working towards prevention of these disease states will improve pregnancy outcomes and the health of mothers, babies, and children throughout their lifespan. For example, maternal diet and body composition have been linked to varying placental sizes (Roland et al., 2014). Larger placentas relative to birth weight have been associated with a higher likelihood that the child will develop cardiovascular diseases later in life like coronary heart disease and stroke (Risnes et al., 2009). Researchers have also identified asthma as having both pre- and perinatal etiologies. When used to control asthma during pregnancy, inhaled glucocorticoids may affect placental vascularity and lead to the child having a higher likelihood of developing asthma (Mayhew et al., 2008). In addition, gestational diabetes mellitus has been linked to limitations in placental and fetal growth as it limits excess maternal glucose and increases vascular resistance (Desoye and Hauguel-de Mouzon, 2007). It has become clear that gestational tissue is an important tool for both prenatal research and research across the lifespan. In order to continue this research, scientists need access to gestational tissues to study the complex system of gestation. Availability of gestational tissues requires efficient collection methods, centralized regulatory protocols, and technical linkages to electronic clinical information for both mother and baby.

This article describes the development of protocols and an electronic alert system associated with creating a new gestational biobank at Vanderbilt University Medical Center (VUMC). Led by the Vanderbilt Institute for Clinical and Translational Research (VICTR) and the Preventing adverse Pregnancy outcomes and Prematurity (Pre³) initiative, PathLink ACquired gEstatioNal Tissue bAnk (Project PLACENTA) was piloted for two years beginning in the winter of 2015. Project PLACENTA was founded from a vision to provide a high quality, sustainable source of gestational tissues customized for usage tailored to the needs of the VUMC researchers. Project PLACENTA adds a unique and valuable
resource to the Vanderbilt community. At VUMC, these samples are linked to the data warehouse through the Research Derivative, a research tool created by VICTR (Danciu et al., 2014). This tool allows for the data to be searched, extracted, and utilized in a customized research-oriented manner, along with the ability to extract discrete data elements, notes, pathology reports, and linkage of maternal-child health data (Bowton et al., 2014). The creation of this tool uniquely connects patient data to the physical tissue samples, enabling researchers to utilize clinical data in their research. Project PLACENTA will enrich knowledge of both maternal and fetal short- and long-term health through gestational tissue research.

Materials and Methods
This pilot study was reviewed and approved by the Vanderbilt University Institutional Review Board (VUMC IRB# 160575 and #172053). After prioritizing gestational tissue needs on campus, we collected, processed, and quality-controlled samples to develop standard operating procedures for sample acquisition. For detailed protocols on DNA, RNA, and protein extraction please see Supplemental Materials.

Exploratory Survey
To investigate the feasibility and needs of Project PLACENTA, we conducted an exploratory survey in September of 2015, which was released to researchers across the Vanderbilt University (VU) and Vanderbilt University Medical Center (VUMC) communities. This survey queried these investigators to understand the research community’s areas of expertise, current, and future uses of gestational tissue, as well as their preferences of sample type and quantity.

Delivery Alert System
In order to facilitate the rapid collection of tissues post-delivery, the Vanderbilt Institute for Clinical and Translational Research (VICTR) created an alert system. This alert utilized data collected at the Labor and Delivery ward at VUMC via the commercially available software “Centricity EMR” by General Electric Healthcare. Our programmer staff received back-end access to the Centricity backup server that is updated within one minute of data entry. Each time a nurse entered a specific code corresponding to a flagged value, an alert was generated and emailed to a Project PLACENTA team member.

Placental Collection
After receipt of a ‘delivery alert’ the Project PLACENTA technologist collected the placenta from the nursing staff outside of the operating or patient’s room at VUMC without interrupting clinical processes. The technologist weighed the placenta using a digital scale (Global Industrial, Port Washington, NY), photographed it using a digital camera (Nikon, New York, NY), and sampled it for DNA (N = 30 samples across 3 placentas), RNA (N = 128 samples across 12 placentas), protein (N = 12 samples across 3 placentas), Formalin Fixed Paraffin Embedded (FFPE; N = 30 samples across 6 placentas) processing, and flow cytometry (N = 3 placentas). All samples included maternal, fetal, and membranous tissue. Figure 1 details the layers and components of the placenta and demonstrates the biopsy punch used to collect tissues.

DNA
Using an Acuderm Acu-Punch (Fisher Scientific, Pittsburg, PA) tool, eight 8.0 mm biopsy punches were sampled per placenta. Each sample was stored individually in a sterile, DNase- and RNase-free 2.0 mL Nalgene long-term storage cryogenic tube (Nalgene, Rochester, NY) with 1 mL of DNAgard preservative solution (Sigma-Aldrich, St. Louis, MO). The samples were snap frozen in a dry ice and isopropanol slurry. Samples were stored at -80°C before DNA was extracted in the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core (see Supplemental Materials 1), and quantified using a NanoDrop 8000 Spectrophotometer (Fisher Scientific, Pittsburg, PA).

RNA
Using an Acuderm Acu-Punch (Fisher Scientific, Pittsburg, PA) tool, eight 4.0 mm biopsy punches were sampled per placenta. Each sample was stored individually in a sterile, DNase- and RNase-free 2.0 mL Nalgene long-term storage cryogenic tube (Nalgene, Rochester, NY) with 1 mL of TRIzol preservative solution (Fisher Scientific, Pittsburg, PA). The samples were snap frozen in a dry ice and isopropanol slurry and then transferred to a -80°C freezer. RNA was extracted either through the VANTAGE core using a modified Fibrous tissue protocol with the QIAsymphony RNA kit (Qiagen, Hilden, Germany) or in a collaborating VUMC laboratory using a modified Qiagen's
Figure 1: This diagram demonstrates the interaction of blood vessels and tissues between the maternal and fetal surfaces. The placenta provides the interface of maternal and fetal connections: myometrium, decidua, maternal spiral artery, villus unit, amnion and captures the communication network between mother and child. The technologist sampled through all layers of the placenta using a biopsy punch (8mm for DNA and Protein, 4 mm for RNA samples).

Figure 2. A) Successful extraction of DNA from snap frozen placenta tissue (N = 30) yields an average of 53.8 µg/µL; B) Successful extraction of RNA from snap frozen tissue (N = 10) yields an average concentration of 679.27 ng/µL.
miRNeasy Mini Kit Protocol (Qiagen, Hilden, Germany; see Supplemental Materials)

**Protein**

Using the Acuderm Acu-Punch (Fisher Scientific, Pittsburg, PA) tool, ten 8.0 mm tissue biopsy punches were harvested from each placenta. Each individual sample was then placed onto a weight boat and into a bucket filled with dry ice for quick freezing, taking special care to ensure that the biopsy core contained all placental layers and froze without curving. After freezing, each sample was stored separately in a sterile, DNase- and RNase-free 2.0 mL Nalgene long-term storage cryogenic tube (Nalgene, Rochester, NY) and placed into dry ice before being stored at -80°C. All samples were transferred to the Mass Spectrometry Research Center at Vanderbilt University where they were prepared for Matrix Assisted Laser Desorption/Ionization (MALDI) analysis (Supplemental Materials) (Cornett et al. 2006). Serial sections of the frozen placenta were obtained on a cryostat. One section was stained with Hematoxylin and Eosin (H and E), scanned (Leica SCN400), and annotated by a pathologist. A serial section had regions that were digested with trypsin and the resulting peptides were analyzed via Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI TOF) mass spectrometry (Bruker UltrafleXtreme). Other serial sections were homogenized, lysed, and resolved approximately 1.5 cm onto a Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel. This gel area was excised and proteins digested using trypsin. Resulting peptides were analyzed via Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS; Thermo-Fisher LTQ) for identification against the human database using SEQUEST. Identifications were collated back to proteins using Scaffold (Proteome Software) and spectral counts were utilized to estimate the relative amounts of each protein.

**Formalin Fixed Paraffin Embedded Tissues**

Using a scalpel, 10 g of fresh placental tissue was taken from each placenta. This sample was then placed into a prefilled 1000 mL formalin container (Fisher Scientific, Pittsburg, PA) and transferred to the Translational Pathology Shared Resource (TPSR) core at VUMC where it soaked for 48-72 hours. Tissue was divided into approximately 2.0 mm thick slices which were then separated and placed into individual histology cassettes (Fisher Scientific, Pittsburg, PA). These cassettes were then soaked in prefilled formalin containers a second time and transferred back to the TPSR core for processing, embedding sectioning and H and E staining.

**Flow Cytometry**

Three placentas were transferred to our laboratory (D.M.A.) for flow cytometry processing and analysis. Villus core (membrane-free) tissue was washed with Dulbecco's phosphate-buffered saline (D-PBS), weighed, minced, and digested in a solution containing 150 mg/ml DNase, 1 mg/ml collagenase, and 1 mg/ml hyaluronidase (Sigma Aldrich, St. Louis, MO) before being layered onto a Percoll gradient (Sigma) as published before (Mason et al., 2013). Cells were stained with a cellular marker panel for T-, B-, Natural Killer (NK)-, NK T-, polymorphonuclear leukocytes (PMN)-, plasmacytoid dendritic- (pDC) and myeloid dendritic- cells (mDC), macrophages and monocytes.

**Consent**

Prior to developing the consent and recruitment workflow, a Community Engagement Studio was convened in Nashville, TN to gather feedback on perspectives surrounding consent and gestational tissue biobanking from community stakeholders (Byrne et al., 2012). These stakeholders included women of diverse age, racial, and ethnic background and parity who had given birth in the last three years. The community members participated by providing input to investigators on how and when consent should take place, thoughts and views on tissue biobanking, and concerns associated with this project. The IRB protocol, consent, study materials, and recruitment workflow were modified based on the results of this studio (VUMC IRB# 172053).

**Results**

Project PLACENTA successfully piloted the gestational tissue biobank at VUMC. After determining which tissues would be most impactful to researchers, quality control measures were conducted on a variety of collection methods, demonstrating the utility of these samples.
Exploratory Survey

Our survey generated 19 responses spanning the VU and VUMC community spanning a range of specialties; 18 of the respondents (94.7%) indicated that they would be interested in using placenta for future research.

Centricity

Our programming staff were able to access Labor and Delivery backup servers where they gained access to the Centricity software system. From this system 45 variables were retrieved including, Medical Record Number (MRN), patient name and delivery status. After a nurse enters information in the Centricity software system, the backup server is updated in real time. The alert system pings the Centricity server every 60 seconds and flags ‘Patient Status OB’ (item number 3149). When this item switches from ‘labor’ to ‘delivery vaginal’ or ‘delivery cesarean’, an alert is created and sent by email to study staff. Over the course of 71 days we received 472 births alerts at VUMC between the hours of 8am and 5pm, Monday through Friday; 238 vaginal births were received within approximately 15.77 ± 0.218 minutes of delivery of the baby. Cesarean section alerts (N=234) arrived an average 15.72 ± 0.2104 minutes after delivery. There was no significant difference in alert time due to the delivery method (P > 0.05).

Placental collection

The technologist attempted collection of 60 deliveries using the Centricity alert system. Of the 60 alerts, 25 (42%) placentas were not available for collection due to being sent to Pathology or assigned to other research protocols. Ten placentas (16%) were placed in the refrigerator by Labor and Delivery prior to arrival of the technologist and thus not usable, and 25 (42%) were available for collection. Due to limited resources, five of the 25 available placentas were not actually sampled for this study. Twenty placentas with an average weight of 601 g ranging from 412 g to 774 g, were collected and averaged 38 minutes of total collection time, ranging from 6 to 55 minutes.

DNA

Ten samples from three separate placentas (N = 30) were extracted at the VANTAGE core for DNA analysis. These samples yielded an average of 53.8 ± Standard Error (SE) 3.19 µg of DNA per sample, ranging from 29.4 µg to 100.6 µg, and an average 260/280 ratio of 1.85 ± SE 0.012 (Figure 2A).

RNA

RNA extraction was not initially successful after preserving samples in RNAlater (N= 62 samples across 4 placentas) or with extraction from the VANTAGE core using TRIzol (N = 28 samples across 4 placentas; data not shown). Ten samples from one placenta were successfully, and consistently, extracted using protocols from a collaborating laboratory. On average, 21.7 ± SE 3.63 µg of RNA per sample were extracted ranging from 7.3 µg to 47.2 µg, and an average 260/680 ratio of 2.04 ± SE 0.007. Samples averaged 679 ng of RNA per µl ± SE 113 µl ranging from 309 µl to 1470 µl (Figure 2B).

Protein

Three cores each from three placentas (for N = 9 samples) were analyzed by MALDI mass spectrometry. MALDI analysis shows robust differences between amnion, decidua and villi (Figure 3A and 3B). Extraction of total protein from the tissue homogenates was successful and good consistency in protein bands are evident between samples, without degradation of the protein. High Performance Liquid Chromatography (HPLC) followed by mass spectrometry was performed on eight of these samples and showed abundant and similar quantities of proteins across samples, especially in serum based proteins. The most abundant signal across the samples corresponded to serum albumin protein (accession # P02768) followed by serotransferrin (accession # P02787). Spectra were mapped to 381 distinct proteins, of which 14 were deemed likely to be false positives. Of the remaining 367 samples, 241 (65.7%) were present at a 95% probability in all three of the placentas. 90 (24.5%) had spectra map to proteins present in all tissues sampled.

Formalin Fixed Paraffin Embedded Tissues

Three placentas were collected to test FFPE procedures (N = 15 samples). The histological quality of each slide was measured with the Leica Microsystem Scoring Systems. Immunohistochemistry for CD31, COX2, and γH2AX was performed (Figures 4A-F). All three placentas showed adequate staining for each
Figure 3. Tissue snap frozen (N = 3) for proteomics shows peptide differentiation between tissue regions. A) Average spectra for maternal and fetal regions of tissue; B) average spectra for fetal and villi tissue regions. Red = maternal, yellow = fetal, green = villi.
Figure 4. Tissues (N = 3) were successfully formalin fixed and paraffin embedded after collection. Staining demonstrates clear histology. A) H and E stained villi (40x); B) CD31 stain in villi with endothelial staining (20x); C) COX2 stain in trophoblasts with no staining in villous macrophages (Hofbauer cells, 40x); D) H and E dense neutrophil infiltrate in the decidua (10x); E) Nuclear γH2AX stain in trophoblast with strong nuclear stain in syncytial knots (40x); F) Nuclear γH2AX stain (20x).

Figure 5. Cell viability (N=3) was 68.1% (±SE= 8.66%) after isolation and analysis through flow cytometry. The majority of cells were leukocytes and lymphocytes in nature, as well as derived from the innate immune system (monocytes, macrophages, neutrophils).
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marker tested. Multifocal chronic villitis, meconium laden macrophages, and stage 2 acute chorioamnionitis were identified. H2A Histone Family Member X (yH2AX) stain showed appropriate patchy nuclear stain with accentuation in syncytial knots in two of the three placentas (Figure 4F). There was no significant yH2AX stain in the third placenta, even in otherwise well preserved areas. This may be due to biologic variability. yH2AX is a marker for DNA damage and has previously been reported in placentae of smokers and in syncytial knots, which are associated with many variables, including length of gestation. Due to the samples being completely de-identified, the smoking status and gestational age of the patients are unknown.

Formalin Fixed Paraffin Embedded Tissues

The three placentas showed cell viability of 68.1% ± 8.66%. The majority of the cells isolated from the villous core of the placenta are hematopoietic (leukocytes and lymphocytes) in nature. The majority of the cells isolated also appeared to be of the innate immune system (monocytes, macrophages, and neutrophils) as shown in Figure 5.

Community Engagement Studio

Ten women who had given birth within the last three years shared their thoughts and suggestions concerning the biobank and consent process. These community experts stressed the belief that in order to increase consent, clear information emphasizing potential benefits to the community would need to be explicit. Familial input on consent is important in the community, so information should be easily accessible and disseminated in a way that facilitates discussion from trusted sources, like providers, well in the advance of delivery. The experts also suggested creating a newsletter that could be disseminated to possible participants once the biobank is being utilized. Though they understood results could not be returned to participants, it would be helpful to understand the findings their tissues have contributed to in the research community.

Discussion

The phrase ‘adverse pregnancy outcomes’ traditionally described immediate complications of pregnancy such as preeclampsia, preterm labor, gestational diabetes mellitus, stillbirth, intrauterine growth restriction, etc (Odibo et al., 2014). Through the fetal-origins theory, also known as the Developmental Origins of Health and Disease (DOHaD) framework, it is increasingly understood that disabilities and diseases across the lifespan of offspring can be influenced by the intrauterine environment, and in this way, can be considered adverse pregnancy outcomes (Eriksson, 2016). Diseases and disorders such as diabetes, obesity, cardiovascular disease, neurocognitive problems, and others, have been linked to the preconception health of parents and the gestational wellbeing of mothers (Pugh et al., 2015; Harreiter, Dovjak, and Kautzky-Willer, 2014).

Such processes as epigenetics and maternal and paternal influences on fetal health (e.g., nutritional status or exposure to environmental toxicants) are able to influence gene expression long beyond the immediate neonatal period (Bianco-Miotto et al., 2017). In this light, there is increased emphasis on the importance of understanding root mechanisms of disease pathogenesis at the maternal-fetal interface. The placenta is a uniquely transient human organ that has long been neglected as an active participant in maternal-child health. Due to its central role in governing fetal development, the placenta provides a unique window into normal and abnormal reproduction.

The ability to obtain high-quality, freshly collected gestational tissue is critical in the understanding of maternal-fetal health and research on diseases that are both gestational-related diseases or develop across the lifespan (Burton, Fowden, and Thornburg, 2016; Coughlan et al., 2001; Mirkovic et al., 2015). Project PLACENTA demonstrated the ability to collect and maintain high-quality placental samples suitable for a diverse range of research studies in a rapid manner after delivery. The ability to complete these collections was due, in part, to cooperation and input from the VU, VUMC, and broader Nashville community. Paired with the success of other gestational tissue banks in the United States and of the BioVU resource, VICTR piloted the creation of a fresh gestational tissue biobank (Roden et al., 2008; Burton, et al. 2014).

In order to determine when placentas were delivered at Labor and Delivery, we established
a linkage to the software system used to track progression of Labor at VUMC (Centricity, GE Healthcare). The largest hurdle in establishing this connection was obtaining access to the server data. The alert system pings Project PLACENTA study personnel within approximately 15 minutes of delivery and our team was able to successfully collect placentas approximately 70% of the time when they were not reserved for the clinical pathology lab. Future work will focus on ensuring a higher proportion of collections, and this may be accomplished with greater awareness of Project PLACENTA by clinical staff. Integration with the clinical pathologic workflow and requirements will allow access to the proportion of placentas from clinically abnormal pregnancies.

Placental DNA has become an increasingly important tool for researchers to understand gestation and early life health (Tjoa et al., 2006; Suter et al., 2011). We established a workflow to collect snap frozen tissue samples immediately after delivery. We successfully collected and extracted DNA and RNA from these samples. During the quality control process we determined that extraction of RNA was much more sensitive to changes in protocol than DNA. In our initial samples, DNA extraction was quite successful, however it was only after multiple iterations of freezing and extraction protocols that we established a successful method for preserving high quality RNA. Snap freezing tissue samples in RNAlater yielded poor results (data not shown), and we found greater success with placing the 4 mm biopsy punches directly into TRIzol reagent. In addition, extracting the RNA using the QIAsymphony RNA kit (fibrous tissue protocol) was not consistently successful. Once we established a specialized protocol and workflow (see supplemental methods) we were able to produce robust RNA samples, making the workflow viable.

We had additional success in snap freezing samples for protein extraction. Downstream analysis showed the ability to visualize differences between the amnion, decidua, and villi using MALDI analysis indicating complete and diverse sampling. LC-MS/MS showed varying abundances of protein across samples, specifically within the serum family of proteins. We reasoned this to be due to highly blood-saturated tissue and potentially moving forward a washing step prior to snap freezing would be advisable (Mushahary et al., 2013).

The work with FFPE tissue was successful at staining using a variety of staining techniques. In one sample however, the placenta stained with yH2AX did not show ubiquitous staining, which may have been due to biological variability. The marker yH2AX signals DNA damage and has previously been reported in placentae of smokers and in syncytial knots, which are associated with many variables, including length of gestation (Slatter et al., 2014). Due to the samples being completely de-identified the smoking status and gestational age of the patient were unknown.

Finally, we established a workflow to transfer fresh (non-frozen) tissue to investigators. These tissues were analyzed using flow cytometry to confirm differing cell types were present and quality of the samples was sufficient for downstream processes. The tissue samples showed good viability after isolation, approximately 70% cellular viability, and a variety of cell types were visualized. Viability may change due to length of labor, and obtaining cesarean delivered placentas may increase cell viability (Burton et al., 2014).

In piloting a fresh gestational tissue biobank there were several takeaway lessons learned. Minimizing the time between birth and collection is important and our collections were much more efficient when study staff received electronic alerts. This reduced burden on the nursing staff as they did not have to contact anyone after delivery, and saved technologist time. We also learned that placental RNA is much more sensitive than the other samples to extraction technique, and optimizing RNA extraction was critical to obtaining usable samples.

Project PLACENTA received IRB approval to consent participants in order to move out of the pilot phase and link tissue samples to electronic medical records. This will allow the use of clinical data in research studies. In conclusion, the collection of fresh gestational tissue is feasible for a variety of downstream analyses and Project PLACENTA was able to develop a protocol for timely collection after delivery, minimizing the burden on clinical and research staff.
Supplemental Materials

DNA extraction at VANTAGE:

After preservation, tissue samples were transferred to the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core where each DNA sample, 220 µl Buffer ATL (Qiagen 19076) and 20 µl proteinase K (used from enzyme rack of the QIA Symphony DNA Mini Kit) were added. Samples were then placed in a water bath at 56°C for 3 hours with intermittent vortexing until tissue was completely lysed. Once tissue was lysed, 220 µl of the supernatant was transferred to a 2.0 mL tube (Sarstedt 72.694) and processed on the QIA Symphony (Qiagen 9001297) using the QIA Symphony DSP DNA Mini Kit (Qiagen 937236) using the High content protocol (Tissue_HC_200_V7_DSP) and eluted to 200 µl in Buffer ATE. Samples were quantified using a NanoDrop 8000 Spectrophotometer (Fisher Scientific ND8000PRO1).

RNA extraction at VANTAGE:

20 mg of tissue samples were thawed in 1.0 mL TRIzol Reagent, and moved to a 2.0 mL microcentrifuge tube with a 5.0 mm stainless steel bead (Qiagen 69989). Samples were then placed on a TissueLyser II (Qiagen 85300) and were run at 25 Hz for 5 minutes, racks were flipped and samples were run again for another 5 minutes at 25 Hz. Samples were briefly spun down to remove liquid from the top of the tube and 100 µL of chloroform was added to each sample. Samples were then spun down at 14,000 RPM for 3 minutes. The aqueous phase was then transferred to a 2.0 mL tube (Sarstedt 72.694) to be processed on the QIA Symphony (Qiagen 9001297). Samples were processed on the QIA Symphony using the QIA Symphony RNA kit (Qiagen 931636) using the Fibrous tissue protocol (RNA_FT_400_V8) and eluted in 50 µl RNase free water. Once the RNA was extracted samples were then quantified using a Qubit 3 fluorometer (Invitrogen Q33216) and quality was measured using an Agilent 2100 Bioanalyzer (Agilent G2939BA). The tissue sections on the ITO slides were Carnoy-washed to remove salts and lipids as follows: 70% ethanol-30 seconds, 100% ethanol-30 seconds, water-30 seconds, Carnoy’s Solution-2 minutes, 100% ethanol-30 seconds. Serial sections were stained with the standard Hematoxylin and Eosin (H&E) method (Leica CM1900). The slides were scanned (Leica SCN400) and the digital images annotated by a pathologist using the PIMS system (Pathology Interface for Mass Spectrometry; Norris et al., 2016). Regions specific to maternal, villi, or fetal morphologies were targeted for MALDI analysis. Tissue

RNA Extraction at collaborative laboratory:

Samples preserved in TRIzol were placed in BeadBug prefilled 2.0 mL tubes (Sigma-Aldrich Z763810) and transferred to a BeadBug beater where they were run at 6,000 RPM for three cycles of 10 seconds. Once removed, the newly homogenized samples were preserved on ice until purification began. For purification, samples were run through Qiagen’s miRNeasy Mini Kit Protocol (Qiagen 217004) diverging on only three occasions (miRNeasy Mini Handbook. Qiagen Sample and Assay Technologies; 2014). First, after the buffers RWT and RPE were mixed with indicated volumes of 100% ethanol to create working solutions, they were kept for either one hour or overnight in a -80°C container. Next, the solution was eluted within a 1.5 mL Eppendorf tube using 2.0 µL of the total 32 µL solution to assess the RNA quantity. This diluted RNA was then used for the bioanalyzer to assess the quality of the RNA. Meanwhile, the remaining 30 µL were used for sequencing or PCR.

Finally, after adding the 32µL RNase-free water to the RNeasy spin column, the solutions were spun at a low speed (2,000 x g) for 2 minutes and then at a high speed (12,000 x g) for 2 minutes to fully elute the RNA. To quantify the volume of RNA eluted, the isolated samples were tested using a Biotek Synergy Mx Take 3 reader. Each 2 µL sample was run through this protocol before it was bioanalyzed. Once quantified, 1.0-2.0 µL of RNA was run through the Agilent RNA 6000 Pico kit protocol using a bioanalyzer (Agilent G2939BA) for analysis.

MALDI Preparation:

All samples were transferred to the Mass Spectrometry Research Center at VU where they kept at -80°C until ready for analysis. Thin sections (12 µm) were obtained on a cryostat (Leica CM1900). Tissue sections were then thaw-mouted onto indium tin oxide (ITO) coated glass slides for MALDI analysis and serial sections were placed on glass slides for staining.

The tissue sections on the ITO slides were Carnoy-washed to remove salts and lipids as follows: 70% ethanol-30 seconds, 100% ethanol-30 seconds, water-30 seconds, Carnoy’s Solution-2 minutes, 100% ethanol-30 seconds. Serial sections were stained with the standard Hematoxylin and Eosin (H&E) method (Fischer et al., 2008). The slides were scanned (Leica SCN400) and the digital images annotated by a pathologist using the PIMS system (Pathology Interface for Mass Spectrometry; Norris et al., 2016). Regions specific to maternal, villi, or fetal morphologies were targeted for MALDI analysis.
sections were returned to the -80°C freezer until ready for analysis.

To complete MALDI analysis, slides were removed from the freezer and allowed to come to room temperature over desiccant. The plate was optically scanned with a desktop scanner. Next, trypsin (Pierce Trypsin Protease MS-Grade) followed by matrix (α-cyano-4-hydroxycinnamic acid) were applied using a robotic spotter (Labcyte Portrait 630) specifically on the regions of interest marked by the pathologist. The samples were analyzed on a Bruker UltraflxXtreme TOF MS in reflectron positive ion mode.

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